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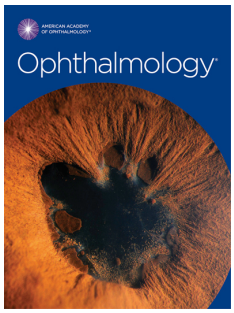

Functional changes of retinal ganglion cells and visual pathways in patients with Leber's hereditary optic neuropathy during one year of follow-up in chronic phase

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TITLE PAGE

Title: Functional changes of retinal ganglion cells and visual pathways in patients with Leber's hereditary optic neuropathy during one year of follow-up in chronic phase.

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Running Head: PERG and VEP changes in LHON patients

Abbreviations
RGCs: retinal ganglion cells
LHON: Leber's hereditary optic neuropathy
PERG: Pattern Electroretinogram
VEP: Visual Evoked Potentials
CL: confidence limit
ANOVA: One-Way Analysis of Variance
mtDNA: mitochondrial DNA
RNFL: retinal nerve fiber layer
PMB: papillo-macular bundle
BCVA: best-corrected visual acuity measurement
ETDRS: Early Treatment Diabetic Retinopathy Study
logMAR: logarithm of the minimum angle of resolution
IOP: intraocular pressure
HFA: Humphrey Field Analyzer
MD: mean deviation
CPSD: corrected pattern standard deviation
ISCEV: International Society for Clinical Electrophysiology of Vision

PERG A: Pattern Electroretinogram Amplitude
VEP IT: Visual Evoked Potentials Implicit time
VEP A: Visual Evoked Potentials amplitude
SNR: Signal to Noise Ratio
SD: standard deviation
PhNR: Photopic Negative Response

This article contains 3 Figures, 3 Tables and additional online-only material. The following should appear online-only: Figure 4

ABSTRACT

Purpose: To assess changes of retinal ganglion cells (RGCs) and visual pathways' function in patients with Leber's hereditary optic neuropathy (LHON) during 12 months of follow-up of chronic phase.

Design: Retrospective case series.

Participants: Twenty-two LHON patients (mean age: 36.3±9.3 years) in the "chronic phase" of the disease, providing 42 eyes (LHON Group) with different pathogenic mitochondrial DNA mutations (Group 11778: 21 eyes; Group 3460: 4 eyes, Group 14484: 13 eyes, and Group14568: 4 eyes) were enrolled. Twenty-five age-similar healthy subjects, providing 25 eyes, served as controls.

Methods: Pattern Electroretinogram (PERG) and Visual Evoked Potentials (VEP), in response to 60' and 15' checks visual stimuli, were recorded at baseline in all subjects and after 6 and 12 months of follow-up in LHON patients. At baseline, in all LHON eyes for each PERG and VEP parameter (amplitude and implicit time), the 95 % confidence limit (CL) of test-retest variability was calculated. PERG and VEP mean values observed in LHON eyes were compared (One-Way Analysis of Variance: ANOVA) to those of controls. During the follow-up, the PERG and VEP differences observed with respect to baseline were evaluated by ANOVA.

Main Outcome Measures: Changes of individual and of mean absolute values of 60' and 15' PERG amplitude and VEP amplitude and implicit time at each time point compared to baseline values in LHON Group.

Results: At baseline, mean values of PERG and VEP parameters detected in LHON Group were significantly ($p<0.01$) different with respect to control ones. In LHON Group, at 6 and 12 months of follow-up, the majority of eyes showed unmodified (within 95% CL) PERG and VEP values and mean absolute values of these measures were not significantly ($p>0.01$) different from baseline ones.

Conclusions. In our untreated chronic LHON patients, with different specific pathogenic mutations, RGCs and visual pathways function were not significantly modified during 12 months of follow-up. This should be considered in the disease natural history when attempts for treatments are proposed in chronic LHON.

Leber's hereditary optic neuropathy (LHON) is a mitochondrial disorder that leads to a bilateral acute loss of central vision, due to variable degree of optic nerve atrophy.[1]

Three pathogenic point mutations affecting mitochondrial DNA (mtDNA) (m.11778G.A/MT-ND4, m.3460G.A/MT-ND1, m.14484T.C/MT-ND6) can be found in the majority of LHON patients.[2,3]

The disease phenotype is highly variable, even within family members carrying the same homoplasmic mutation (all mtDNA copies are mutated)[4] and it is more frequent in males, with a female/male ratio ranging from 1:3 to 1:8, depending on the mutation type.[1]

In the "acute phase" of the disease, the ophthalmoscopic examination of the optic nerve shows typical signs such as telangiectaties and tortuous peripapillary vessels (peripapillary microangiopathy) and retinal nerve fiber layer (RNFL) swelling (pseudoedema). In this phase, there is an early and selective involvement of the central retina and specifically of the papillo-macular bundle (PMB),[5] which rapidly progresses to axonal loss in the temporal sector, responsible for the sudden loss of central vision with cecocentral scotoma. Over a period of about one year, LHON patients enter the "chronic phase" in which they develop pallor of the optic disc that is prominent on the temporal side, thus indicating various degree and extension of optic atrophy.[6]

Functionally, in LHON patients and carriers, it is possible to track the main dysfunction of retinal ganglion cells (RGCs) and of the optic nerve by electrophysiological methods. In particular, the dysfunction of the RGCs and relative nerve fibers can be studied by using Pattern Electroretinogram (PERG) recordings,[8-10] and abnormal PERG responses in both LHON patients[11,13] and in asymptomatic carriers[12,13] have been found. To verify the extent of the functional impairment along the axons forming the optic nerve, Visual Evoked Potentials (VEP) recordings in response to pattern[14] or multifocal stimuli[15] can be used. Indeed, by using pattern or multifocal VEP it was detected that LHON patients present an optic nerve dysfunction[16-17] with a predominant, but not exclusive, involvement of axons driving responses from the central retina (small axons) when compared to those serving the mid-peripheral retina (large axons).[16]

In carriers, instead, the finding of normal VEP suggested a functional integrity of the optic nerve.[12]

However, all above cited studies[11-13,16,17] were performed exclusively in the disease "chronic phase" with various degrees of optic atrophy and with no follow-up.[6]

Actually, there is a lack of exhaustive information in the literature about the possible functional changes in RGCs (evaluated by PERG) and in visual pathways (evaluated by VEP recordings) that may occur during the "chronic phase" in a wide cohort of LHON patients. In fact, functional changes have been evaluated by electrophysiological methods only in few works, reporting isolated cases or in subsets of single mtDNA mutation.[18-22]

Therefore, the aim of the present study was to assess, in a large cohort of LHON patients carrying different mtDNA mutations, possible functional changes in RGCs and their fibers (by PERG recordings) and in visual pathways (by VEP recordings) during 12 months of follow-up in the "chronic phase" of the disease.

Since all enrolled LHON patients were under no type of treatment, our study may provide useful information on the natural functional history of the disease, that should be considered when attempts for experimental treatments are planned in chronic LHON.

MATERIALS AND METHODS

Participants

Twenty-two patients (mean age 36.3±9.3 years, range: 20-46 years) from 20 families with a molecularly confirmed diagnosis of LHON harbouring either the m.11778G.A/MT-ND4, or m.3460G.A/MT-ND1, or m.14484T.C/MT-ND6, or m.14568C.T/MT-ND6 mutation, were studied (LHON Group). Each pedigree was also assessed for the mtDNA haplogroup,[23] which confirmed that they were unrelated. The male/female ratio was 4.5:1. The mean disease duration was 18.8± 9.9

years (range 6-34 years) and therefore all patients were studied in the "chronic phase" of the disease.

Twenty-five eyes from 25 normal age-similar subjects (mean age 37.2 ± 8.8 years, range: 19-48 years) served as Controls.

All Controls and LHON patients underwent extensive ophthalmologic characterization, including best-corrected visual acuity (BCVA) measurement, slit-lamp biomicroscopy, intraocular pressure (IOP) measurement, indirect ophthalmoscopy, optic nerve head 30˚ color standard photography, and Humphrey 24-2 automated visual field test [Humphrey Field Analyzer (HFA) 740; Zeiss, San Leandro, CA].

Normal subjects had a IOP less than 18 mmHg; BCVA of 0.0 logMAR with a refractive error between -2.00 and +2.00 spherical equivalent; 24-2 threshold visual field with a mean deviation (MD) of ±0.5 dB and corrected pattern standard deviation (CPSD) <1 dB; and no evidence of optic disc or retinal disease.

At baseline and during follow-up (see below), inclusion criteria for LHON patients were:

1) age ranging from 20 to 60 years;

2) diagnosis of LHON, confirmed by identifying one of the pathogenic mutations;

3) LHON duration no less than two years in both eyes;

4) HFA 24-2 with MD between -0.5 and -10 dB and CPSD between +1 and +10 dB; enlargement of the blind spot, cecocentral scotoma, paracentral defect around fixation more commonly temporal rather than nasal, central defects enclosing the physiologic blind spot; ability to maintain a stable fixation comparable to that of normal subjects (fixation loss rate ranging between 4% and 6%)

5) ability to clearly perceive a fixation target of PERG and VEP stimuli (see below) on a screen at a viewing distance of 114 cm;

6) BCVA between 0.00 and 1 logarithm of the minimum angle of resolution (logMAR);

7) refractive error (when present) between -3.00 and +3.00 spherical equivalent;

8) IOP less than 18 mmHg;

9) no previous history or presence of any ocular disease involving cornea, lens and retina/ macula or detectable spontaneous eye movements (i.e., nystagmus);

10) absence of any type of treatment, including gene therapy, idebenone [24] or citicoline, [25] during the 12 months preceding the enrolment or during the entire period of follow-up.

We excluded from the present study all eyes showing any sign of optic nerve pathology other than LHON.

LHON patients carried the following specific mutations:

1) m.3460/MT-ND1: 2 patients, providing 4 eyes (LHON-3460 Group),

2) m.14484/MT-ND6: 7 patients, providing 13 eyes that completed the follow-up and 1 eye excluded during the follow-up due to dense cataract (LHON-14484 Group),

3) m.11778/MT-ND4: 11 patients, providing 21 eyes that completed the follow-up and 1 eye excluded during the follow-up due to dense cataract (LHON-11778 Group),

4) m.14568/MT-ND6: 2 patients, providing 4 eyes (LHON-14568 Group).

Controls and LHON patients were evaluated at baseline and LHON patients after 6 and 12 months of follow-up.

All participants signed the informed consent. The research followed the tenets of the Declaration of Helsinki and the local Institutional Review Board/Ethics Committee approval was obtained (Azienda Santaria Locale Roma A, Rome, Italy).

Instrumentation and Procedures

Visual Acuity assessment

BCVA was evaluated by the modified Early Treatment Diabetic Retinopathy Study (ETDRS) Table (Lighthouse, Low vision products, Long Island City, NY, USA) at the distance of 4 meter. VA was measured as LogMAR values.

Electrophysiological examinations

In agreement with our previously published studies,[8,12,26-29] simultaneous PERG and VEP recordings were performed using the following methods.

Subjects were seated in a semi-dark, acoustically isolated room, in front of the display and surrounded by a uniform field of luminance of 5 candelas per meter squared. Prior to the experiment, each subject was adapted to the ambient room light for 10 minutes, with a pupil diameter of approximately 5mm. No mydriatic or miotic drugs were used. Stimulation was monocular after occlusion of the fellow eye. Visual stimuli were checkerboard patterns (contrast 80%, mean luminance 110 cd/m^2) generated on a TV monitor and reversed in contrast at the rate of 2 reversals per second. At the viewing distance of 114 cm, the check edges subtended 60 minutes (60') and 15 minutes (15') of visual angle. We used two different checkerboard patterns as suggested by the International Society for Clinical Electrophysiology of Vision (ISCEV)'s standards [30] to obtain a prevalent activation of larger (60' checks) or smaller (15' checks) axons.[12,26,31-33] The monitor screen subtended 23 degrees. A small fixation target, subtending a visual angle of approximately 0.5 degrees (estimated after taking into account spectacle-corrected individual refractive errors), was placed at the centre of the pattern stimulus. At every PERG and VEP acquisition, each patient positively reported that he/she could clearly perceive the fixation target. The refraction of all subjects was corrected for viewing distance.

PERG Recordings

The bioelectrical signal was recorded by a small Ag/AgCl skin electrode placed over the lower eyelid. PERG was bipolarly derived between the stimulated (active electrode) and the patched (reference electrode) eye using a previously described method.[34] As the recording protocol was extensive, the use of skin electrodes with interocular recording represented a good compromise between the signal-to-noise ratio and signal stability. A discussion on PERG using skin electrodes and their relationship to the responses obtained by corneal electrodes can be found elsewhere.[35,36] The ground electrode was in Fpz.[37] Interelectrode resistance was lower than 3000 ohms. The signal

was amplified (gain 50000), filtered (band pass 1-30Hz) and averaged with automatic rejection of artefacts (100 events free from artefacts were averaged for every trial) by BM600 (Biomedica Mangoni, Pisa, Italy). Analysis time was 250 msec. The transient PERG response is characterized by a number of waves with three subsequent peaks of negative, positive, and negative polarity, respectively. In visually normal subjects, these peaks have the following implicit times: 35, 50 and 95 msec (N35, P50, N95). In the analysis of PERG responses, we considered the peak-to-peak amplitude between the P50 and the N95 peaks: PERG P50-N95 amplitude (PERG A) measured in microvolt.

VEP recordings

Cup shaped electrodes of Ag/AgCl were fixed with collodion in the following positions: active electrode in Oz, [37] reference electrode in Fpz,[37] ground in the left arm. Interelectrode resistance was kept below 3000 ohms. The bioelectric signal was amplified (gain 20000), filtered (band-pass 1-100 Hz) and averaged (200 events free from artefacts were averaged for every trial) by EREV 2000. Analysis time was 250 msec. The transient VEP response is characterized by a number of waves with three subsequent peaks of negative, positive, and negative polarity, respectively. In visually normal subjects, these peaks have the following implicit times: 75, 100 and 145 msec (N75, P100, N145). In the analysis of VEP responses, we considered the implicit time of the peak P100, VEP P100 Implicit time (VEP IT) measured in milliseconds, and the peak-to-peak amplitude between the N75 and the P100 peaks, VEP N75-P100 amplitude (VEP A) measured in microvolt.

During the recording sessions performed at baseline and after 6 and 12 months of follow-up, simultaneous PERG and VEP were recorded at least twice (2 to 6 times) and the resulting waveforms were superimposed to check the repeatability of results. For all PERG and VEP, implicit times and peak-to-peak amplitudes of each of the averaged waves were directly measured on the displayed records by means of a pair of cursors.

On the basis of previous studies[12,29,] we know that intra-individual variability (evaluated by

test-retest) is approximately ± 2 ms for VEP IT and approximately ± 0.25 microvolts for PERG A and VEP A. During the recording session, we considered as "superimposable" and therefore repeatable, two successive waveforms with a difference in msec (for VEP IT) and in microvolts (for PERG A and VEP A) less than the above reported values of intra-individual variability. Sometimes the first two recordings were sufficient to obtain repeatable waveforms; other times, however, further recordings were required (but never more than 6 in the cohort of patients or controls). For statistical analyses (see below), we considered PERG and VEP values measured in the recording with the lowest PERG A.

In each subject or patient, the signal-to-noise ratio (SNR) of PERG and VEP responses was assessed by measuring a 'noise' response while the subject fixated at a not modulated field of the same mean luminance as the stimulus. At least two "noise" records of 200 events each were obtained, and the resulting grand average was considered for measurement. The peak-to-peak amplitude of this final waveform (i.e., the average of at least two replications) was measured in a temporal window corresponding to that at which the response component of interest (i.e., VEP N75-P100, PERG P50-N95) was expected to peak. SNRs for this component were determined by dividing the peak amplitude of the component by the noise in the corresponding temporal window. An electroretinographic noise <0.1 microvolts (mean 0.074 microvolts, range 0.063 to 0.094 microvolts, resulting from the grand average of 400-1200 events), and an evoked potential noise < 0.15 microvolts (mean 0.087 microvolts, range 0.076 to 0.114 microvolts, resulting from the grand average of 400-1200 events) was observed in all subjects tested. In all subjects and patients, we accepted PERG and VEP signals with a signal-to-noise ratio > 2.

Statistics

We calculated the sample size by using mean ± 1 standard deviation (SD) data from 20 patients with LHON (17 from our previous report[12] and 3 from unpublished data).

Groups' sample size was calculated based on LHON 60' and 15' PERG P50-N95 amplitude data (60': 0.95±0.64 μV; 15': 1.10±0.68 μV) and VEP P100 implicit time (60': 141.91±24.15 ms; 15': 145.35±20.78 ms).

We sized our group based on the expected changes that allow statistically significant changes of the values detected at follow-up with respect to baseline. At $\alpha = 0.05$ and $\beta = 0.20$, the changes and SD at follow-up, calculated as a percentage (%) with respect to the baseline values, were the following: for 60'PERG P50-N95 amplitude: % of mean ±38.90, % of SD ±47.30; for 15' PERG P50-N95 amplitude: % of mean ±29.10, % of SD ±12.73; for 60' VEP P100 implicit time: % of mean ±10.50, % of SD ±13.61; for 15' VEP P100 implicit time: % of mean ±12.20, % of SD ±17.14.

Based on this data, we obtained the following sample size for each parameter: 60' PERG P50-N95 amplitude: 36 eyes; 15' PERG P50-N95 amplitude: 38 eyes ; 60' VEP P100 implicit time: 35 eyes; 15' VEP P100 implicit time: 38 eyes. To reach the required number of eyes, and considering a possible drop-out lower than 15 %, we enrolled 22 LHON patients providing a sample of 44 eyes.

Therefore, it was mandatory to consider, in all statistical evaluation of this study, the group of all LHON patients entirely. Consequently, no inferential statistic could be applied to mutation-specific LHON Groups, since a number of eyes lower than that required was available (LHON-3460 Group: 4 eyes; LHON-14484 Group: 13 eyes; LHON-11778 Group: 21 eye; LHON-14568 Group: 4 eyes).

Test-retest data (obtained in LHON eyes evaluated in this study) of PERG and VEP results were expressed as the mean difference between two recordings obtained in separate sessions performed on two different days (the time elapsed form the first and the second sessions of recordings was between 2 and 4 days) ± 1 SD of this difference. A 95% confidence limit (CL, mean ± 2 SD) of test-retest variability in LHON eyes was established assuming a normal distribution.

At baseline, mean values of PERG and VEP parameters observed in LHON Group were compared to those of Controls by the one-way analysis of variance (ANOVA).

During the follow-up, the differences of PERG and VEP values observed in individual LHON eyes with respect to the baseline values (values detected at 6 and 12 months minus those detected at baseline) were calculated performing a logarithmic transformation. The changes of absolute values of PERG and VEP respect to the baseline, observed in LHON Group, were also evaluated by ANOVA.

In all ANOVA analyses, a conservative p value of 0.01 was considered as statistically significant, to compensate for multiple comparisons: (p = 0.05/number of comparison: baseline vs 6 months and baseline vs 12 months = 2; p= 0.05/2 = 0.025 significance level).

During the follow-up, Pearson's correlation was used to evaluate the relationship between the changes (6 and 12 months with respect to baseline) of electrophysiological (PERG and VEP) data. PERG and VEP changes detected at 12 months were correlated with the corresponding changes of BCVA. A p value of 0.05 was considered as statistically significant for this correlation.

All statistical analyses were performed using MedCalc V.13.0.4.0 (MedCalc, Mariakerke, Belgium).

RESULTS

Figure 1 shows representative traces of unmodified, improved or worsened PERG and VEP responses observed in LHON eyes after 6 and 12 months of follow-up with respect to baseline condition.

Table 1 reports the mean values of PERG, VEP, HFA and BCVA detected at baseline in Controls and LHON eyes and relative statistical analysis.

Table 2 lists the number of individual functional changes using 60' and 15' checks stimuli expressed in absolute values and percentages with respect to the total number of eyes belonging to LHON Groups at months 6 and 12 of follow-up.

Individual 15' and 60' PERG and VEP changes during follow-up observed in LHON eyes at 6 and 12 months are shown in Figure 2.

Mean data of absolute values of PERG and VEP parameters observed in LHON Group at baseline and after 6 and 12 months and the relative statistical analyses with respect to baseline are shown in Table 4.

The correlations between PERG and VEP changes (12 months with respect to baseline) detected in all LHON eyes are reported on Figure 3.

1) Retinal Ganglion Cells functional changes: PERG data

At baseline, all LHON eyes showed a reduction in 60'and 15' PERG A. Mean values observed in LHON Groups were significantly ($p<0.01$) different with respect to control ones (see Table 1).

When considering the individual changes concerning the 95% CL, the majority of eyes of LHON Group showed unmodified PERG A recorded with 60' checks after 6 and 12 months of follow-up (78.57 and 76.19% respectively) or with 15' checks during the same times of follow-up (78.57 and 64.29%, respectively). The individual changes detected in mutation-specific LHON Groups and in LHON Group are reported on Table 2 (see "Differences 6 and 12 months minus baseline") and Figure 2.

In LHON Group, the mean of absolute values of 60' and 15' PERG A detected at 6 and 12 months of follow-up was not significantly ($p>0.01$) increased and/or reduced when compared with those observed at baseline (see Table 3: "60' and 15' PERG P50-N95 Amplitude").

In LHON Group, the 60' and 15' PERG A changes were not significantly ($p>0.05$) correlated with BCVA data. The correlation is reported in Figure 4 (available at www.aaojournal.org).

2) Neural Conduction along the visual pathways changes: VEP data

At baseline all LHON eyes showed an increase in 60'and 15' VEP IT and a reduction in 60' and 15' VEP A; the values observed in LHON Groups were significantly ($p<0.01$) different with respect to control ones (see Table 1).

When considering the individual changes regarding the 95% CL, the majority of LHON eyes showed unmodified VEP IT recorded with 60' checks after 6 and 12 months of follow-up (83.33 and 80.95 % respectively) or with 15' checks during the same time points (76.19 and 71.43% respectively). The VEP A values were unmodified in the great percentage of LHON eyes (from 88.10% of eyes for 15' VEP at 12 months of follow-up to 97.62% of eyes for 60' VEP at 6 months of follow up). The individual changes detected in each mutation-specific LHON Group and in LHON Group are reported on Table 2 (see "Differences 6 and 12 months minus baseline") and Figure 2.

In LHON Group, the mean of absolute values of 60' and 15' VEP IT and A observed at 6 and 12 months of follow-up were not significantly ($p>0.01$) modified when compared with those observed at baseline (see Table 3: 60' and 15' VEP P100 Implicit times and N75-P100 Amplitude).

In eyes of LHON Group, at 6 months of follow-up, the changes in 60' and 15' VEP IT were independent ($p>0.01$) from the corresponding changes in 60' and 15' PERG A. At 12 months of follow-up, not significant ($p>0.01$) correlation between the changes in 60' VEP IT and 60' PERG A were found. The changes in 15' VEP IT were weakly dependent ($r =0.5428$, $p= 0.0263$) from the changes in 15' PERG A (see Figure 3).

In LHON Group, the 60' and 15' VEP IT changes were not significantly ($p>0.05$) correlated with BCVA data. This correlation is reported on Figure 4 (available at www.aaojournal.org).

DISCUSSION

Our study aimed to evaluate the possible functional changes of RGCs and related fibers and of visual pathways in untreated LHON patients, affected by different mtDNA mutations

306 (11778/ND4; 3460/ND1, 14484/ND6, and 14568/ND6), along 12 months of follow-up of the
307 disease "chronic phase".

308 *1) Retinal Ganglion Cells functional changes: PERG data*

309 In our study, the function of RGCs and of their fibers was assessed by PERG recording.[8-10]
310 As in our previous study,[12] the enrolled patients in the present study were aged between 20 and 45
311 years (mean age 36.3±9.3 years) and thus they are "not old". This is important when considering
312 that several factors (i.e. cataract or age-related maculopathy) can influence PERG responses.

313 With respect to our aim, at different time points (baseline, 6 and 12 months), we considered
314 exclusively the P50-N95 amplitude of PERG responses, since, actually, this parameter is considered
315 as "more specific" to evaluate the function of RGCs and their fibers.[38,39] The PERG P50 implicit
316 time was not considered, based on previous documented evidences suggesting that also the
317 functional integrity of preganglionic elements is necessary in order to generate a normal P50
318 implicit time response.[38,40]

319 At baseline, a significant reduction of PERG A in all LHON eyes was found when compared
320 to Controls. Considering the specific mtDNA mutations: about the 11778/ND4, the present baseline
321 PERG results are in agreement with those of our previous study[12] and with other Authors' findings,
322 [11,13,19,22] where PERG was assessed as recruitment criteria or for the evaluation of the effects of
323 gene therapy. Similar PERG abnormalities, detected in our LHON patients with 3460/ND1
324 mutation, were observed also by others.[11,12,18, 20,21] And also for the 14484/ND6 mutation, PERG
325 abnormalities found in our present and previous study[12] are in agreement with those observed by
326 others.[20, 21] On the PERG abnormalities detected in LHON patients with 14568/ND6 mutation, the
327 present study represents a novel finding since LHON patients carrying this mutation were never
328 studied previously through an electrophysiological approach.

329 The observed reduction in PERG A can be ascribed to a dysfunction of the innermost retinal
330 layers (RGCs and their fibers), similarly to that observed in other diseases (i.e glaucoma,[29,41-44] or

ischemic optic neuropathy [45]). Nevertheless, abnormal PERG responses were also detected by using high-contrast checks, subtending 60 minutes of visual arc (60'). By using this type of visual stimuli, a complex electrophysiological response is generated, with contributions of both contrast- and luminance-sensitive retinal generators (ganglion and preganglionic cells).[8] Therefore, in presence of abnormal 60' PERG responses observed in LHON eyes, a functional contribution of the pre-ganglionic elements needs to be considered, although the evidence is slim in support of pre-ganglionic dysfunction[11,17] and previous histological studies documented sparing of photoreceptors and retinal pigmented epithelium in affected LHON.[46]

Actually, the so-called Photopic Negative Response (PhNR) of the light-adapted electroretinogram is another electrophysiological method available for assessing the RGCs functional integrity. It is interesting to consider that RGCs dysfunction in LHON eyes with 11778/ND4, 3460/ND1 and 14484/ND6 mutations was detected also by using this new electrophysiological approach.[21,47]

At 6 and 12 months of follow-up, in the analysis of individual changes, the great percentage of LHON eyes showed unmodified PERG A. Nevertheless, in each mutation-specific Group there were cases with an improvement or a worsening of PERG responses (see Tables 2 and Figure 2).

In particular, in LHON-11778, it was observed that in 29% of eyes there was a change in RGCs function. This may be a consequence of the large number of eyes belonging to this Group. Our findings in LHON-11778 are in contrast with those observed by Yang et al., [19] who found unmodified PERG responses in 8 LHON-11778 eyes during 12 months of follow-up. In LHON-3460, a worsening of 15' PERG amplitude in 2 out of 4 eyes and of 60' PERG amplitude in 1 out of 4 eyes were found; no PERG improvement was detected. This is in contrast with Sharkawi et al.,[18] who reported PERG improvement in only one case in which PERG was recorded by using similar visual stimuli. About the 14484/ND6 mutation, we observed PERG improvement in 1 out of 13 eyes (7.69%) in contrast to Jarc-Vidmar et al.,[20] who reported no PERG changes in the only patient

enrolled with this mutation after 30 months of follow-up. About the LHON-14568 patients, we observed a reduction in 60' PERG A in 1 out of 4 eyes, while by using 15' checks, no eyes showed changes in PERG responses. There is no comparative information in the literature for this mutation.

Mean values of PERG A detected in LHON Group were similar with respect to baseline (see Table 3), thus suggesting that the RGCs function evaluated in a global cohort of LHON eyes is not significantly modified during 12 months of follow-up. Since in each mutation-specific LHON Group the number of eyes was lower than required for a correct statistical analysis, we could not provide statistical data referred to PERG changes observed at 6 and 12 months of follow-up with respect to baseline.

Data on RGCs dysfunction detected by PERG abnormalities in our LHON Group are consistent with the reported RNFL layer thinning evaluated by Optical Coherence Tomography.[48]

2) Neural Conduction along the visual pathways changes: VEP data

In this study, as for as in our previous published work,[12] VEP responses were obtained by using different spatial frequencies with larger or smaller checks, subtending respectively 60 minutes (60') and 15 minutes (15') of visual angle. This approach was used to obtain information on the function of both large and small axons forming the visual pathways. In fact, it is well known that the stimulation of different size of the retinal receptive fields (that can be obtained by varying the spatial frequencies of visual stimuli) induces a predominant activation of different neural components of the visual pathways that evoke responses driven to the cortical areas by different axons' populations with variable neural conduction velocity.[31,32] Thus, by using the 60' checks, we could mainly activate the large retinal receptive fields, thereby driving responses to the cortex by large axons and by using the 15' checks (spatial frequency with smaller checks), we could preferentially activate the smaller retinal receptive fields with the bioelectrical signal being driven to the visual cortex by small axons.[49]

At baseline, significant abnormal VEP responses (IT delay and A reduction using both visual stimuli of 60' and 15' checks) were observed in all LHON eyes when compared to Controls. Considering the specific mutations: about the 11778/ND4 our baseline VEP results are in agreement with those reported by Ziccardi et al.[12] and by Yang et al., who considered VEP parameters in the recruitment for gene therapy and their changes in the evaluation of its effects.[19,22] The VEP abnormalities found in our LHON patients with 3460/ND1 mutation are consistent with those previously observed in our study[12] and in other works.[18,20,21] Also LHON eyes with 14484/ND6 mutation showed abnormal VEP responses similarly to that observed in our previous study,[12] and to that reported by Jarc-Vidmar et al.[20] and by Majander et al..[21] LHON eyes with 14568/ND6 were never studied by electrophysiological methods and therefore the detected VEP abnormalities represent a novel finding.

Our baseline VEP findings obtained in responses to both 60' and 15' checks, confirming our previous data,[12] can be explained considering that in the "chronic phase" of the disease there is a not selective dysfunction for the smaller fibers of the papillo-macular bundle, but also an involvement of the larger axons. This is also supported by the electrophysiological evidences obtained by using more selective visual stimuli such as the multifocal VEP stimuli.[16] Our VEP findings are consistent with morphological studies in which it has been reported that the smaller fibers of the papillo-macular bundle are selectively damaged in the initial phase of the acute disease and later the morphological changes extend to the rest of the axons of the optic nerve, when the optic atrophy occurs.[2,6]

After 6 and 12 months of follow-up, VEP responses were unmodified in a great percentage of LHON eyes and a small percentage of them showed both improvement or worsening of VEP responses (see Tables 2 and Figure 2).

In particular, in a percentage of about 28% and 19% (with 15' and 60' of visual stimuli, respectively) of LHON-11778, a shortening of the VEP IT was found and in a percentage of 14%

(for both 15' and 60' of visual stimuli) a further VEP IT delay was observed. As for PERG results, this may be a consequence of the large number of eyes belonging to this Group. Our VEP results are in contrast with those of Yang et al., [19] who detected unmodified VEP responses , during 12 months of follow-up.

In the Group with 3460/ND1 mutation, all eyes showed unmodified VEP responses in agreement with previous reports,[20] while Sharkawi et al.[18] observed an improvement in only one case in which the VEP, found "undetectable" at baseline, became "detectable but delayed" after 18 months of follow-up.

About the 14484/ND6 mutation, a shortening in VEP IT in about 15% and 8% (with 15' and 60' of visual stimuli, respectively) and a further delay in VEP IT in about 8% and 0 (with 15' and 60' of visual stimuli, respectively) were observed. By contrast, Jarc-Vidmar et al.,[20] observed unmodified abnormal VEP responses in only one of enrolled patient during 30 months of follow-up. In LHON patients with 14568/ND6 mutation, VEP responses were unmodified in all eyes and so far there are no similar information in the literature.

Mean values of VEP parameters detected in LHON Group were not significantly different when compared to baseline (see Table 3). As for PERG results, in each mutation-specific LHON Group (see above), we were not able to provide statistical analysis referred to VEP changes observed at 6 and 12 months of follow-up with respect to baseline.

Our VEP findings let us to believe that the neural conduction along both large and small axons of the visual pathways is substantially unmodified in the global cohort of LHON eyes during 12 months of follow-up. In addition, the observed improved/worsened/unmodified neural conduction (for both large and small axons) along the visual pathways is not entirely dependent from the modification in RGCs function as suggested by barely significant correlation between the changes (12 months minus baseline) in PERG A and VEP IT (see Figure 3). The variations in neural conduction did not influence the changes in BCVA, as derived by the lack of correlation

between the changes (12 months minus baseline) in VEP IT and BCVA (see Figure 4, available at www.aaojournal.org).

Conclusions

In our cohort of LHON patients, with specific mitochondrial mutation, RGCs and visual pathways function were, on average, not statistically modified through 12 months of follow-up of the chronic phase of the disease.

In our study, we used an electrophysiological approach to assess the RGCs function (PERG recordings) and to evaluate the neural conduction along the visual pathways (VEP recordings). On the basis of our results, we suggest that, when these methods are applied, it is crucial to well establish the range of variability of the electrophysiological responses. Only in this manner, it is possible to distinguish between true worsened or ameliorated responses that are those that exceed the limits of the inter-individual variability.

We believe that it is very important also to consider that in a variable percentage of LHON eyes, in relationship to the specific mutation (i.e. 11778/ND4), there is the possibility that worsening or improvement of RGCs and visual pathways function can spontaneously occur during the disease natural history. All this should be taken in account when attempts for treatments are proposed in the chronic phase of LHON disease.

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569 **FIGURE LEGEND**

570 **Figure 1.** Examples of Pattern Electroretinogram (PERG) and Visual Evoked Potentials (VEP)
571 recordings, performed in 3 Leber's hereditary optic neuropathy (LHON)-11778 eyes at baseline
572 condition and after 6 and 12 months of follow-up. In these patients, with respect to baseline, at 6
573 and 12 months, it is possible to detect values of Pattern Electroretinogram (PERG) P50-N95
574 Amplitude (A, ↕), Visual Evoked Potentials (VEP) P100 Implicit Time (IT, ↔) and VEP N75-
575 P100 Amplitude (A, ↕) unmodified (Implicit Time and Amplitudes modified within the intra-
576 individual limits of variability), worsened (increased values of Implicit Time and reduced values of
577 Amplitudes exceeding the intra-individual limits of variability) or improved (increased values of
578 Amplitudes and reduced values of Implicit Time exceeding the intra-individual limits of
579 variability).

580 **Figure 2.** Individual differences of Pattern Electroretinogram P50-N95 Amplitudes (PERG A),
581 Visual Evoked Potentials P100 implicit times (VEP IT) and N75-P100 amplitudes (VEP A) in
582 patients with Leber's hereditary optic neuropathy (LHON) detected at 6 and 12 months of follow-up
583 (6m/bas and 12m/bas, respectively). 60' and 15' refers to visual stimuli in which each checks
584 subtended 60 and 15 minutes of visual arc, respectively. 3460, 14484, 11778 and 14568 refers to
585 specific mitochondrial DNA mutations. The percentage of unmodified (within the 95% confidence
586 test-retest limit), improved (values over the 95% confidence test-retest limit, solid line) and
587 worsened (values lower the 95% confidence test-retest limit, dashed line) eyes are reported on
588 Table 2. CL: Confidence limit.

589 **Figure 3.** Pattern electroretinogram (PERG) P50-N95 amplitude, in response of 60' and 15' checks
590 (60' and 15') individual differences between baseline and 6 (6 months) and 12 (12 months) months
591 of follow-up detected in all Leber's hereditary optic neuropathy (LHON) eyes plotted as a function
592 of the values of the corresponding differences in Visual Evoked Potentials (VEP) P100 Implicit
593 Time. Pearson's test was used for regression analysis and correlations.

Figure 4. Pattern electroretinogram (PERG) P50-N95 amplitude and Visual Evoked Potentials (VEP) P100 Implicit Time, in response of 60' and 15' checks (60' and 15'), individual differences between baseline and 12 (12 months) months of follow-up detected in all Leber's hereditary optic neuropathy (LHON) eyes plotted as a function of the values of the corresponding differences in visual acuity. Pearson's test was used for regression analysis and correlations.

Table 1. Mean values of Pattern Electroretinogram P50-N95 Amplitudes (PERG A), Visual Evoked Potentials P100 Implicit times (VEP IT) and N75-P100 Amplitudes (VEP A) Humphrey 24-2 perimetry (HFA) Mean Deviation (MD) and LogMAR best-corrected visual acuity (BCVA) measurement expressed as a logarithm of the minimum angle of resolution (logMAR), detected in Controls (C, 25 eyes) and in patients with Leber's hereditary optic neuropathy (LHON Group, 42 eyes) at baseline. Statistical evaluation by a One-way Analysis of Variance (ANOVA). Abbreviations: SD: 1 standard deviation. 60' and 15':visual stimuli in which each check subtended 60 and 15 minutes of visual arc respectively; µV: microvolt; Nr: number of eyes inside the normal limits; Ab: number of eyes outside the normal limits. Normal limits were obtained from control subjects by calculating mean values +2 standard deviations for VEP P100 implicit time and mean values –2 standard deviations for PERG P50-N95 and VEP N75-P100 amplitudes. MD was considered as Ab for values less than -2dB. BCVA was considered as Ab for values greater than 0.0.

	Group	**Mean**	**SD**	**ANOVA: LHON vs : f (1,66);**		**Nr**	**Ab**
				f=	P=		
60' PERG A (µV)	**C**	2.39	0.15				
	LHON	1.29	0.48	123.43	<0.001	0	42
60'VEP IT (msec)	**C**	102.37	3.41				
	LHON	123.72	14.3	614.33	<0.001	0	42
60' VEP A (µV)	**C**	11.56	1.87				
	LHON	3.65	1.99	258.78	<0.001	0	42
15' PERG A (µV)	**C**	2.48	0.18				
	LHON	1.15	0.27	478.40	<0.001	0	42
15'VEP IT (msec)	**C**	104.42	3.86				
	LHON	127.70	13.31	72.44	<0.001	0	42
15' VEP A (µV)	**C**	10.62	2.15				
	LHON	3.14	1.60	263.98	<0.001	0	42
HFA MD (dB)	**C**	0.18	0.46				
	LHON	-7.89	3.23	153.27	<0.001	0	42
BCVA (LogMAR)	**C**	0.00	0.00				
	LHON	0.44	0.56	15.34	<0.001	0	42

Table 2. Six and 12 months of follow-up in patients with Leber's hereditary optic neuropathy (LHON). Changes of Pattern Electroretinogram (PERG) P50-N95 Amplitudes, Visual Evoked Potentials (VEP) P100 Implicit Times and N75-P100 amplitudes. 3460, 14484, 11778 and 14568 refers to the specific mitochondrial DNA mutation. 60' and 15': visual stimuli in which each checks subtended 60 and 15 minutes of visual arc respectively. Unmodified: values of PERG and VEP Amplitudes and VEP Implicit Time within the 95% confidence test-retest limit; Improvement: increase in values of PERG and VEP amplitudes and decrease in values of VEP Implicit Time that exceeded the 95% confidence test-retest limit; Worsening: reduction in values of PERG and VEP Amplitudes and increase in values of VEP Implicit Times that exceeded the 95% confidence test-retest limit; N: number of eyes.

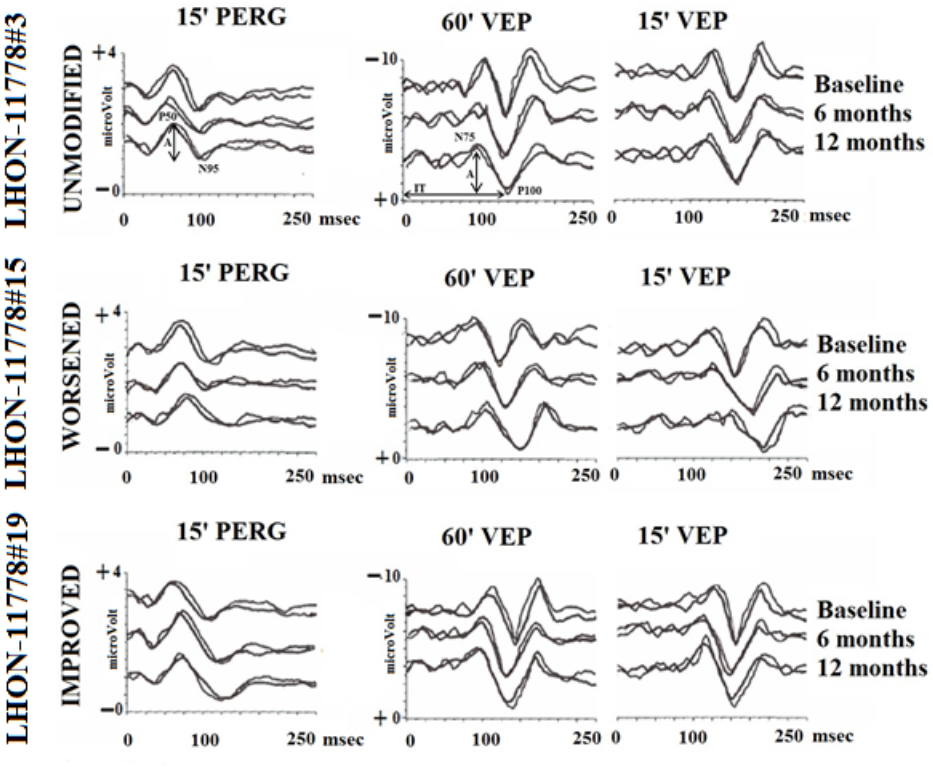
60' PERG P50-N95 Amplitude												
	Difference 6 months minus baseline						**Difference 12 months minus baseline**					
	unmodified		**Improvement**		**worsening**		**unmodified**		**Improvement**		**worsening**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
LHON-3460 (N=4)	3	75.00	0	0.00	1	25.00	3	75.00	0	0.00	1	25.00
LHON-11484 (N=13)	10	76.92	1	7.69	2	15.38	11	84.62	1	7.69	1	7.69
LHON-11778 (N=21)	17	80.95	3	14.29	1	4.76	15	71.43	4	19.05	2	9.52
LHON-14568 (N=4)	3	75.00	0	0.00	1	25.00	3	75.00	0	0.00	1	25.00
LHON Group (N=42)	33	78.57	4	9.52	5	11.90	32	76.19	5	11.90	5	11.90
60' VEP P100 Implicit Time												
	Difference 6 months minus baseline						**Difference 12 months minus baseline**					
	unmodified		**Improvement**		**worsening**		**unmodified**		**Improvement**		**worsening**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
LHON-3460 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON-11484 (N=13)	12	92.31	1	7.69	0	0.00	12	92.31	1	7.69	0	0.00
LHON-11778 (N=21)	15	71.43	4	19.05	2	9.52	14	66.67	4	19.05	3	14.29
LHON-14568 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON Group (N=42)	35	83.33	5	11.90	2	4.76	34	80.95	5	11.90	3	7.14

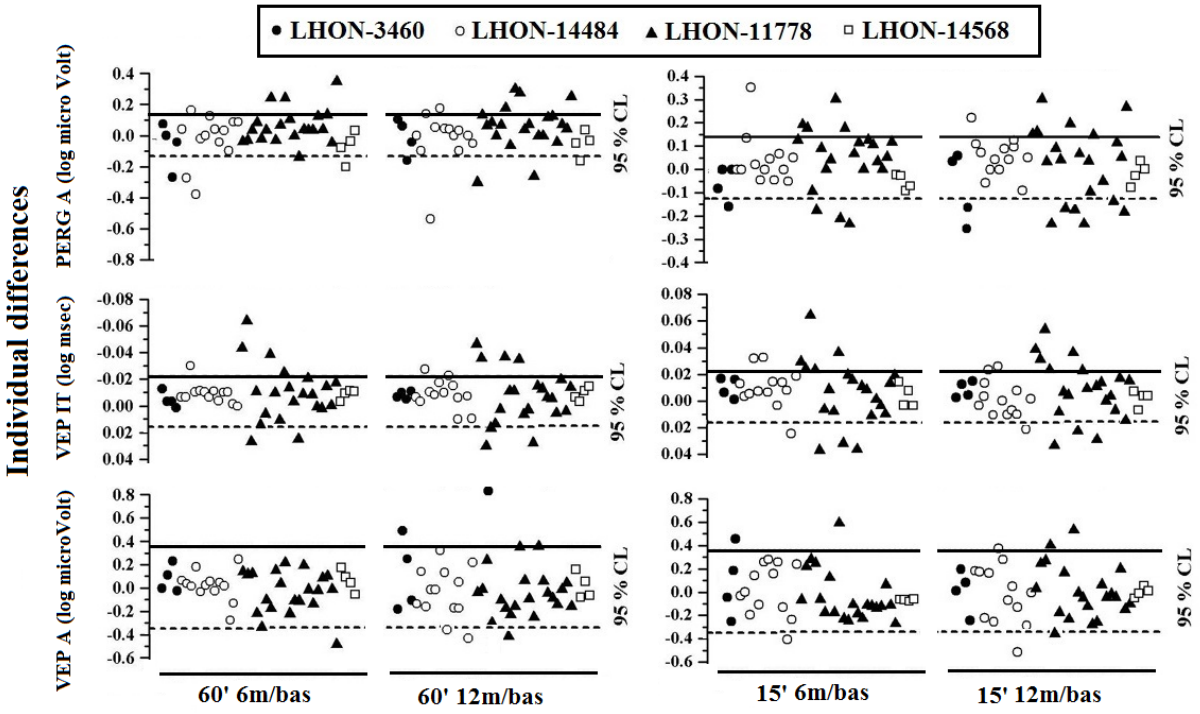
60' VEP N75-P100 Amplitude												
	Difference 6 months minus baseline						Difference 12 months minus baseline					
	unmodified		improvement		worsening		unmodified		improvement		worsening	
	N	%	N	%	N	%	N	%	N	%	N	%
LHON-3460 (N=4)	4	100.00	0	0.00	0	0.00	3	75.00	1	25.00	0	0.00
LHON-11484 (N=13)	13	100.00	0	0.00	0	0.00	11	84.62	0	0.00	2	15.38
LHON-11778 (N=21)	20	95.24	0	0.00	1	4.76	20	95.24	0	0.00	1	4.76
LHON-14568 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON Group (N=42)	41	97.62	0	0.00	1	2.38	38	90.48	1	2.38	3	7.14
15' PERG P50-N95 Amplitude												
	Difference 6 months minus baseline						Difference 12 months minus baseline					
	unmodified		Improvement		worsening		unmodified		Improvement		worsening	
	N	%	N	%	N	%	N	%	N	%	N	%
LHON-3460 (N=4)	3	75.00	0	0.00	1	25.00	2	50.00	0	0.00	2	50.00
LHON-11484 (N=13)	12	92.31	1	7.69	0	0.00	12	92.31	1	7.69	0	0.00
LHON-11778 (N=21)	14	66.67	4	19.05	3	14.29	9	42.86	6	28.57	6	28.57
LHON-14568 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON -Group (N=42)	33	78.57	5	11.90	4	9.52	27	64.29	7	16.67	8	19.05
15' VEP P100 Implicit Time												
	Difference 6 months minus baseline						Difference 12 months minus baseline					
	unmodified		Improvement		worsening		unmodified		Improvement		worsening	
	N	%	N	%	N	%	N	%	N	%	N	%
LHON-3460 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON-11484 (N=13)	10	76.92	2	15.38	1	7.69	10	76.92	2	15.38	1	7.69
LHON-11778 (N=21)	14	66.67	4	19.05	3	14.29	12	57.14	6	28.57	3	14.29
LHON-14568 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON Group (N=42)	32	76.19	6	14.29	4	9.52	30	71.43	8	19.05	4	9.52
15' VEP N75-P100 Amplitude												
	Difference 6 months minus baseline						Difference 12 months minus baseline					
	unmodified		improvement		worsening		unmodified		improvement		worsening	

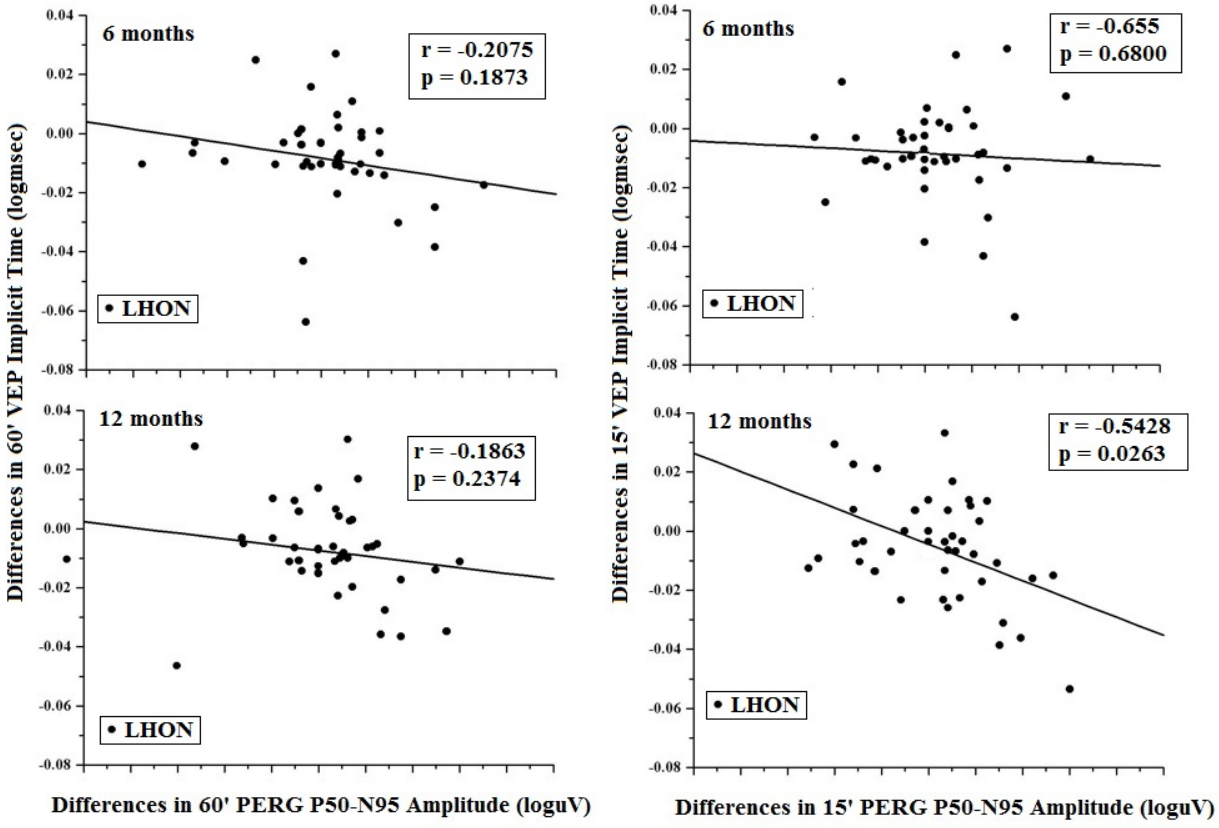
	N	%	N	%	N	%	N	%	N	%	N	%
LHON-3460 (N=4)	3	75.00	1	25.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON-11484 (N=13)	12	92.31	0	0.00	1	7.69	11	84.62	1	7.69	1	7.69
LHON-11778 (N=21)	20	95.24	1	4.76	0	0.00	18	85.71	2	9.52	1	4.76
LHON-14568 (N=4)	4	100.00	0	0.00	0	0.00	4	100.00	0	0.00	0	0.00
LHON Group (N=42)	39	92.86	2	4.76	1	2.38	37	88.10	3	7.14	2	4.76

Table 3. Baseline, 6 and 12 months of follow-up in all patients with Leber's hereditary optic neuropathy (LHON Group, 42 eyes). Mean of absolute values of Pattern Electroretinogram (PERG) P50-N95 Amplitudes, Visual Evoked Potentials (VEP) P100 Implicit Times and N75-P100 Amplitudes. Abbreviations: ANOVA: One-way Analysis of Variance. SD: 1 standard deviation; 60': visual stimuli in which each checks subtended 60 minutes of visual arc; 15': visual stimuli in which each checks subtended 15 minutes of visual arc. A: Amplitude; IT= implicit time

	60' PERG P5-N95 A (microVolt)		**60' VEP P100 IT (msec)**		**60' VEP N75-P100 A (microVolt)**		**15' PERG P5-N95 A (microVolt)**		**60' VEP P100 IT (msec)**		**15' VEP N75-P100 A (microVolt)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Baseline	1.29	0.482	126.2	15.6	3.65	1.99	1.15	0.277	127.7	13.3	3.14	1.60
6 months	1.30	0.355	123.7	14.3	3.62	2.02	1.23	0.354	125.5	12.4	3.03	1.52
ANOVA vs baseline	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**
	0.022	0.881	0.621	0.432	0.005	0.944	1.360	0.246	0.604	0.439	0.101	0.750
12 months	1.34	0.462	124.0	14.8	3.39	1.73	1.23	0.406	125.9	11.8	3.17	1.50
ANOVA vs baseline	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**	**f: (1,83)**	**P=**
	0.231	0.632	0.466	0.496	0.415	0.521	1.07	0.303	0.398	0.530	0.007	0.931






6 months
r = -0.2075
p = 0.1873
• LHON
12 months
r = -0.1863
p = 0.2374
• LHON
Differences in 60' VEP Implicit Time (logmsec)
Differences in 60' PERG P50-N95 Amplitude (loguV)
6 months
r = -0.655
p = 0.6800
• LHON
12 months
r = -0.5428
p = 0.0263
• LHON
Differences in 15' VEP Implicit Time (logmsec)
Differences in 15' PERG P50-N95 Amplitude (loguV)

Precìs

Retinal ganglion cells and visual pathways' function is substantially unmodified during 12 months period of follow-up independently from the pathogenic mutation in patients affected by LHON in the "chronic phase".