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Leber's hereditary optic neuropathy (LHON) in an Apulian cohort of subjects

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Leber's hereditary optic neuropathy (LHON) is a maternally inherited disorder that causes severe loss of sight in young adults, and is typically associated to mitochondrial DNA (mtDNA) mutations. Heteroplasmy of primary LHON mutations, presence of 'ancillary' mtDNA mutations, and mtDNA copy number are probably correlated with the penetrance and the severity of the disease. In this study, we performed a mutational screening in an Apulian cohort of LHON patients and we found that 41 out of 54 subjects harbored the m.11778G>A mutation, and 13 harbored the m.3460G>A mutation. Whole mtDNA sequencing was performed in three affected subjects belonging to three unrelated m.11778G>A pedigrees to evaluate the putative synergistic role of additional mtDNA mutations in determining the phenotype. Our study suggests to include haplogroup T as a possible genetic background influencing LHON penetrance and to consider the increase of mtDNA copy number as a protective factor from vision loss regardless the hetero/homoplasmic *status* of LHON primary mutations.

Key words: *LHON; heteroplasmy; homoplasmy; Mitochondrial DNA mutation; mtDNA copy number*

Introduction

Leber hereditary optic neuropathy (LHON, MIM#535000) is the most frequent inherited mitochondrial disorder due to mitochondrial DNA (mtDNA) mutations, with a prevalence ranging between 0.2 to 0.4 cases per 100,000 in Europe (1-5). Typically, LHON arises in males (6) during young adulthood with painless loss of central vision in one eye followed by loss of vision in the second eye within a short time (7, 8). LHON is most frequently associated to one of three mtDNA point mutations affecting NADH-ubiquinone oxidoreductase (complex I; EC 1.6.5.3) subunits, *i.e.* m.3460G>A in MT-ND1; m.11778G>A in MT-ND4; m.14484T>C in MT-ND6 (9). Fifteen further mutations have been identified as pathogenic for LHON (http://www.mitomap.org/MITOMAP) with some of them affecting MT-ND subunits of complex I, and associating with phenotypes overlapping MELAS (10), Leigh syndrome (11) and deafness (12-14). Unlike the majority of mtDNA mutations which are heteroplasmic (a mixture of both mutant and normal molecules) in mitochondrial diseases, LHON mutations are frequently homoplasmic (only mutant mtDNA); nonetheless, heteroplasmic mutations, particularly the m.3460G>A, have been detected in about 14% of the families observed (15-19). Although LHON has been the first disease to be identified as caused by mutations in the mtDNA, there are several puzzling features of LHON, *i.e.* the incomplete penetrance. All the matrilineal members of a LHON pedigree harbor mtDNA mutations, but only some individuals develop blindness implying that the primary mutation is a necessary but not sufficient condition to develop optic neuropathy. Among the genetic factors affecting penetrance, the homo/heteroplasmic condition is one of the pathological features which still needs to be completely elucidated (20). It was suggested that a contributing factor to the complexity of LHON is determined by additional mtDNA mutations, defined as 'secondary', that may well act in synergy with the primary ones – so far several nucleotide variants have been reported as such (21-25) – though the significance of such variants still remains controversial. Furthermore, as mtDNA is a multicopy genome, it has been proposed that the fine-tuning of mtDNA 'copy number' (20-26) may respond to an alteration of the bioenergetics request (27). Other genetic

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risk factors as well as environmental triggers – e.g. smoking – have been reported as significantly associated with increased risk of visual loss (28, 29).

Herein, we present molecular and genetic data of a cohort of LHON patients collected from the Apulia Region and we propose some 'ancillary' mtDNA mutations and mtDNA copy number as putative factors that may significantly affect LHON penetrance.

Materials and methods

Ophthalmologic examination and sample collection

A total of 54 subjects were enrolled in the study: 46 subjects were from the Ophthalmology Clinic, Policlinico Hospital of Bari, and 8 subjects were from the I.R.C.C.S. Casa Sollievo Della Sofferenza Hospital, San Giovanni Rotondo, Italy. Prior written and informed consent was obtained from each subject according to Institutional Guidelines. Among 54 subjects, 31 had already been partially analyzed in previous studies (20, 26). The control group consisted of 90 unrelated Italian subjects. Slit-lamp biomicroscopy dilated stereoscopic examination of the optic nerve head and *fundus*, visual field (VF) test (when possible), optical coherence tomography (OCT) (when possible) and fluorescein angiography were performed. The peripapillary RNFL thickness was measured using a spectral-domain Cirrus HD-OCT. The results from the VF tests were considered reliable if the fixation losses were less than 20% and false positive and false negative rates were less than 15%.

Mitochondrial genetics and statistical analysis

Total genomic DNA was extracted by standard methods from peripheral blood of the patients and their relatives with suspicion of LHON and from healthy control subjects. To detect the m.3460G>A, m.11778G>A and m.14484T>C mutations, convenient fragments were amplified by PCR performing a final last cycle of super-extension for 5 min at 72°C, to minimize the possible formation of heteroduplexes between mutant and wild-type strands. The presence of mutations was detected by PCR-RFLP (30) in all the subjects and, when present, confirmed by direct sequencing (ABI prism 310, Applied Biosystems). Entire mtDNA sequencing was performed in three affected individuals as described previously (31). All sequences were analyzed by comparison with mitochondrial reference sequence (*Reconstructed Sapiens Reference Sequence – RSRS* (32). Mitochondrial haplogroups were defined by the web-based bioinformatic platform Mitochondrial Disease Sequence Data Resource (https://mseqdr.org/). Relative quantification of mtDNA copy number was performed (33). All the data were analyzed by GraphPad Prism and Medcalc (MedCalc Statistical Software version 17.5.5 (MedCalc Software bvba, Ostend, Belgium) applying the chi-square test or the Fisher's exact test as appropriate to compare percentages of independent groups. To compare quantitative variables ANOVA test in conjunction with Bonferroni test was used and description was done by means and standard deviation if data approach Gaussian distribution. If data were not Gaussian distributed description was done by median and interquartile range, Kruskal-Wallis non parametric analysis of variance were applied to compare groups and non-parametric test according to Conover was used for post-hoc comparisons. Statistical significance was set at $P < 0.05$.

Results

Population and clinical features

The cohort consisted of 54 subjects including 42 subjects belonging to 12 families, and 12 unrelated subjects. Male: female ratio was overall 28:26, 19: 8 for patients and 9: 18 for unaffected subjects. We counted 28 LHON affected subjects, representing the 52% of the entire cohort, in which the molecular genetic diagnosis was positive for one of the primary mutations (Table 1). All the clinical findings, sex, age and age at onset, molecular genetics test, and therapy as Idebenone administration, risk factors and recovery of vision for all the subjects, when available, are reported in Table 1. Among the 54 subjects tested, 6 (II-1 FAM-A1; II-1 FAM-A8; I-1 FAM-A14; I-1 FAM-A14; III-1 FAM-B2; I-1 FAM-B3) were examined at the acute phase and the diagnosis of LHON was clinically based on the unilateral and severe visual decline followed by a declining vision in the contralateral eye within a few weeks. Alteration of the visual field for the presence of a centrocecal scotoma, an ophthalmoscopic appearance of the *fundus* with the presence of an edematous, hyperemic optic nerve head, vascular tortuosity and telangiectasia were examined. Fluorescein angiography was performed in all affected subjects, and highlighted vascular telangiectasia in those examined during the acute phase without phenomena of leakage or staining, whereas it showed non-specific alterations in those observed during the atrophic stage. Interestingly, three m.11778G>A patients and one m.3460G>A patient experienced visual recovery: one (II-1 FAM-A2) homoplasmic and one (II-5 FAM-A4) heteroplasmic patient without Idebenone treatment, and two (I-1 FAM-A14; I-1 FAM-B3) homoplasmic patients, including an m.3460G>A, out of the 13 Idebenone treated-affected subjects. In all the cases, the diagnosis was confirmed by mtDNA genetic analysis.

Since LHON patients were characterized by a sudden and devastating vision loss, the asymptomatic carriers, who had normal vision, were considered unaffected by the disease. Hereafter, we will refer to the subjects as *Carriers*, *Affected* and C*ontrols*. The control group consisted of 90 (male: female *ratio* 47:43) unrelated subjects who did not show any sign of optic neuropathy. The mean age resulted significantly different among LHON subjects, aged 45.3 ys ± 15.9, Carriers aged 47.8 ± 20.5 and *Controls* aged 37.9 ys ± 11.9 (F = 5.675; p = 0.004).

Genetic analysis of mtDNA

If we consider a total of 54 LHON mutation-positive subjects, they represent an overall observed prevalence of 1: 75,503 in the Apulia population with sex proportion of 29 males (1: 68,250 male) *versus* 25 females (1: 83,916 female) (December 31, 2016). On the basis of clinical features and genetic mitochondrial analysis, we diagnosed 28 subjects as *Affected* and 26 as *Carriers*. Among the 54 subjects, 76% harbored the m.11778A mutation and 24% carried the m.3460G>A mutation. Moreover, the 95% of subjects who harbored m.11778G>A were homoplasmic (51% *Affected*; 49% *Carriers*) and 5% were heteroplasmic (all *Affected*); whereas among the subjects who harbored m.3460G>A, 70% were homoplasmic (56% *Affected*; 44% *Carriers*) and 30% were heteroplasmic (25% *Affected*; 75% *Carriers*). The difference of frequency of homoplasmic subjects between m.11778G>A and m.3460G>A resulted statistically significant at Fisher exact test (p=0.0248). Among the heteroplasmic *Affected*, I-1 FAM-A5 and II-5 FAM-A4 (*Family11*) were both heteroplasmic for m.11778G>A with mutant load estimated to be ~75% (34) and ~60%, respectively; I-1 FAM-B4 was heteroplasmic for m.3460G>A with an estimated load of ~15%. Among the heteroplasmic *Carriers,* I-2, II-2, III-4 all belonging to FAM-B2 harbored the m.3460G>A with a mutant load estimated as ~30%, ~40%, and ~40%, respectively. None of them carried the m.14484T>C primary mutation. Among all 54 LHON subjects, 33 (Table 1) were reported in our previous study (20, 26) and the relatives of FAM-B2 (Table 1) will be described in the near future (Manuscript in preparation).

The penetrance rate of both LHON mutations in our cohort was: 52% (28/54), *i.e.* 71% (20/28) in males and 29% (8/28) in females. Among the 41 subjects (22 males; 19 females) who harbored the m.11778G>A mutation, 24 (16 males; 8 females) developed typical optic neuropathy thus showing 59% phenotype penetrance. In the remaining 13 subjects (7 males; 6 females) who harbored m.3460G>A mutation, 6 (4 males; 2 female) were *Affected* thus showing 46% phenotype penetrance. The penetrance rate did not result significantly different (chi-square = 1.375; p = 0.2409), but there was a difference of percentage of affected between males and females respectively 32% (8/25) vs 68.97% (20/29) that resulted statistically significant (chi-square = 7.348, p = 0.0067).

We performed Sanger sequencing of the entire mtDNA genome of three *Affected* carrying homoplasmic m.11778G>A mutation: II-1 FAM-A1; II-1 FAM-A2; I-1 FAM-A3. The criterion of selection of these three patients was based on the fact that they showed a quite peculiar manifestation. II-1 FAM-A1 reported a history of alcohol, tobacco, and drug abuse and he had been diagnosed as psychotic following psychiatric examination. II-1 FAM-A2 had reported visual recovery without having been treated with Idebenone; I-1 FAM-A3 had experienced an early onset of LHON with an acute and painless loss of central vision already at 4 ys of age.

All the mtDNA nucleotide variants identified in the three patients were analyzed by MtoolBox which performs prioritization taking into account the pathogenicity of each mutated allele with different algorithms, and the nucleotide variability of each variant site (34). For all three patients the m.11778G>A mutation was prioritized with a high score of pathogenicity (Supplemental Table 1). II-1 FAM-A1 showed 56 additional variants of which 45 mutational events help to define the sample haplogroup (K1a). The prioritization process recognized 7 variants annotated in Mitomap and they were predicted as not having a deleterious effect (Supplemental Table 1A). II-1 FAM-A2 showed 61 variants, of which 38 contributed to defining the sample haplogroup (T2e2a) and 8 further variants were prioritized (Supplemental Table 1B). We focused on two out of the eight variants: m.4136A>G/*MT-ND1* (p.Y277C) and m.9139G>A/MT-ATPase6 were predicted to have a deleterious effect (Supplemental Table 1B) and already reported as associated to LHON disease (http://www.mitomap.org/MITOMAP). We noticed that the patient carried two additional mutations, m.4216T>C/*MT-ND1* (p.Y304H) and m.4917A>G/*MT-ND1* (p.N150D), previously defined as 'secondary' LHON mutations (35), that were not prioritized because they are indeed polymorphic variants and also T2e2a haplogroup markers (http://www.phylotree.org/). I-1 FAM-A3 showed 78 variants, of which 64 contributed to defining the sample haplogroup (T2e2a) and 9 variants were further prioritized (Supplemental Table 1C). Among the nine mutations, the m.4136A>G/*MT-ND1* and m.9139G>A/*MT-ATPase6* mutations are the same deleterious variants also identified in the above mentioned II-1 FAM-A2. In the I-1 FAM-A3 patient we noticed the presence of m.4216T>C and m.4917A>G mutations which define the same haplogroup T2e2a. Interestingly, the same MT-ND1 variants defining haplogroup T2e2a had been identified also in I-1 FAM-A5 (36). None of the *Affected* fitted to haplogroup J previously suggested as increasing the penetrance of the m.11778 LHON mutation (37).

Table 1. Clinical and genetic findings in subjects carrying the primary LHON mutations. Ophthalmological findings, sex, age, possible exposure to environmental triggers (i.e. alcohol, smoking and illicit drugs) and mtDNA LHON mutations, percentage of heteroplasmy and copy number are indicated. M, male; F, female; RE, right eye; LE, left eye; LP, light perception; N, no; Y, yes; n.a., not available; HOM, Homplosmic; HET, Heteroplasmic. Number in brackets near Subject ID indicates the reference of papers where the mtDNA copy number was previously reported.

Subject ID	Family ID	Relation	Sex	*Age (ys)*	Visual condition at the first examination		Currently visual condition		Level of Visual Loss	Recovery	Idebenone treatment	*Age at test (ys)*	*Age at onset (ys)*	Clinical Features	Risk factors	Primary mtDNA mutation	mtDNA Mutation type (%mut)	Copy number (mtDNA/nDNA)
					RE	LE	RE	LE										
I-1 (26)	FAM-A1	Relative	F	62	20/20	20/20	20/20	20/20	Normal vision	-	-	56	-	Normal vision	-	m.11778G>A	HOM	431 ± 49
II-1 (26)		Proband	M	38	20/200	20/40	LP	LP	Profound	N	Y	32	32	Bilateral optic atrophy; RNFL: LE 80.17μm; psychotic	Illicit drug and tobacco abuse	m.11778G>A	HOM	196 ± 16
II-2 (26)		Relative	M	36	20/20	20/20	20/20	20/20	Normal vision	-	-	30	-	Normal vision RNFL: RE 87μm; LE 88μm	-	m.11778G>A	HOM	256 ± 47
I-1 (26)	FAM-A2	Relative	F	61	20/20	20/20	20/20	20/20	Normal vision	-	-	58	-	Normal vision	-	m.11778G>A	HOM	685 ± 271
II-1 (26)		Proband	M	35	20/200	20/200	20/200	20/200	Moderate	Y	N	24	n.a.	Bilateral optic atrophy	-	m.11778G>A	HOM	348 ± 35
I-1	FAM-A3	Proband	F	66	na	na	20/40	LP	Moderate	N	Y	54	4	RE optic subatrophy LE optic atrophy	-	m.11778G>A	HOM	177 ± 46
II-1 (26)		Relative	M	30	20/20	20/20	20/20	20/20	Normal vision	n.a.	n.a.	29	n.a.	Normal vision	-	m.11778G>A	HOM	316 ± 62
II-5 (20)	FAM-A4	Proband	M	51	n.a.	n.a.	20/20	20/20	Normal vision	Y	N	47	n.a.	Bilateral optic subatrophy	Alcohol	m.11778G>A	HET (60%)	149 ± 40
III-2 (20)		Proband	M	n.a.	n.a.	n.a.	LP	LP	Profound	n.a.	N	n.a.	n.a.	Bilateral optic subatrophy	-	m.11778G>A	HOM	n.a.
I-1 (20)	FAM-A5	Proband	M	67	20/300	20/300	LP	LP	Profound	N	Y	60	56	Bilateral optic subatrophy	-	m.11778G>A	HET (70%)	n.a.
I-1 (26)	FAM-A6	Proband	F	51	20/25	20/32	20/25	20/32	Mild	N	Y	39	30	Bilateral optic subatrophy with hemeralopia; psychiatric signs	-	m.11778G>A	HOM	311 ± 30
II-1 (26)		Proband	F	25	20/40	20/32	20/40	20/40	Mild	N	Y	13	11	RE optic atrophy, LE optic subatrophy	-	m.11778G>A	HOM	366 ± 43
I-1 (26)	FAM-A7	Proband	M	66	LP	LP	LP	LP	Profound	n.a.	N	65	28	Bilateral optic atrophy	-	m.11778G>A	HOM	183 ± 35
I-2 (26)		Proband	M	45	LP	20/1000	LP	20/800	Severe	n.a.	N	44	25	Bilateral optic atrophy	-	m.11778G>A	HOM	429 ± 75
I-1	FAM-A8	Proband	F	52	n.a.	n.a.	20/40	20/200	Moderate	N	N		n.a.	Bilateral optic subatrophy	-	m.11778G>A	HOM	486 ± 56
II-1		Proband	M	21	20/200	20/200	LP	20/800	Severe	N	Y	20	20	Bilateral optic atrophy; RNFL: RE 95.12μm; LE 98.34μm	-	m.11778G>A	HOM	190 ± 33
II-2		Relative	F	15	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	221 ± 48
I-1	FAM-A9	Relative	F	61	20/20	20/20	20/20	20/20	Normal vision	-	-	-	n.a.	Normal vision	-	m.11778G>A	HOM	510 ± 77
II-1		Proband	M	45	20/200	20/40	LP	20/200	Severe	n.a.	n.a.	-	n.a.	Bilateral optic atrophy	-	m.11778G>A	HOM	327 ± 45
II-2		Relative	F	n.a.	20/20	20/20	20/20	20/20	Normal vision	n.a.	-	-	n.a.	Normal vision	-	m.11778G>A	HOM	430 ± 94

Subject ID	Family ID	Relation	Sex	*Age (ys)*	Visual condition at the first examination		Currently visual condition		Level of Visual Loss	Recovery	Idebenone treatment	*Age at test (ys)*	*Age at onset (ys)*	Clinical Features	Risk factors	Primary mtDNA mutation	mtDNA Mutation type (%mut)	Copy number (mtDNA/nDNA)
					RE	LE	RE	LE										
I-2	FAM-A10	Relative	F	89	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	453 ± 52
II-2		Relative	F	59	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	562 ± 68
III-1		Proband	M	34	n.a	n.a.	LP	LP	Mild	n.a.	n.a.	-	n.a	Bilateral optic subatrophy	-	m.11778G>A	HOM	334 ± 29
III-2		Relative	M	28	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	484 ± 85
I-1	FAM-A11	Relative	M	74	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	165 ± 47
I-2		Relative	F	68	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	161 ± 39
I-3		Relative	F	66	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	167 ± 25
I-4		Relative	F	58	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	241 ± 73
I-5		Relative	F	55	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	182 ± 59
II-1		Relative	M	34	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	380 ± 18
II-2		Proband	M	38	n.a.	n.a.	LP	LP	Profound	n.a.	Y	-	n.a.	Bilateral optic atrophy	-	m.11778G>A	HOM	201 ± 45
II-3		Relative	F	38	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	263 ± 55
I-1 (26)	FAM-A12	Proband	F	55	20/40	20/25	20/40	20/25	Mild	N	N	51	26	Bilateral optic subatrophy with hemeralopia	-	m.11778G>A	HOM	246 ± 31
I-1 (26)	FAM-A13	Proband	F	42	20/25	20/32	20/25	20/32	Mild	N	Y	40	30	Bilateral optic subatrophy with hemeralopia	-	m.11778G>A	HOM	187 ± 26
I-1 (26)	FAM-A14	Proband	M	41	20/80	20/160	20/200	20/40	Mild	Y	Y	28	28	Bilateral optic subatrophy	Tobacco and alcohol abuse	m.11778G>A	HOM	739 ± 121
I-1	FAM-A15	Proband	M	34	n.a.	n.a.	n.a.	n.a.	Severe	n.a.	n.a.	21	n.a.	Bilateral optic atrophy	-	m.11778G>A	HOM	n.a.
I-1 (26)	FAM-A16	Proband	M	41	20/20	20/200	20/200	20/800	Severe	Y	Y	33	33	Bilateral optic Subatrophy	-	m.11778G>A	HOM	136 ± 36
I-1 (26)	FAM-A17	Proband	M	26	20/20	20/200	LP.	LP.	Profound	N	Y	19	19	Bilateral optic atrophy	-	m.11778G>A	HOM	313 ± 44
I-1 (26)	FAM-A18	Proband	M	53	20/800	20/800	20/800	20/800	Profound	N	N	50	20	Bilateral optic atrophy	-	m.11778G>A	HOM	268 ± 108
I-1 (26)	FAM-A19	Unrelated	M	n.a.	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	582 ± 76
I-1 (26)	FAM-A20	Unrelated	F	n.a.	20/20	20/20	20/20	20/20	Normal vision	-	-	-	-	Normal vision	-	m.11778G>A	HOM	303 ± 78
I-1 (26)	FAM-B1	Proband	F	61	20/32	20/28	20/32	20/28	Mild	N	N	54	40	Bilateral optic subatrophy	-	m.3460G>A	HOM	279 ± 36
I-2 (26)		Proband	F	70	20/40	20/32	20/40	20/32	Mild	N	Y	63	35	Bilateral optic subatrophy	-	m.3460G>A	HOM	259 ± 27
II-1 (26)		Proband	M	49	20/40	20/40	20/40	20/40	Mild	N	Y	42	27	Bilateral optic subatrophy, severe visual impairment with hemeralopia	-	m.3460G>A	HOM	305 ± 38

Subject ID	**Family ID**	**Relation**	**Sex**	***Age* *(ys)***	**Visual condition at the first examination**		**Currently visual condition**		**Level of Visual Loss**	**Recovery**	**Idebenone treatment**	***Age at test* *(ys)***	***Age at onset* *(ys)***	**Clinical Features**	**Risk factors**	**Primary mtDNA mutation**	**mtDNA Mutation type (%mut)**	**Copy number (mtDNA/nDNA)**
					RE	**LE**	**RE**	**LE**										
I-2	FAM-B2	Relative	F	71	20/20	20/20	20/20	20/20	Normal vision	-	-	70	-	Normal vision	-	m.3460G>A	HET (35%)	206 ± 15
II-1 (26)		Relative	F	47	20/20	20/20	20/20	20/20	Normal vision	-	-	39	-	Mild mental retardation	-	m.3460G>A	HOM	636 ± 74
II-2		Relative	F	46	20/20	20/20	20/20	20/20	Normal vision	-	-	38	-	Borderline mental functioning	-	m.3460G>A	HET (40%)	713 ± 144
II-3 (26)		Relative	M	44	20/20	20/20	20/20	20/20	Normal vision	-	-	43	-	Normal vision	Tobacco abuse	m.3460G>A	HOM	213 ± 9
III-1 (26)		Proband	M	25	20/200	20/200	20/800	L.P.	Profound	N	Y	17	n.a.	Bilateral optic atrophy epilepsies, mild mental retardation	-	m.3460G>A	HOM	240 ± 86
III-2 (26)		Relative	M	21	20/20	20/20	20/20	20/20	Normal vision	-	-	17	-	Hyperemic optic disk, borderline mental functioning	-	m.3460G>A	HOM	604 ± 149
III-3 (26)		Relative	F	18	20/25	20/25	20/25	20/25	Normal vision	-	-	12	-	Borderline mental functioning	-	m.3460G>A	HOM	667 ± 72
III-4		Relative	M	18	20/20	20/20	20/20	20/20	Normal vision	-	-	10	-	Borderline mental functioning	Tobacco abuse	m.3460G>A	HET (40%)	739 ± 184
I-1 (26)	FAM-B3	Proband	M	16	20/80	20/200	20/40	20/80	Moderate	Y	Y	13	13	Bilateral optic subatrophy; several maternal relatives with optic atrophy	-	m.3460G>A	HOM	140 ± 38
I-1 (20)	FAM-B4	Proband	M	77	20/32	20/25	20/32	20/25	Mild	N	Y	69	30	Bilateral optic subatrophy with hemeralopia	Illicit drug Alcohol and tobacco abuse	m.3460G>A	HET (15%)	285 ± 24

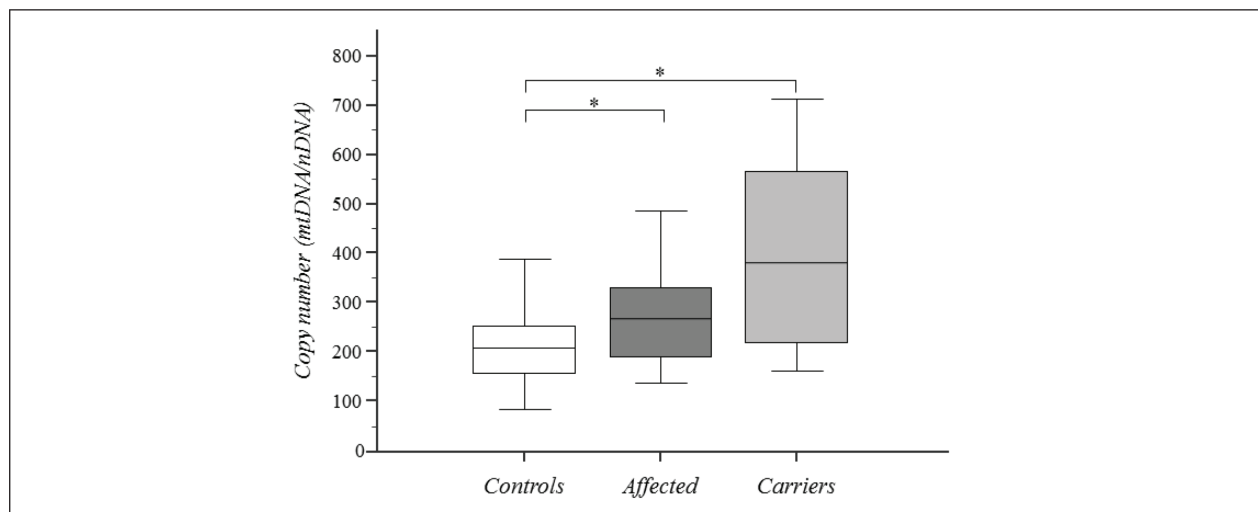

Figure 1. Analysis of mtDNA content in LHON subjects. Box-plot of mtDNA copy number (mtDNA/nDNA) by Affected, Carriers and Controls. Experiments were performed in triplicates for all samples; for thirty-one LHON mutation carriers mtDNA content was evaluated in previous works (20, 26) and herein included. Asterisks indicate statistical significance (p-value<0.05) at post-hoc comparisons.

Analysis of mtDNA copy number in LHON subjects

The evaluation of mtDNA copy numbers was performed independently from the type of primary mutations in 25 *Affected* (23 homoplasmic; 2 heteroplasmic) and 26 *Carriers* (24 homoplasmic; 3 heteroplasmic) (Table 1). mtDNA copy number of thirty-one LHON subjects was evaluated in previous works (20, 26). Mitochondrial copy number values were measured either using MT-*ND1* or MT-*ND4* as target genes. The analysis of the MT-*ND4* region was indeed implemented after assessing the presence of an ancient polymorphism, m.3480A>G/*ND1*, just within the annealing region of *ND1* primers in I-1, II-1, II-2 (FAM-A1), I-2, II-2, III-1, III-2 (FAM-A10) I-1 FAM-B4 LHON subjects, and unexpectedly led to very low values of copy number. Three *Affected* (I-1 FAM-A5, III-2 FAM-A4 and I-1 FAM-A15) were diagnosed, but we could not perform the quantitation of the mtDNA content due to the scarcity of DNA samples. The values of the mtDNA copy number showed a statistically significant difference (KW = 24.828, p = 0.000004) and the peak of mtDNA content shifted progressively towards higher values from *Controls* (median 207; interquartile range 155.5-251.25) to *Affected* (median 268; interquartile range 189.25-328.75) to *Carriers* (median 380; interquartile range 219-567). The post-hoc analysis showed a statistically significant difference between *Controls versus Affected* and *Controls versus Carriers* (Fig. 1). Among the *Carriers*, we noticed that the relatives belonging to *Family11* showed a quite low mtDNA content below the mean of *Carriers*. Indeed, the age of the *Family11* members is relatively higher than the age of the other *Carriers* and we reasoned that this may explain the lower amount of mtDNA according to the decrease of mtDNA copy number with aging (38); additionally, the age of the oldest women of the family (FAM-A11) was over 60ys which is compatible with a low estrogens condition thus confirming *in vivo* the loss of the protective role by estrogens in activating mitochondrial biogenesis and mtDNA content in LHON (39). On the other hand, the comparison between the mtDNA copy number of *Affected* subjects, whose blood samples were obtained in the acute phase of the disease (II-1 FAM-A8, I-1 FAM-A14, I-1 FAM-A17, III-1 FAM B2), and mtDNA copy number of those subjects already affected by optic atrophy in the chronic stage (II-1 FAM-A2, I-1 FAM-A7, I-2 FAM-A7, I-1 FAM-A18), did not reveal any difference.

Discussion

In the present study, we report on the genetic and molecular characterization of LHON subjects born and living in Apulia, Southern Italy. From our data, the prevalence of subjects carrying the LHON primary mutations can be estimated to be approximately 1 case in 75,503 thus, if compared to the prevalence in the North of England (1/31,000), the Netherlands (1/39,000) (2) and Finland (1/50,000) (19,40), it may indicate that in our Region the disease is very low or remains underestimated. Probably, the reason is that some patients may not be adequately diagnosed or are misdiagnosed or are diagnosed outside the Region. Our mutational screening disclosed two out

of the three most common primary LHON mutations, i.e. m.11778G>A and m.3460G>A, either homoplasmic or heteroplasmic. Interestingly, we found that heteroplasmy is present for both primary mutations but it is more frequent for the m.3460G>A than for m.11778G>A mutation, according to a previous estimation in different countries (2, 41). Among the mitochondrial diseases, LHON is notable for the incomplete penetrance since not all the *Carriers* will develop loss of sight and it is expected that additional genetic and environmental factors may play a role in LHON penetrance (42). As in the majority of mitochondrial disorders, it has been suggested that a certain amount of wild-type mtDNA can compensate for the mutant mtDNA in a cell of a LHON individual (18). In our cohort we found no difference in the LHON manifestations among the *Affected*, either homoplasmic or heteroplasmic, since there was no clear-cut segregation with either more severe or benign clinical course of the disease, respectively. This finding is supported by a study performed specifically in a LHON heteroplasmic population (20).

With the aim of better investigating LHON penetrance we performed the whole mtDNA sequencing of three *Affected* who had a different course of disease: the first one was a man (II-1 FAM-A1) who was diagnosed as psychotic and, although after the disease onset he had started Idebenone treatment, he did not experience any visual recovery; the second one (II-1 FAM-A2) was a man who experienced visual recovery without Idebenone treatment; the third one (I-1 FAM-A3) was a woman who had an early acute and painless loss of central vision at four years of age. Unexpectedly, the latter two cases who manifested a less severe and a more severe LHON phenotype respectively, shared the co-occurrence of two mutations (m.4136A>G, m.9139G>A), thus suggesting that both mutations do not have an unequivocal effect in worsening the LHON manifestation, although both mutations had been previously reported to play a synergistic role when occurring with the LHON primary mutation (43). Furthermore, we identified the co-occurrence of the m.4136A>G with two haplogroup markers, m.4216T>C and m.4971A>G, in two out of three sequenced patients; the same genotype with the three variants had been previously reported in other three unrelated patients harboring the m.11778G>A mutation, coming from the Apulia Region, described by La Morgia et al. and Torroni et al. (43, 44). Interestingly, the three Apulian patients previously described (43, 44), similarly to our patients, belonged to haplogroup T2. The shared Apulian origin of these families supports the hypothesis that the m.11778G>A together with m.4136A>G, 4216T>C and 4917A>G are indeed associated and acquired by descent from a common maternal ancestor. Several studies have identified the haplogroup J as a risk genetic background for patients with m.11778G>A and m.14484T>C (44-46) and haplogroup K for patients with m.3460G>A; conversely, haplogroup H seemed to have a protective role in patients with m.11778G>A (47). On the basis of the data herein discussed, we suggest that also haplogroup T may play a role in LHON penetrance; this might not be unexpected since haplogroup T, like haplogroup J, belongs to the same macro-haplogroup JT (http://www.phylotree.org/tree/index.htm). Regarding the II-1 FAM-A1 case, the patient did not show any significant or prioritized mutation implying that his extraocular signs (i.e. psychotic trait) might be probably due more to his drug and alcohol addiction.

According to recent evidences (20, 26, 27) supporting the concept that the increase in mitochondrial mass differentiates the LHON unaffected from the affected, we measured mtDNA content in either heteroplasmic or homoplasmic LHON subjects. With the aim of evaluating the mtDNA copy number of the overall cohort of LHON subjects, we also included thirty-one subjects already reported (20, 26), and we found an increase in the number of mtDNA molecules in peripheral blood of *Carriers versus Affected* despite the homo- or heteroplasmy of LHON mutations. We may claim that such increase can be considered as a compensatory response to the decline in the respiratory function and a way to protect from vision loss. On the other hand, in agreement with previous studies (39, 48) we found that environmental triggers such as tobacco, alcohol, as well as low estrogen conditions and age itself are all contributing factors that affect mitochondrial biogenesis. We cannot rule out that among the *Carriers* those subjects showing low content of mtDNA might be considered at a high risk of developing the disease. But, of course, this should be monitored over time.

In conclusion, our study on Apulian LHON subjects, despite the small number, suggests the haplogroup T as a possible genetic background influencing LHON penetrance. Furthermore, mtDNA content may be considered as a protective factor from vision loss regardless the hetero/homoplasmic *status* of LHON primary mutations.

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Supplemental Table 1. List of mtDNA mutations prioritized by MToolBox in three LHON patients. All variants identified in each LHON subject were prioritized as potentially deleterious (score closer to 1 is more likely to be damaging) the mutations which do not contribute to the macro-haplogroup definition and, if non-synonymous, can be predicted as disease-associated by at least one of the pathogenicity prediction methods. A. II-1 FAM-A1; B. II-1 FAM-A2; C. I-1 FAM-A3.

A. II-1 FAM-A1

Variant Allele	Locus	Nt Variability	Codon Position	AA Change	AA Variability	Disease Score	Mitomap Associated Disease(s)	Somatic Mutations	dbSNP ID	Haplogroup
11179G	MT-ND4	0.00021	3	syn	1.0					K1a
15653T	MT-CYB	0.00149	1	M303L	0.0028	0.124				
309.CCT	MT-DLOOP	0.00196								
11778A	MT-ND4	0.00368	2	R340H	0.0605	0.853	LHON/Progressive Dystonia		rs199476112	
513.CA	MT-DLOOP	0.0681								
5046A	MT-ND2	0.079	1	V193I	0.21	0.127				
310C	MT-DLOOP	0.283						Normal buccal swab	rs66492218	
150T	MT-DLOOP	0.419					Longevity / Cervical Carcinoma / HPV infection risk	Elderly fibroblasts/leukocytes, lung, thyroid, prostate tumors	rs62581312	

B. II-1 FAM-A2

Variant Allele	Locus	Nt Variability	Codon Position	AA Change	AA Variability	Disease Score	Mitomap Associated Disease(s)	Somatic Mutations	dbSNP ID	Haplogroup
309.CT	MT-DLOOP	0.00196								T2e2a
11778A	MT-ND4	0.00368	2	R340H	0.0605	0.853	LHON/Progressive Dystonia		rs199476112	
9139A	MT-ATP6	0.00431	1	A205T	0.0051	0.893	LHON			
4136G	MT-ND1	0.00641	2	Y277C	0.0226	0.76	LHON		rs199476121	
6026A	MT-CO1	0.0479	3	syn	0.0				rs41474553	
16293G	MT-DLOOP	0.106						Glioblastoma		
310C	MT-DLOOP	0.283						Normal buccal swab	rs66492218	
150T	MT-DLOOP	0.419					Longevity / Cervical Carcinoma / HPV infection risk	Elderly fibroblasts/leukocytes, lung, thyroid, prostate tumors	rs62581312	

C. I-1 FAM-A3

Variant Allele	Locus	Nt Variability	Codon Position	AA Change	AA Variability	Disease Score	Mitomap Associated Disease(s)	Somatic Mutations	dbSNP ID	Haplogroup
11778A	MT-ND4	0.00277	2	R340H	0.0402	0.853	LHON / Progressive Dystonia		rs199476112	T2e2a
9139A	MT-ATP6	0.00376	1	A205T	0.0043	0.893	LHON			
309.CT	MT-DLOOP	0.0043								
4136G	MT-ND1	0.00639	2	Y277C	0.0232	0.76	LHON		rs199476121	
8222C	MT-CO2	0.0147	1	syn	0.0021					
16153A	MT-DLOOP	0.038							rs2853512	
6026A	MT-CO1	0.0718	3	syn	0.0				rs41474553	
16293G	MT-DLOOP	0.098						Glioblastoma		
310C	MT-DLOOP	0.215						Normal buccal swab		
150T	MT-DLOOP	0.414					Longevity / Cervical Carcinoma / HPV infection risk	Elderly fibroblasts/leukocytes, lung, thyroid, prostate tumors	rs62581312	