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A novel homozygous change of *CLCN2* (p.His590Pro) is associated with a subclinical form of LeuKoencePhalopathy with Ataxia (LKPAT).

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Conflict of interest: none to declare

ClC-2 is a plasma membrane chloride channel with widespread expression in the human body, including the brain. Its function is still being studied, although it is thought to have a role in ion and water homoeostasis in the brain. ClC-2 is part of a complex containing GlialCAM and MLC1. Both these genes are associated with autosomal recessive human leukodystrophies with intramyelinic edema. Biallelic mutations in CLCN2, encoding the ClC-2 channel, have been reported in patients with a rare form of LeuKoencePhalopathy with Ataxia (LKPAT; MIM # 615651). No peculiar neurological features have been reported for this disease, although slight visual impairment due to chorioretinopathy or optic atrophy, mild ataxia, learning disabilities and headaches are recurrent symptoms in patients. However, MRI shows a typical diagnostic pattern that consists of white matter signal abnormalities in the posterior limbs of the internal capsules, cerebral peduncles, pontine pyramidal tracts and in the middle cerebellar peduncles, associated with lower Apparent Diffusion Coefficient (ADC) values in most cases. Specific anomalies of Brainstem Auditory Evoked Potentials (BAEP) have also been described [1-3].

Here, we report on a 52 year-old Moroccan woman presenting with mild and asymptomatic bilateral optic atrophy detected at a routine ophthalmological examination for presbyopia. Best-corrected high-contrast visual acuity was 20/20 in both eyes. Anterior segment and intraocular pressures were normal, and pupillary reflexes were present. Upon fundus biomicroscopy, mild pallor and excavation of the optic disc were evident in both eyes. Moreover, in her left eye, myelinated retinal nerve fibers were present, while her right eye was normal (Figure 1). No alterations were evident upon examination with optical coherence tomography (Supplementary Figure 1) or fundus autofluorescence in either eye. A chronic simple glaucoma and retinal vessel pathology were excluded. Her familial and personal clinical history was unremarkable, apart from a moderate headache, and blood exams were normal. The neurological examination was normal except for bilateral mild optic disc pallor at

funduscopy (Figure 1). At the neurophysiological study, a normal latency and amplitude of the visual evoked potential (VEP) responses were documented, while BAEPs were characterized by a marked increase in the III-V interval, representative of ponto-mesencephalic conduction time, a normal latency of I wave, normal I-III interval values, and V wave amplitude (Supplementary Figure 2). MRI displayed an unusual pattern of symmetrical white matter anomalies suggestive of LKPAT (Figure 1A). Post contrast MRI did not demonstrate any enhancement.

Informed consent was obtained from the patient, and the Internal Review Board committee of the Department of Medical Sciences (University of Turin) approved this study. We analyzed all coding exons and flanking intron boundaries of CLCN2 (NM_004366) by PCR amplification of blood-extracted genomic DNA (Qiagen, Mannheim, Germany; conditions available upon request), followed by Sanger sequencing (Life Technologies, Carlsbad, CA, USA). We identified the homozygous c.1769A>C (p.His590Pro) missense variant in exon 16 (Figure 1B). This change was not reported in any genetic database (ExAC v.0.3.1, dbSNPs147), and was shown to affect a highly conserved residue (Figure 1C), predicted to be pathogenic based on bioinformatics analyses (SIFT, PolyPhen2, I-Mutant, Mutation taster, PHD-SNP, SNAP-F; Table 1). To ascertain the role of this mutation on the protein, we compared the subcellular localization of the wild type and mutated ClC-2 protein after transient transfection of VERO cells with the V5-His6-tagged ClC-2 expression plasmid [1]. We demonstrated that the p.His590Pro ClC-2 protein barely reaches the plasma membrane and accumulates within the endoplasmic reticulum, with a pattern similar to the positive control plasmid carrying the known pathogenic p.Ala500Val variant (Figure 1D) [1]. These *in vitro* findings corroborate a pathogenic role for the p.His590Pro missense change.

There have only been eight LKPAT patients described in the literature (Supplementary Table 2). Depienne et al. reported six unrelated cases with homozygous or compound heterozygous mutations in

the *CLCN2* gene [1]. A subclinical form of leukodystrophy associated with infertility was also reported in a man with a novel homozygous *CLCN2* mutation [2]. More recently, Hanagasi et al. described a 22 year-old woman with paroxysmal kinesigenic dyskinesia due to a homozygous *CLCN2* mutation [3]. Our case resembles other LKPAT cases, characterized by a very mild clinical phenotype despite a clear MRI abnormality involving the pyramidal tracts. This clinical/radiological discrepancy suggests that white matter anomalies could cause intramyelinic edema, which does not impair the axonal function. Conversely, the compromised chloride channel function may be harmful for the visual and auditory systems, as suggested by the optic atrophy and the BAEP anomalies found in our patient, as well as in the animal model of the disease [4]. The animal model displays a "pure" slowing conduction with preservation of the synchronous activation of potential generators. This pattern is distinct from those observed in the vast majority of genetic or acquired myelin pathologies in which slowing conduction and potential dispersion occur concomitantly. Moreover, myelin disorders are usually characterized by a prevalent slowing conduction in the I-III interval, both in murine models of myelination defects [5] and in human leukodystrophies.

However, a selective increase in the III-V interval is far more common in acquired disorders that determine a brainstem focal lesion.

In conclusion, we report on a novel case of subclinical leukodystrophy associated with *CLCN2* mutations. Our case confirms the extreme variable clinical presentation of LKPAT and emphasizes the importance of recognizing specific MRI patterns as a guidance to genetic tests in adult leukoencephalopathies.

Figure legends

Figure 1. MRI, molecular and *in vitro* analyses.

Panel A: MRI abnormalities. FLAIR (1-3) and DWI (4-6) images show hyperintensity of the pyramidal tracts in the posterior limbs of internal capsules (arrowheads in 1, 4), midbrain cerebral peduncles (arrowheads in 2, 5), and middle cerebellar peduncles and pons (light green and light blue arrowheads, respectively, in 3, 6). No restriction at the ADC map was present (7-9). **Panel B:** fundus biomicroscopy signs. Mild pallor and excavation of the optic disc in both eyes (L and R) and myelinated retinal nerve fibers in the left eye (L). **Panel C**: **molecular analysis**. The p.His590Pro change is shown. **Panel D: *in vitro* studies.** Subcellular localization of the ClC-2 wild type (WT), p.Ala500Val (with a known pathogenic change [1]) and p.His590Pro variant proteins is shown (green). A marker of the endoplasmic reticulum (anti-calreticulin, red) was used to verify co-localization by confocal microscopy. Note that wild type ClC-2 protein typically reaches the plasma membrane, whereas both the ClC-2(p.Ala500Val) and ClC-2(p.His590Pro) proteins are retained in the endoplasmic reticulum. A 10X inset is shown. Scale bar = 25 μM.

Acknowledgements

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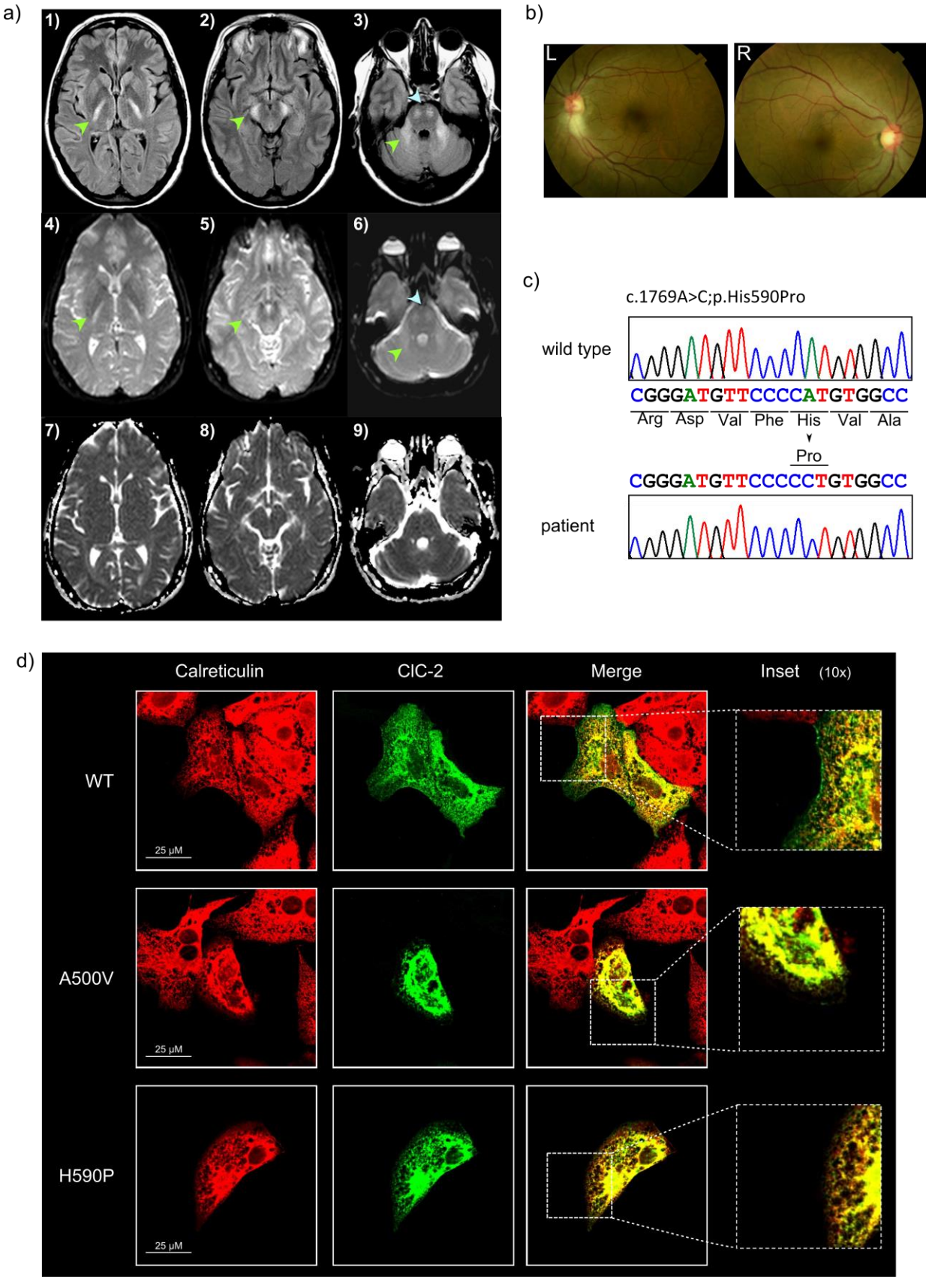
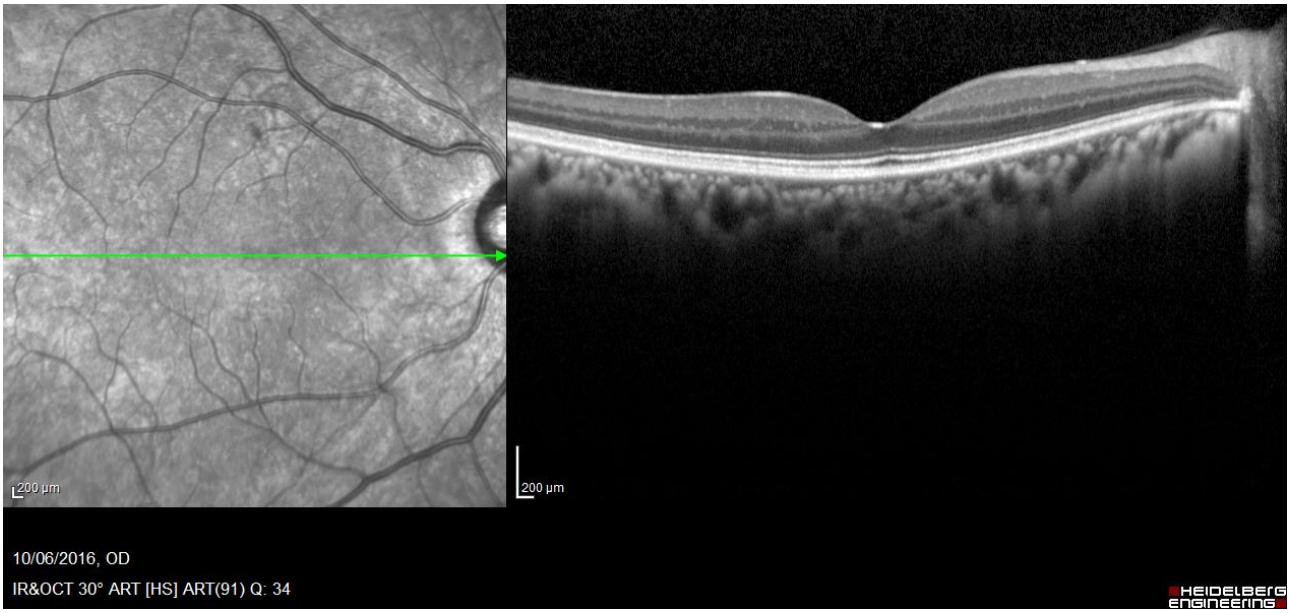
Figure 1


a)
1)
2)
3)
4)
5)
6)
7)
8)
9)
b)
L
R
c)
c.1769A>C;p.His590Pro
wild type
CGGGATGTTCCCCATGTGGCC
Arg Asp Val Phe His Val Ala
Pro
CGGGATGTTCCCCCTGTGGCC
patient
d)
Calreticulin
ClC-2
Merge
Inset (10x)
WT
A500V
H590P
25 µM
25 µM
25 µM

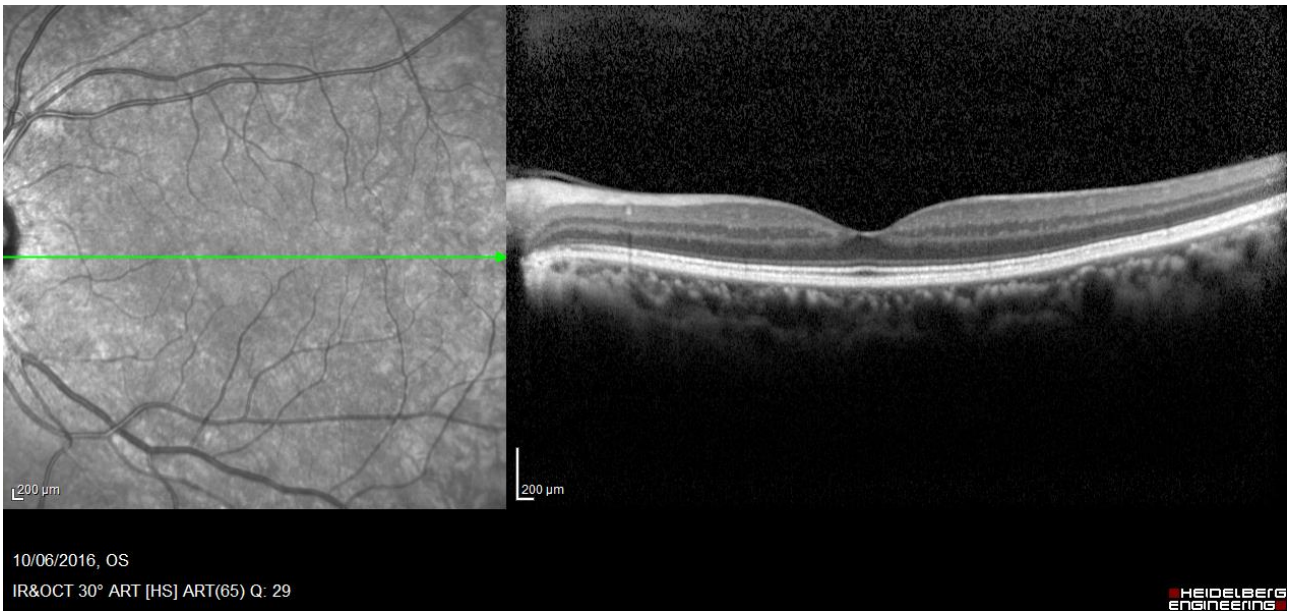
Supplements

Supplementary Figure 1: optical coherence tomography.

Right Eye: no alteration

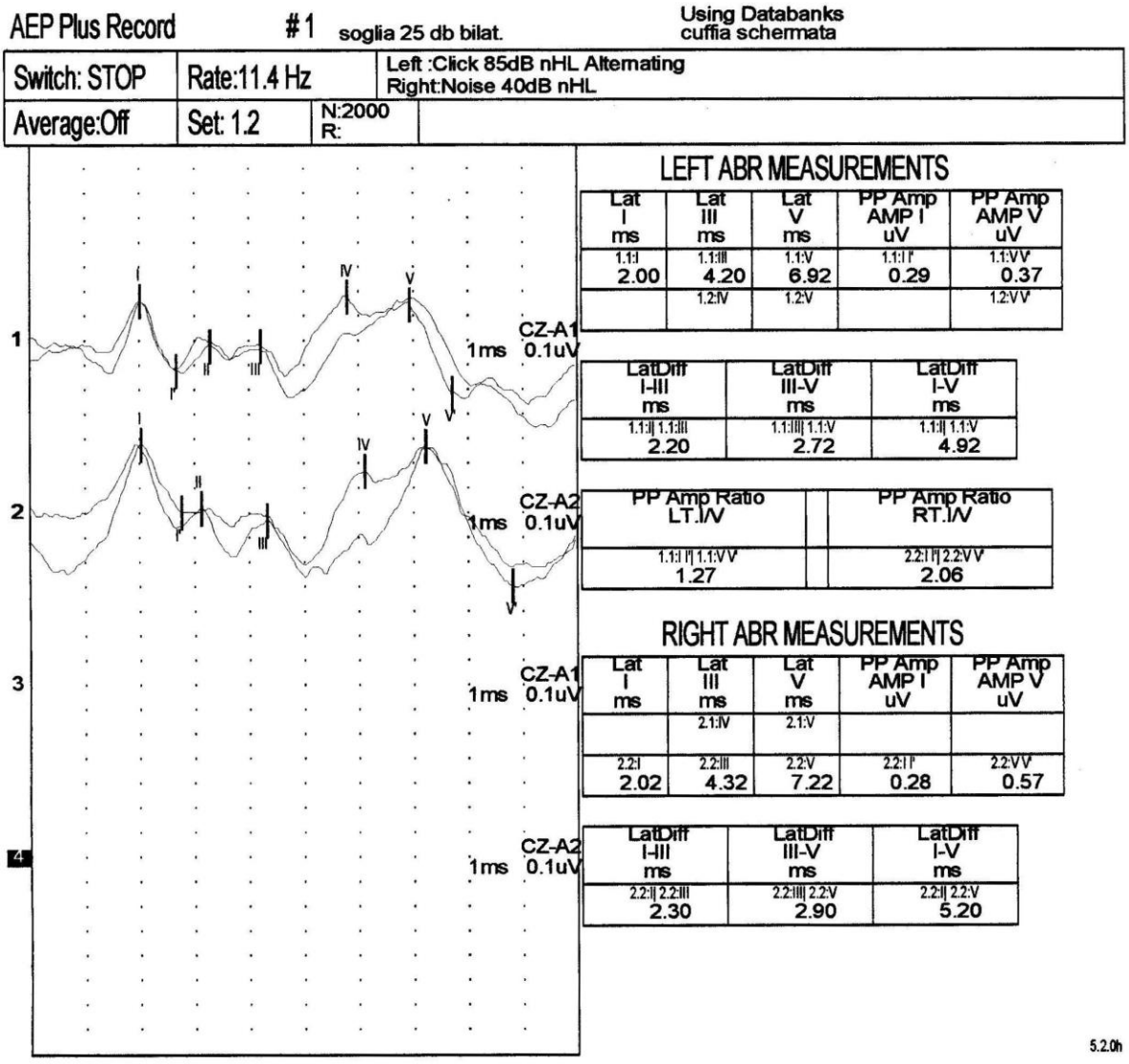


Left Eye: no alteration



Supplementary Figure 2: BAEP and VEP findings.

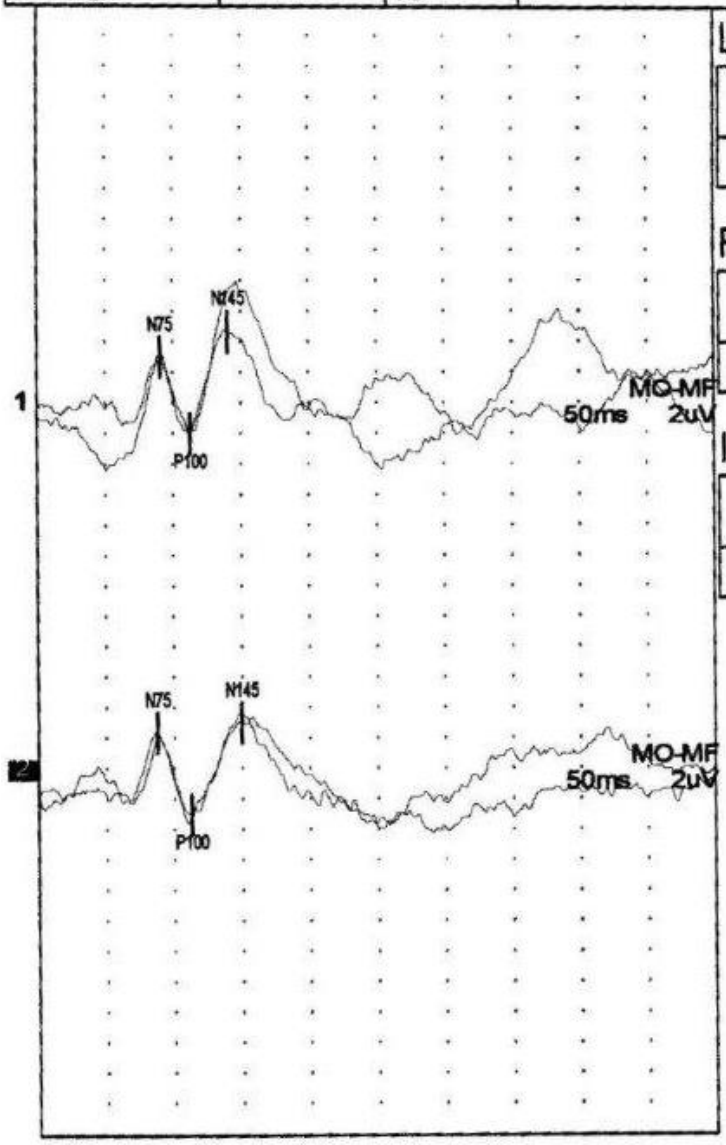
BAEP: marked increase in the III-V interval



VEP: normal and symmetrical (P100) latency and amplitude.

VEP Plus Record #1 Visual Angle=0.27' 1Ch PRVEP Master Montage 216A & Databanks

Switch: STOP	Rate: 1.6Hz	1015 Stimulator
Average:Off	Set: 1.1	N: 100 R:



LEFT EYE MEASUREMENTS

Text	Lat N75 ms	Lat P100 ms	Lat N145 ms	PP Amp 75-100 uV
	1.1:N75	1.1:P100	1.1:N145	1.1:N75 P100
MO	89.5	112	140	5.24

RIGHT EYE MEASUREMENTS

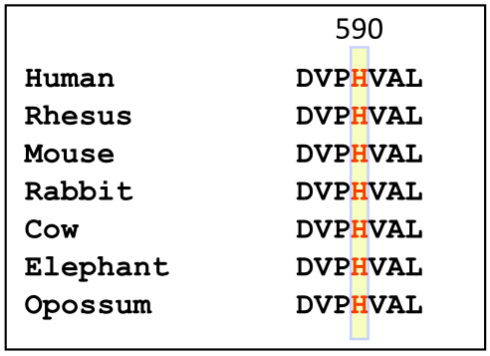
Text	Lat N75 ms	Lat P100 ms	Lat N145 ms	PP Amp 75-100 uV
	2.1:N75	2.1:P100	2.1:N145	2.1:N75 P100
MO	86.5	112	149	5.39

INTEROCULAR MEASUREMENTS

Text	LatDiff LT-RT ms	PP Amp Ratio LT-RT %
	1.1:P100 2.1:P100	1.1:N75 P100 2.1:N75 P100
MO	0.50	103

Software	Information	Description	Value output	Bibliography
SIFT	Pathogenic or not predictor	damaging	0	Ng and Henikoff (2001)
PolyPhen-2	Pathogenic or not predictor	Possibly damaging	0.77	Adzhubei IA et al. (2010)
I-Mutant	Stability changes predictor	Increase stability	-0.01	Capriotti et al. (2005)
Mutation Taster	Pathogenic or not predictor	damaging	0.97	Schwarz et al. (2014)
SNAP-F	Pathogenic or not predictor	Non-neutral	3	Bromberg and Rost (2007)
PHD-SNP	Pathogenic or not predictor	Disease	5	Capriotti et al. (2006)

Supplementary Table 1. *In silico* prediction of variant pathogenicity and the evolutionary conservation among mammals of the histidine 590 residue using the "Multiz Alignment of 100 Vertebrates" track of the UCSC genome browser (www.genome.ucsc.edu)



Supplementary Table 2: LKPAT patients described in the literature.

	Depienne et al., 2013 (1)	Depienne et al., 2013 (2)	Depienne et al., 2013 (3)	Depienne et al., 2013 (4)	Depienne et al., 2013 (5)	Depienne et al., 2013 (6)	Di Bella et al., 2014	Hanagasi et al., 2015	Our patient
Gender/ ancestry	F/North Africa	F/North Africa	F/North Africa	F/Europe	M/Europe	F/Europe	M/Europe	F/Middle East	F/ North Africa
Age at first neurological sign	44 yrs.	57 yrs.	30 yrs.	12 yrs.	6 yrs.	3 yrs.	42 yrs.	7 yrs.	52 yrs.
Symptom at onset	Action tremor Mild gait ataxia	Tinnitus Vertigo	Chorioretinopathy Psychosis	Learning disability Headache	Headache	Action tremor Mild gait ataxia	Postural hand tremor Palmomental reflex Increased deep tendon reflexes Bilateral Hoffman sign	Mild bilateral hand tremor	Presbiopia Headache
Disease course	Stable	Progressive	Stable	Stable	Stable	Stable	Stepwise	Stable	Stable
Vision	Normal	20/80 RE 20/400 LE	1/10 RE 1/50 LE	Normal with glasses	Normal with glasses Strabismus	Normal	Normal	Normal	Presbyopia
Fundoscopy and optic nerve	Normal	Retinoschisis Bilateral optic neuropathy	Retinal atrophy Choroidal neovascularisation Bilateral optic neuropathy	Normal	Normal	Normal	Normal	Normal	Bilateral mild pallor and excavation of the optic disc Myelinated retinal nerve fibers in left eye
Hearing	Normal	Perceptive hearing loss	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Pyramidal	No	No	No	Mild spasticity	No	Mild spasticity	Increased deep tendon reflexes Bilateral Hoffman	No	No

signs							sign		
Cerebellar signs	Mild ataxia	Mild ataxia	Mild ataxia	Mild ataxia	Mild ataxia Nystagmus	Mild ataxia	Postural hand tremor	Mild dismetria Dysdiadochokinesia	no
Other neurological signs							Palmomental reflex	Paroxysmal kinesigenic dyskinesia	
Headhache	No	No	Yes	Severe	Severe	No	No	No	Mild
Other							Azoospermia		