UNIVERSITA' DEGLI STUDI DI GENOVA
Dottorato in Scienze Pediatriche

Tesi di Dottorato
XXXIII ciclo

"Clinical features, disease evolution, ocular phenotype and electroretinogram abnormalities in Italian Lafora disease patients: a road to precision therapy"

Candidato: Dr. Alessandro Orsini

Relatore: Chiar.mo Prof. Carlo Minetti
CoRelatore: Chiar.mo Prof. Pasquale Striano

SUMMARY

7. REFERENCES

ABSTRACT:

INTRODUCTION:

Lafora disease (LD) is characterized by progressive myoclonus, refractory epilepsy, and cognitive deterioration,associated with pathogenic variants in *EPM2A*/*EPM2B*, encoding laforin and malin, two glycogen metabolism enzymes. Long-term follow-up data of this rare disease are lacking. So we collect the entire Lafora Disease italian population describing the clinical features and genetic findings. Moreover In order to identify a safe non invasive and rapid biological marker of Lafora disease we examined retina anatomy, FAF, SD, OCT, visual acuity and color vision of a particular subgroup of these patients. .

METHODS:

Patients with *EPM2A/EPM2B* pathogenic variants were identified by direct gene sequencing or gene panels with targeted re-sequencing. Disease progression, motor functions, and mental performance were assessed by a simplified disability scale .Spontaneous/action myoclonus severity was scored

by the Magaudda Scale. In a subgroups of patients we examined retina color and visual acuty in the ocular department at San Martino‘s institute, in Genova

RESULTS:

Age range was 12.2-46.2 years (mean:25.53±9.14) for the italian Lafora's population. Age at disease onset ranged from 10 to 22 years (mean:14.04±2.62). Mean follow-up period was 11.48±7.8 years. Twelve out of the 26 (46%) patients preserved walking ability and 13 (50%) maintained speech.An overall slower progression with preserved ambulation and speech after ≥4 years of follow-up was observed in 1 (11%) out of the 9(35%) *EPM2A* patients and in 6 (35%) out of the 17 (65%) *EPM2B* patients. Follow-up was >10 years in 7 (41.2%) *EPM2B* individuals, including two harbouring thehomozygous p.(D146N) variant.

In ten patients Full field ERG analysis revealed generalized ROD bipolar involvement and photoreceptor involvement.

CONCLUSIONS:

We confirm an overall worse disease outcome with severe deterioration of ambulation and speech in patients harbouring *EPM2A* variants. However, a slower onset of disabling symptoms in *EPM2B*subjects harbouring the p.(D146N) variant suggests that the LD disease severity canindeed be related to the underlying specific causative variant. Moreover we confirm that LD patients show retinal impairment regardless of their disease stage. The dysfunction grade may be related to disease duration. ERG may be an important

tool to detect early stage LD, to evaluate disease progression and eventually a biomarker to assess the efficacy of future treatments.

2.0 LAFORA DISEASE

2.1 INTRODUCTION:

Lafora disease (LD) is a severe autosomal recessive progressive myoclonus epilepsy with onset in early adolescence in otherwise neurologically normal individuals. (1) It was identified for the first time over 100 years ago by Gonzalo Lafora, student of Cajal, Alzheimer and Kraeplin. His descriptions of the neurological features, the recessive inheritance, and the disease course were so complete that they were never really significantly complemented since. The

Spanish neurologist identified intracytoplasmic abnormally branched glycogen inclusions occupying the whole neuronal cells. Later on the pathology was named by him and these aberrant inclusions became known as Lafora bodies (LBs). It was over half a century later, while Lafora was still alive and working, that the community of pathologists started calling the disease by his name and the inclusions Lafora Bodies (LB). LD can be found in any population and particularly with consanguinity. It is particularly frequent in Mediterranean countries (Spain, Italy, France), Northern Africa, Southern India, Pakistan and Middles East. Initial symptoms are invariably and rapidly followed by progressive dementia, refractory status epilepticus, psychosis, cerebellar ataxia, dysarthria, mutism, and respiratory failure. Symptoms usually lead to a severe burden of disability or death within 10 years (2-5).

2.2 Clinical features and diagnosis.

Clinically, LD is a fairly homogenous disease with onset in adolescence and neurological decline soon after, but the timing and severity of symptoms can be variable, even within families. Patients present with an epileptic event in their early to mid-teen years, and these episodes along with the cognitive decline and myoclonus progressively increase until death approximately 10 years later because of respiratory complications, sudden unexpected death (SUDEP), or a massive epileptic event (6, 7) (2, 3, 5, 8)

The onset of the disease occurs between the ages of 6 and 19 years, in otherwise neurologically normal individuals. most typically at 14–15 years. The age of onset and the course of the disease itself can be variable even between siblings (Tassinari et al., 1978). (9)The first symptoms are often beheadaches and school difficulties; subsequently started myoclonic seizures , generalized tonic-clonic seizures, absences or drop attacks, making difficult to differentiate from other forms of generalized epilepsies syndrome (10-12). (6)

When focal visual seizures, that was present in almost 50 % in early stage of patients with Lafora started LD was recognised . Visual seizures are caracterised by as transient blindness, or simple or complex visual hallucinations that are not only epileptic but also part of psychotic manifestation and sometimes they initially respond to antipsychotic rather than antiepileptic medication. (6)

In many cases, the disease shows an insidious near-simultaneous, or closely consecutive, appearance of headaches, difficulties at school, myoclonic jerks, generalized seizures, and visual hallucinations.

Electroencephalogram (EEG) at onset is no different from that of a juvenile myoclonic epilepsy (JME) or another generalized form showing a normal background and spike wave discharge or polyspike and wave discharges. Anomalies over time (usually within a few months, sometimes only after several years) become more characteristic and shows shows a slowing background and spike-wave discharges that do not have the regularity of the

JME's spike. The occipital discharges on EEG, arising from a slowed posterior dominant rhythm are, in the proper clinical context, highly suggestive of the disease (6, 12). (7, 10)

Moreover focal, particularly occipital epileptiform discharges begin to show up. The paroxysmal abnormalities are not increased during sleep in LD as they are in IGE and over time The physiological sleep patterns tend to disappear. Photosensitivity is usually present The somatosensory can reveal aberrant integration of somatosensory stimuli and cortical hyperexcitability .

Myoclonus can be symmetric, asymmetric, partial or generalized and it can accur at rest but it usually desappears during the sleep and it increases with action, photic stimulation or emotional excitment (1) (7, 12). (6)

Myoclonus becomes continuous, progressive, generalized and difficult to control over time, and sometimes erratic and not associated with the paroxysmal EEG anomalies. Myoclonus is usually the reason of walking disability and wheelchair dependency in this patients.

The seizures, and especially the myoclonus, initially respond to medications (valproic acid, perampanel, levetiracetam, zonisamide), but with time they become intractable.

Within a few years after disease onset, the patients are out of school unable to walk, mainly because of frequent myoclonic and atonic attacks. (5) (6, 10) (1, 12)

Behaviourial changes, depression and apathy are frequent neuropsychiatric symptoms, and they commonly emerge in the realm of a disinhibited dementia. Important symptoms in the late stages of the disease are progressive dementia, refractory status epilepticus, psychosis, cerebellar ataxia, dysarthria, mutism and respiratory failure. In the course of LD, the myoclonus worsens and the frequency of the epileptic seizures increases, alternating with periods of apparent remission; episodes of cortical blindness may occur. The prognosis of LD is invariably progressive and fatal, leading to total disability or death 5-10 years after clinical onset, but long surviving cases, though bedridden, are increasingly observed due to improvement of long-term care.

Visual evoked potentials (VEPs), may demonstrate increased latencies or absence of response. Brain MRI is usually unremarkable at onset, however two reported cases FDG-PET revealed posterior hypometabolism during the early stages of the disease. During the late stages of the disease, MRI may reveal mild cerebellar or corical atrophy.(1, 3, 6, 12)

LD is primarily caused by mutationsof two genes: *EPM2A* and *EPM2B* (*NHLRC1*), both located on chromosome 6 at q24.3 and p22.3, respectively. The *EPM2A*gene encodes the laforin dual specificity phosphatase, whilst the *EPM2B* encodes the malin ubiquitin E3 ligase. Laforin and malin are involved in glycogen metabolism, thus causing deposition offibrillary polysaccharides composed of poorly-branched glucose polymers, which are called Lafora bodies (LBs). Neuronal LBsmainly localize in dendrites but not in axons,

possibly explaining the cortical hyperexcitability reported in LD (2, 13). (3) (1, 2, 6, 10, 11, 14)

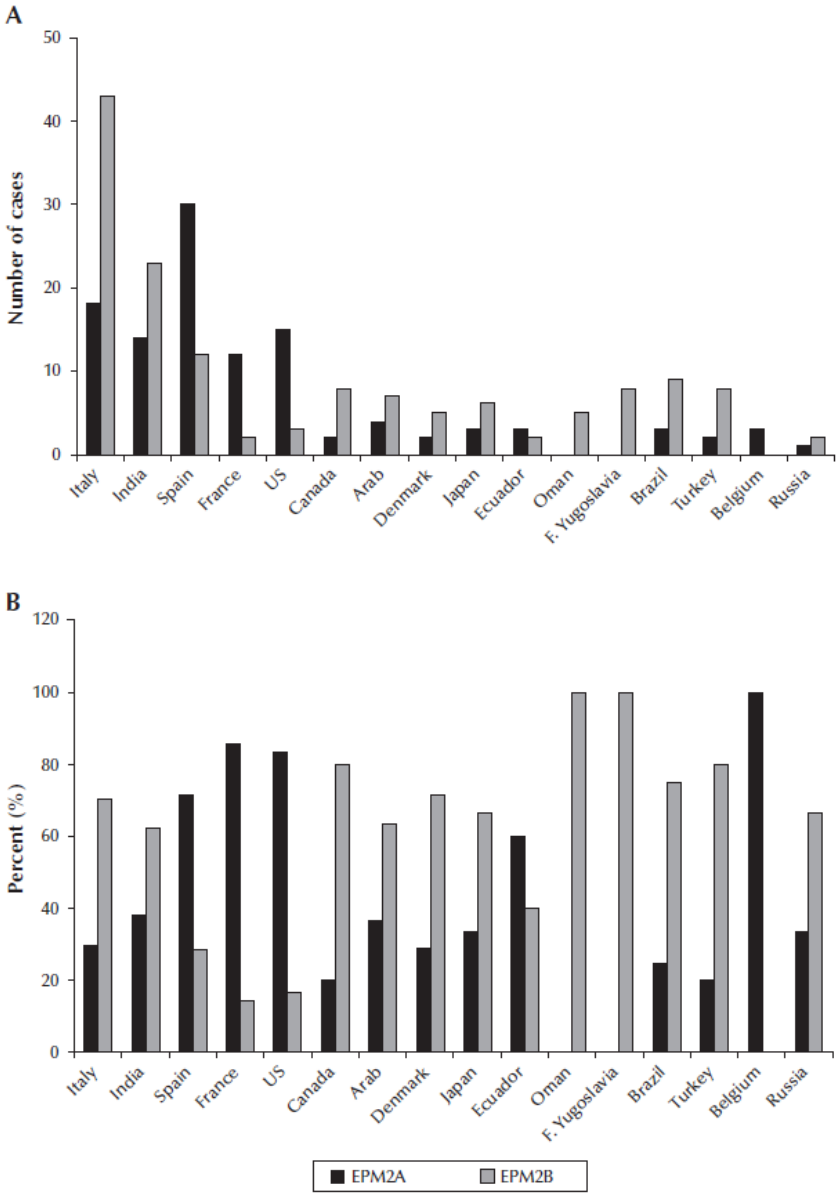

Figure 1. Number (A) and percentage (B) of EPM2A and EPM2B cases according to ethnicity/country, known to us at the time this article was prepared. Only ethnicities/countries with more than one case are shown here. Turnbull et al (10)

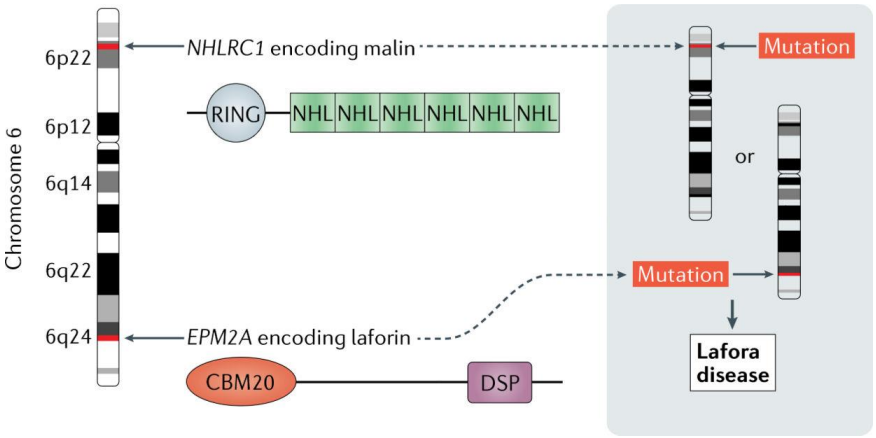

Figure 2. Causative mutations in Lafora disease.
EPM2A and NHLRC1 encode laforin and malin, respectively. Both genes are located on chromosome 6. Laforin contains an amino- terminal family 20 carbohydrate-binding module (CBM20) and a carboxy-terminal dual-specificity phosphatase (DSP) domain. Malin contains RING and NHL domains, which are typical for E3 ubiquitin ligases. Mutations — usually missense, nonsense or frameshift — in either of the two genes cause Lafora disease.**Nitschke et al (11)**

Skin biopsy shows the presence of Lafora Bodies: characteristic periodic acid-Schiff PAS-positive glycogen-like intrecellular inclusion bodies. Lafora himself, and all studies subsequent, showed that the larger LB are in neuronal cell bodies, usually juxtanuclear, but in addition to the large somatic LB, there are countless small LB in numerous cell processes. LB can be found in the myoepithelial cell of the secretory acini of the apocrine sweat glands and in the eccrine and apocrine sweat duct cells. The presence of LB can be

confirmed by Electron Microscopy These polyglucosan accumulationsare profuse in all brain regions and in the majority of neurons, specifically intheir cell bodies and dendrites.(5, 6, 15)

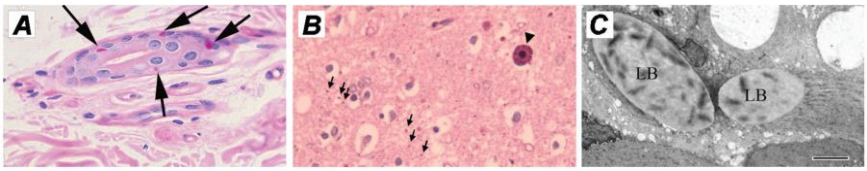

Figure 2. Skin biopsy specimen of a patient with Lafora disease. (A) PASD-stained section of an eccrine duct. Note the numerous Lafora bodies in the ductile cells (arrows). (B) Light micrograph of neuropil stained with periodic acid–Schiff reagent following diastase digestion (PASD). Both type I LBs (thin arrows) and type II LB (thick arrow) are present. (C) Low power electron micrograph of the myoepithelial cells surrounding an apocrine sweat gland. Lafora bodies are seen in the cytoplasm. Bar equals 2µm. Abbreviations: PASD, periodic acid–Schiff diastase. Courtesy of Striano et al (6).

From a clinical standpoint, a slower disease course has been reported in the *EPM2B* mutated patients, with delayed age at death.Nowadays, it is becoming clearer that there may exist one relatively common, milder, *EPM2B* mutation: patients with either heterozygous or homozygous p.(D146N) mutation, invariably show atypical milder LD, with delayed disease onset, longer disease course, and extended preservation of daily living activities.

The most pathogenic variants include the following loss of function mutations: splice site, missense, nonsense and small intragenic deletions and insertions. An additional A third gene, PRDM8(Pr Domain-Containing Protein 8,

* 616639), has been reported and it's associated with early childhood onset phenotype. (16-18)

The most common *EPM2A* mutation is the R241X mutation, that has been found in the 17% of the patients with EPM2A mutation, and large delections make up 10-15 %. The remaining *EPM2A* mutations span the entire gene and are rare.The most common *EPM2B* mutations are the missense mutation P69A and the frameshift mutation G158fs16.Certain mutations appear to be more frequent in specific ethnic/geographic regions; for expample *EPM2A* mutations are more common in Spain and *EPM2B* mutations, in Italy and France, (Lesca *et al.*, 2010). Moreover for example it was demostrated that in Oman, all cases from five separate, unrelated families resulted from a single ancestral mutational event in *EPM2B* this could be explained by a single mutated common ancestor (19) (6). (12, 15)

Mutations within exon 1 of the *EPM2A* gene may produce a different phenotype, with childhood-onset learning difficulties and only later the more classical course. Exon 4 mutations were mainly associated with classic LD with no childhood-onset educational difficulties. The authors suggested that exon 1 mutations may be associated with the complete loss of laforin function, whereas exon 4 mutations may preserve some of its functionality

Moreover, it is clear that there exists one relatively common, milder, EPM2Bmutation: patients with either heterozygous or homozygous D146N mutation in EPM2B, in all cases in the literature, have an atypical milder LD

consisting of a later onset of symptoms, longer disease course, and extended preservation of daily living activities (16, 19).(1, 6, 12)

2.2 DISEASE MANAGEMENT AND TREATMENT

At present, Lafora treatment remains palliative, with the best current therapies having limited success in the modulation of symptoms. AED are the only options for seizures control[80]. The most used is valproic acid, that is usually effective in suppressing, momentarily, most GTCS, the symptoms associated with photic sensitivity, and some of the m oclonus. Other AEDs used are: lamotrigine (LTG)but it is not very advisable in the context of a myoclonic epilepsy; phenobarbital (PB) and primidone (PRM) are effective, but at high doses they have cognitive effects that worse those of the condition; and levetiracetam (LEV). Other helpful drugs include topiramate (TPM) and zonisamide (ZNS), which both have marked antimyoclonic effects. Additional relief can be obtained, often transiently, with ethosuximide, felbamate, and benzodiazepines (BZD). Finally, there have been two recent single case reports of rather dramatic beneficial effects of perampanel, , one of the newer antiepileptic drugs,and a selective noncompetitive antagonist of the AMPA-type glutamate receptors [81] and a group of ten patients . Piracetam (PIR), a more specific antimyoclonic agent, can alleviate the burden of seizures and myoclonus. Besides AEDs, vagal nerve stimulation resulted in temporary

cessation of generalized tonic–clonic seizures and status epilepticus in two single- case studies (1, 11, 20, 21)

The ketogenic diet has been tried, but without success, in The ketogenic diet has been studied in human LD, but only as a pilot in 5 patients with the diet initiated years after onset well into the disease's neurodegenerative course. The authors did not detect clinical improvement, however they concluded that further studies are need thus the variability of cases and stage disease and answer, and the small number of cases that did not allow stratification. Moreover a recent animal model study detect a 22% reduction in LB in LD mouse model under ketogenic diet, thus presumably reducing the neuronal glucose availability for glycogen (and Lafora body) synthesis.

With the progression of LD, AED treatment progresses to polytherapy, with a combination of several of the drugs quoted above (with the exclusion of LTG); the commonly used combinations are VPA+TPM or ZNS or LEV, with an additional BZD, a 3 to 5 drug combination is common.

There is no evidence that carbamazepine (CBZ), oxcarbazepine (OXC), phenytoin (PHT), eslicarbazepine, gabapentin, pregabalin, vigabatrin, or lacosamide are of any benefit and a worsening effect may observed. However, in some cases status epilepticus responds well to phenytoin, but it should not be kept as maintenance medication.

(1, 11, 21, 22)

2.3THE ROAD TO PRECISION THERAPY

In 2016, the European Commission granted orphan designation and permission to use metformin for the treatment of Lafora disease. Metformin as an activator of AMPK largely used as an antidiabetic disease seems to reduce LB and seizure susceptibility (ref). Aminoglycoside antibiotics and supplement sodium selenate has been used for proposed treatment in LD. Sodium selenato seems to reduce neurodegeneration, gliosis, seizure and memory loss in mouse mode. Aminoglycoside antibiotics however was gravied by hard adverse effect so the use is controversial.

The most exciting approach remains and will be gene therapy for the treatments of the hereditary diseases. Gene therapy to deliver a functional copy of the defective gene would thus be an obvious option for Lafora disease treatment. Such therapies could be with the use of virus vector for gene replacement. Viral vectur are secure and have the capability to veiculate sequences of Epm2a or EPM2b gene directly in cell. These vectors also have the ability to cross the the blood–brain barrier BBB, which makes intravenous delivery possible; even if the administration in the cerebrospinal fluid (CSF) is however currently preferable to avoid neutralizing antibodies and the hepatic first pass effect . This type of approach has been used in trials on other pathologies but currently not yet in LD. Moreover rna interfere is potential option for post- transcriptional suppression of Lafora disease- related therapeutic targets and other

neurodegenerative disorder and have been used in gene knockdown in Huntington disease.

Another possible approach is to reduce LB with inhibitors of GS at both the mRNA and protein levels.ASO (Antisense oligonucleotides), are emerging as an excellent therapy platform, already used with success in other neurodegenerative disease such as SMA (spinal muscular atrophy); this kind of molecules have the effect to target the mRNA encoding GYS1, PP1 subunit R5 and/or other PP1 subunits with a considerably good effect in mouse model. In particular in LD mouse models has been shown that a mere genetic 50% reduction in GS activity in the brain dramatically reduces, to near completelyeliminating, LB. This is accompanied with absence of the neurological abnormalities and neurodegeneration that areotherwise present in these mice. An alternative treatment may be the degradation of LBs by delivery of α amylase fused to a cell membrane penetrating monoclonal antibody.

All of these treatment are gravied by the difficulty to perform double blind trial due to the severity and the rapid progression of the disease. So another line of research will be the finding of a possible biomarker for LD. A few years ago, perform a study with ERG in eyes find a finding a significant alteration in ERG of patients with Lafora disease, which can also be used as an indicator of the stage of the disease. (1, 11, 12)

3.0 MATERIALS AND METHODS

3.1 MATERIALS AND METHODS:

Patients with LD were recruited from 14 Italian Epilepsy centers through the collaborative network of the Italian League Against Epilepsy (LICE). The diagnosis of LD was based on the clinical and electrophysiological features, as well as on the identification of typical LBs in skin, liver, or muscle samples. Clinical findings, neurophysiologic features,genetic results, and brain MRI and treatment data were retrospectively collected from medical charts provided by the referring clinicians.

To assess disease progression, a simplified disability scale evaluating the residual motor function, mental performance activities of daily living (ADL),and social abilities was used. Scores ranged from 1 to 4 as follows:1) mild cognitive impairment (MoCA>25), mild gait ataxia (scale 4), preserved ADL, and maintained interpersonal and family interactions; 2)moderate cognitive impairment(MoCA<25), moderate gait ataxia (scale 3 to 2), limited ADL, and preserved but limited social interaction; 3) severe mental impairment (MoCA<10), severe gait ataxia (scale 2 to 1), impaired ADL, and poor social interaction; 4)severe mental impairment (MoCA<10), severe gait ataxia (scale 0), wheelchair-bound or bedridden, no significant ADL, no social interaction, and gastrostomy/ tracheostomy.

The severity of spontaneous and action myoclonus was also evaluated using

the Magaudda Simplified Myoclonus Rate Scale: 0) no myoclonus; 1) minor myoclonus with no interference with ADL; 2) mild myoclonus with interference with fine movements or speech, butno interference with walking; 3) moderate myoclonus, but preserved ambulation without support; 4) moderate to severe myoclonus with preserved ability to stand and supported ambulation; 5) severe myoclonus with patient wheelchair-bound or bedridden.

Different targeted re-sequencing gene panels for epileptic disorders (or direct single gene sequencing of the coding regions of *EPM2A*and*EPM2B* were performed to investigate the gene defects. Segregation analysis of candidate variants was undertaken in all families.

Moreover we proposed complete opthalmological evaluation to all Lafora patients that are able to reach our centre in Genova.

A sub group of patients (group B) underwent opthalmological evaluation at the ocular department, San Martino's Institute, Genoa, Italy. Firstly, the visual acuity was measured with the ETDRS Visual Acuity Charts. The integrity of colour vision was assessed using Ishihara's test, evaluating the red-green vision. Then, full-field ERG analyzed the rods' and cones' electrophysiological responses. ERG measurements were performed following the International Society for Clinical Electrophysiology of Vision (ISCEV) standard protocol. After twenty minutes of dark adaptation (DA), patients underwent scotopic ERG, using a 0,01 cd s/m^2 flash, which triggers a positive B-wave, representing rod bipolar cells' activity. The second stimulation was a DA ERG, tested using

a 3.0 cd s/m^2 flash, eliciting a negative a-wave, due to rods photoreceptors activation and which is followed by the positive b-wave reflecting rod bipolar cell depolarisation. Then, the DA oscillatory potentials test was performed to evoke responses from amacrine cells and, after completion, patients were light-adapted (LA) for ten minutes through a background luminance of 30 cd.m^2. Lastly, the cones system was tested using a 3.0 cd s/m^2 flash stimulus at two different frequencies: 2 Hz photopic ERG and 30 Hz flicker ERG. The 2 Hz frequency aroused an a-wave followed by a b-wave; in this case, the a-wave is driven by cones photoreceptors and cones Off-bipolar cells, whereas the b-wave by On- and Off-bipolar cells. Otherwise, the 30 Hz frequency evoked a flicker response originating in the cones system reflecting post-receptoral responses of cones On- and Off-pathways. Upon conclusion of the ERG, retinal examination, spectral domain optical coherence tomography (SD-OCT) and fundus autofluorescence (FAF) was performed.

3.2 STATISTICAL ANALYSIS

Patients were divided into two groups, those harbouring variants in *EPM2A* and those carrying *EPM2B* variants. Categorical data were summarised in terms of absolute frequencies and percentages. Quantitative variables were summarised in terms of medians with 1^{st} and 3^{rd} quartiles (1^{st} – 3^{rd} q), as the data were not normally distributed. The normality of the distributions wascalculatedby the

Shapiro-Wilk test. The association between categorical data was evaluated by the Chi-square test or Fisher's Exact test in case of expected frequencies <5. The comparison of quantitative variables between the 2 groups of patients was evaluated by the Mann-Whitney U test. Non-parametric analysis of variance (Kruskal-Wallis test) was used to assess the relationship between quantitative and categorical polynomial variables. To avoid the "multiple comparison error", the Bonferroni's correction was applied, with the P-value indicated as "P_B". The software "Statistica", release 9 (StatSoft Inc., Tulsa, OK, USA), was used for all univariate and bivariate analyses. The software "Stata", release 11.0 (StataCorp, College Station, TX, USA),was used to calculate Fisher's Exact test for tables with more than 2 rows or columns.Spearman's rank correlation coefficient (r_S) was applied to evaluate the statistical dependence between the rankings of two independent variables.

Moreover to investigate the relationship between LD stage and the rods' and cones' dysfunction we calculated the Pearson's correlation coefficient. We compared also the mean level of rods' and cones' dysfunction by dividing patients into two subgroups (*EPM2A* vs *EPM2B* mutated patients) and the Student's t-test for undifferentiated samples was applied.

4.0 RESULTS

4.1 **CLINICAL FEATURES OF THE COHORT**

Twenty-six patients (16 females and 10 males) with LD from 24 different Italian families were investigated. Age range was 12.2-46.2 years (mean, 25.53±9.14). Age at disease onset ranged from 10 to 22 years (mean, 14.04±2.62) (**Table 1**).

Patients with *EPM2B*variants showed a mean age at onset of 14.2±2.5 years, whilst the mean age at onset was 13.6±2.9 years in those with *EPM2A*variants. Overall, the mean follow-up period was 11.48±7.8 years, being 12.3±8.2 and 9.8±7.0 years in *EPM2B* and *EPM2A* patients. Ambulation and speech abilities at last follow-up were evaluated. Twelve (46%) patients retained and 14 (54%) lost ambulation. Thirteen (50%) patients preserved speech, while the other half showed absence of speech at last follow-up. Thirty-five percent of *EPM2B* patients showed loss of both walking and speech abilities after a mean of 15.1 years of follow-up. Isolated loss of ambulation or speech was instead observed in only two patients (#10, #24)at the mean age of 13.3 years from disease onset. As for*EPM2A*patients, 78% were non-ambulatory and 67% had no speech, with a mean follow-up period of 12.7 years. The mean score of the disease stage was 3.2. Sub-analysis between *EPM2A* and *EPM2B*individuals showed a mean disease stage score of 3.4 and 3.0 points, respectively.

The severity mean scores for spontaneous and action myoclonus in 21 patients were 3.5 and 3.7 points, respectively:3.9 and 3.7 points in *EPM2A* subjects;3.4 and 3.6 in *EPM2B* subjects. Comparison between spontaneous/action myoclonus severity and age at disease onset showed a linear correlation with

an r_S= -0.44 for spontaneous myoclonus and an r_S= -0.61 for action myoclonus (**Figure 1**).Mean age at tonic-clonic and myoclonic seizuresonset was 14.65±3.7 and 14.8±2.5 years. Tonic-clonic seizures mainly occurred monthly in 70% of cases, 78% of *EPM2B* patients and 50% of *EPM2A* subjects. Weekly or yearly seizures were reported in the remaining cases. Patient #2 experienced a single tonic-clonic seizure. Seventy-seven percent of patients had ataxia, with a mean age at onset of 17.3 years. Dementia was also frequent (75% of cases), with a mean onset of 16±1.7years and occurring 2.5-3.0 years after disease onset in *EPM2A* patients or up to 4 years in the *EPM2B* subjects.

Brain MRI was normal or revealed slight to moderate cerebellar atrophy in most cases. In one case (#2b), diffuse cortical atrophy after 7.6 yearsfrom disease onset was identified. Most individuals manifested refractory epilepsy and myoclonus despite treatment with a combination of antiseizure medications (ASMs), including valproate (VPA), levetiracetam (LEV), carbamazepine, clonazepam, perampanel, and zonisamide. Notably, two patients (#2a, #15) achieved seizure control (isolated or monthly tonic-clonic seizures) with VPA or LEV monotherapy.

4.2 *GENETIC FINDINGS*

Nine (35%) patients harbouredpathogenic variantsin *EPM2A* and 17 (65%) in *EPM2B*. Compound heterozygousvariants were detected in 6 (35%) and 2 (22%) patients in the *EPM2A*and *EPM2B*groups.The remaining individuals

were found to harbour homozygous variants. Seven distinctvariants(four missense, one truncating, one frameshift, and one large exonic deletion) in *EPM2A* and ten different variants in *EPM2B* (six missense, one truncating, and three frameshift) were identified. Three novel variants were detected, including the p.(D82RfsTer7) in *EPM2A* and the p.(F204LfsTer28) and p.(A277DfsTer23) in*EPM2B*. The p.(P69A) and p.(D146N) variants were the most frequent, beingdetected in 6 (35%) and 4 (24%) *EPM2B* patients, respectively. In patients #11 and #12, a deletion involving the exon 2 of *EPM2A* was identified.

Commentato [MOU1]: Utile avere intervallo

4.3 GENOTYPE-PHENOTYPE CORRELATIONS

The mean age at onset was 13 years in*EPM2A*patients and 14.5 years in*EPM2B*subjects. In the *EPM2B*group, the follow-up period was >10 years in 7 (41.2%) patients, including two subjects (#20, #23) homozygous for the p.(D146N) variant. Patient #23 showed a remarkably late-onset (22 years) of symptoms and a very long follow-up (24.2 years)with a disease stage of 2.Amoderatetosevere disease stage was observed in patients withthe homozygous variants p.(P69A) (4 patients),p.(F204LfsTer28) (1 patient),p.(D146N)(1 patient),p.(E67Ter)(1 patient), and p.(D308V)(1 patient). In the *EPM2A* group, the follow-up period was >10 years in four (44%) patients: #4, harbouring the compound heterozygous p.(R241Ter)andp.(G279S)variants; #9, carrying thehomozygous p.(R241Ter)

variant; #7,homozygous for the p.(D82RfsTer7)variant; #12, harbouring the exon 2 deletion.

Ataxia was observedin 11 out of 14 (78.6%)*EPM2B*patients, with a mean age at onset of 17years. The *EPM2B*variantsp.(P69A)and p.(D146N)were associated with both early (#21, #23) and late-onset ataxia (#8, #10), as well asthe absence of ataxia after ≥ 5 years from disease onset (#14, #20). Particularly, patient #20 did not show any ataxia after 29 years of follow-up. In the *EPM2A*group, ataxia occurred in 5 out of 6 (83.3%) patients,with a mean age at onset of15 years. Deletions involving the exon 2 of *EPM2A*were associated with ataxia within the first year of follow-up,whereas delayed ataxia onset was observed in the patient with the p.(R241Ter)variant (#5).

Dementia occurred at 3.3±2.0 years and 2.4±1.5 years after disease onset in *EPM2A*and *EPM2B* patients, respectively. Dementia occurred in 10 out of 15 (66.7%)*EPM2B*patients, with a mean age at onset of 17 years. The p.(P69A)variant in *EPM2B*was associated with dementiain the first year of follow-up (#14) as well as dementia after >4 years(#1).In the*EPM2A* group, 83% of patients showed dementia, with a mean age at onset of 15 years. Patient #7 (harbouringthe p. D82RfsTer7)showed dementia within one yearfrom disease onset, whereas patients #5 and #9 (carryingthe homozygous p.R241Ter mutation) showed late-onset dementia.

The mean spontaneous myoclonus scoreswere4.5 [4-5] [n=6] and 3 [3-4] [n=13] points in the *EPM2A* and *EPM2B* groups, respectively. Six (35.3%)

*EPM2B*patients had severe spontaneous myoclonus. Patients #1 and #21 (harbouring the p.P69A) showed severe spontaneous myoclonus after <5 years since disease onset. Patients #20and #23, carryingthe homozygousp.(D146N),showed mild spontaneous myoclonus after >20 years of follow-up.Three patients showed severe action myoclonus: patient #1 (p.D146N), patient #17a (p.G331EfsTer3), and patient #21 (p.D146N). Patient #3 (p.D146N) showed mild action myoclonus after 8.8 years of follow-up.

Mean disease stage score was 3.5±1.5 points in the *EPM2A*group,whereas the mean score was3.0±0.9 points in *EPM2B* patients.Disease stage was severe in eight (88.9%) *EPM2A*subjects and mild in only 1 case (11%). One *EPM2A* patient (#2b), carrying the homozygous p.(R108L)variant, showed a disease stage score of 3 points after 9 years of follow-up. Seven out of 9 (77.8%) *EPM2A* patients lost ambulation and 6 (66.7%) speech ability. Patient #2b (homozygous for the p.R108L variant) remained able to walk and speak after 9 years fromdisease onset. Patient #12 (*EPM2A* exon 2 deletion) maintained speech but lost ambulation after >12 years of follow-up. Seven (41.2%) *EPM2B* patients lost both ambulation and speech. Loss of ambulation occurred <10 years since disease onset in patient #10 (p.P69A), patient #14 (p.P69A; A277DfsTer23), and patient #17b (p.G331EfsTer3). However, patient #10 preserved speech. Three*EPM2B* patients preserved ambulation after >20 years from disease onset: #20, (p.D146N); #22, (p.P111L; p.E280K); #24 (p.D308V).

Patients #20 and #23, harbouring the p.(D146N) mutation,preserved speech after >20 years of follow up.

In general, *EPM2B* patients showed a milder disease stage. In particular, patients #20, #23, and #24 showed a slower disease progression, with a follow-up period nearly to 20 years. Two of them (#20, #23)carried the G to A missense change at nucleotide 436, whilst #24 harbouredan A to T transition at nucleotide 923. No *EPM2A* patient in our cohort showed such a prolonged course. *EPM2B*patients also showed, on average, more preserved motor and cognitive functions. In particular, 10 out of 17 (59%) *EPM2B* patients showed moderate to severe disease stage, and just 70% of them lost speech or ambulation. A similar disease course was instead observed in eight (89%) *EPM2A* patients, with seven (88%) and six (75%) of them losingspeech and ambulation, respectively. Remarkably, not all *EPM2B* patients showed a milddisease course. Indeed, the patient harbouring the p.G331EfsTer3(#17a)presented with dementia and ataxia within one year after disease onset, as well as severe spontaneous and action myoclonus after less than 4 years of follow-up. Of note, an earlier disease onset correlatedwith the early loss of ambulation in the *EPM2B* group, whereas a correlation was not observed between preserved ability to ambulate andlater diseaseonset (P=0.013) (**Figure 4**).

Specific missense mutations in *EPM2A* and *EPM2B* were associated with a slower disease course. Patients carrying the *EPM2B* p.(D146N)mutation (#8,

#20, #23) showed mild disease stage and myoclonus severity, with a follow-up period of 20 and 5 years, respectively. Moreover, patient #20 and #23 preserved speech and ambulation. Similarly, the p.(P69A) variant in*EPM2B* was associated with preserved ambulation and speech ability in patient #3 after 9 years from disease onset. The *EPM2A* missense variant p.(R108L)was associated with milder disease stage: patient #2b preserved walk and speech capabilities after 8 years of follow-up. No significant differences emerged between *EPM2A* and *EPM2B*variantsconcerningdisease duration (P=0.89), presence of ataxia (P=1.0) anddementia (P=0.62), disease stage (P=0.07), absence of ambulation (P=0.11) or speech (P=0.41), and spontaneous (P=0.28) or action (P=0.44) myoclonus severity. However, earlier disease onset was observed in the *EPM2A* group, witha value of P= 0.039 (**Table 2**). Furthermore, patients with *EPM2A*variants (r_S=0.29)showeda faster disease progression as compared to *EPM2B*subjects (r_S=0.44) (**Figure 5**).

Results Subgroup B

Six patients (4 females),(#2a,#2b,#7,#9,#14,#21) 50% carrying an *EPM2A* gene mutation were investigated for the ophthalmological point of view. Age at evaluation ranged from 13 to 26 years (mean, 19.5 years), while age at disease onset ranged from 11 to 16 years (mean, 12.5 years) with a mean disease duration of 7 years (range, 2-13 years). **(table 3)** The myoclonus severity scored of these patients was between 0 and 4 points (mean, 2.50 points) and the disease stage ranged from 0 and 4 points (mean, 2.67 points). The retinal anatomy, FAF, SD-

OCT, visual acuity, and colour vision tests were unremarkable in all individuals. No patients did show retinitis pigmentosa, excluding retinal pigment epithelial atrophy and any significant structural abnormality of the photoreceptor outer segments in the central retina (50°). We did not detect any structural alterations in the macula on SD-OCT. Full-field ERG analysis revealed a global mild-to-severe cones' dysfunction in all patients; specifically, mild cones' dysfunction was detected in one patient (#P2; amplitude deviation RE/LE: -3.3/-3.5), whereas a moderate dysfunction was found in patients #(mean amplitude deviation RE/LE: -5.5/-5.6) and severe cones' dysfunction emerged in three patients (mean amplitude deviation: -9.2/-8.6) (**Table 3**). A positive linear correlation (y=1.47+0.32*x) between LD stage and the degree of cones' and rods' dysfunction at the ERG analysis **(Figure 7)** was found; moreover, a relationship between the type of mutation and the cone and rod dysfunction using a t-test **(Table 4, Table 5)**.

5.0 DISCUSSION

LD is a well-known but extremely complex neurological condition and definite correlations between the underlying genetic variants and phenotypic features remain to be elucidated.

In our cohort of Italian LD patients, *EPM2A*variantswere associated with a more severe disease course,whereas*EPM2B*patients generally have milder

disease course and longer survival. The type of genetic variant also appears to influence the disease course, as highlighted by the milder phenotype observed in the patient harbouring the missense mutation p.(D146N) in *EPM2B* (#20). Noteworthy, Italian patients showed a higher prevalence of *EPM2B*variants, which may indicate a founder effect in this population. However, no predominant mutations could be identified, likely due to the highly heterogeneous genetic background. In line with previous reports, the p.(P69A) and p.(D146N) were the most common *EPM2B* variants, whereas the p.(R241Ter) and exon 2 deletion were frequent in the *EPM2A* group.(23-26)

Clinical onset appeared to be earlier in *EPM2A*patients, with a lower age at onset of myoclonic and tonic-clonic seizures. Mean latency of 2 years between disease onset and the diagnosis of dementia was observed in both *EPM2A* and *EPM2B* patients. However, on average, subjects harbouring *EPM2A*variants were diagnosed with dementia 1 year before*EPM2B*-mutated patients. The observation that patients in the *EPM2B* group had a longer follow-up duration and different *EPM2B* variants were associated with different follow-up periods suggests the existence of possible correlations. In particular, a slower disease progression might be associated with specific variants in *EPM2A/EPM2B* or even with different types of genetic variants within the same gene.

Long term follow-up of LD patients is extremely rare, due to invariably severe disease progression within a few years from the diagnosis. Accordingly, MoCA assessment timelines need to be pointful and shared through the scientific community to achieve a reliable characterization of the clinical course.Based

on follow-up duration and disease stage, we observed that there may be a more aggressive disease in patientsharbouring *EPM2A*variants. Indeed, a higher prevalence of loss of speech and ambulation, as well as a higher disease stage at the last follow-up, were observed in this group. Patients with *EPM2B* variants exhibited instead a longer follow-up period (more than 5 years)and a milder disease stage. Interestingly, 4 of these subjects were also included in the study published in 2006, suggesting a prolonged disease course, whereas no subject carrying *EPM2A* variants was reported. None of the *EPM2A* patients in the current cohort showed such a comparably long survival. In particular, two out of the four previously reported patients preserved ambulation and exhibited a low disease stage after more than 20 years of follow-up. Moreover, patients harbouring the p.(D146N) variant presented with a milder disease course in one heterozygous (#8) and two homozygous cases (#20; #23). Of note, the same mutation was associated with later disease onset, around the age of 20 years. (24, 26)

Moreover we evaluated patients ocular phenotype in order to find a possible biomarker of the disease. Only six patients agreed to undergo the eye examination. The other patients either did not accept or were unable to reach the San Martino's hospital in Genoa to carry out the eye examination and related instrumental examinations.

The study of the CNS is limited by arduous accessibility; however, the retina may serve as a unique window into the CNS. The retina and the optic nerve share

their embryological origin and vasculature with the brain, and the inner blood-retinal barrier and aqueous humour are similar to blood-brain barrier and cerebrospinal fluid. Eyes have been used as a non-invasive instrument for visualization of neural integrity in a large number of neurological conditions. Moreover, recently, the OCT was used to identify specific markers of prediction, diagnosis, and progression of neurological and neurodegenerative disorders such as Alzheimer's and Parkinson's Diseases, Neuromyelitis Optical Spectrum Disorders (NMOSD), and Multiple Sclerosis (MS). Recent studies have identified potentially useful ophthalmological biomarkers and suggested electroretinogram (ERG) alterations in LD patients. ERG is a clinical technique measuring the electrical activity generated by neural cells in the retina in response to a light stimulus. This could provide important diagnostic information on a variety of retinal acquired and congenital disorders , including retinitis pigmentosa, Stargardt disease and Leber congenital amaurosis.(27-32)

To identify a safe, non-invasive, and rapid biological marker of LD we examined the retinal anatomy, FAF, SD-OCT, visual acuity, and colour vision of six patients with a genetically confirmed diagnosis of LD. During the phtalmological evaluation, the retinal anatomy was unremarkable; FAF, SD-OCT, visual acuity and colour vision were normal. Otherwise, full-field ERG did show global cones and rods photoreceptors' dysfunction in all the patients, confirming preliminary results of the previous study conducted by Vincent et al in 2018. In our cohort, the cones' dysfunction ranged from moderate to severe,

with only one patient experiencing mild dysfunction. Bipolar cell dysfunction observed at the ERG may thus reflect the histological bipolar cells' atrophy described in LD (32)(**Table 3**).

Furthermore, we identified a positive linear correlation between the disease stage and the degree of "dysfunction" found at the ERG analysis, with statistical significance for the full-field ERG data considering the grade of cones' dysfunction and the decreased rods photoreceptors A-wave amplitude function in left eye (**Table 4**). Results show a trend toward linear correlation between decreased rods photoreceptors A-wave amplitude function in the right eye and the disease stage, but statistical significance was not reached. We could argue that a bigger sample of patients would have allowed to reach statistical significance. Additionally, considering the small size of the sample due to the extreme rarity of the disease, we were not able to identify a statistical significance for the rods photoreceptors A-wave amplitude function in right eye; hence, these preliminary data must be confirmed and expanded with more patients.

The Student's t-test used to compare the type of mutation with the cones' and rods' dysfunction showed that the *EPM2A* gene mutation seems to correlate with more severe dysfunction of both cones and rods, giving much more evidence to data in the Literature showing more severe phenotypes in patients with *EPM2A* mutations as compared to patients with *EPM2B* (**Table 5; Fig 8; Fig 9**).

In conclusion, ERG, a minimally invasive method, could be used as a marker of disease stage in LD and could also be used to identify disease progression, serving as a biomarker to future precision medicine treatments like gene therapy.

6.0 CONCLUSION

In summary, we report a large cohort of Italian LD patients, focusing on the possible genotype-phenotype correlations in this severe condition, and in a Subgroup of 6 patients, we perform a complete ophtalmological assessment.. Some limitations can be recognized in our study, including the small number of patients carrying *EPM2A* variants and the limited duration of follow-up in some cases (#2a; #15), requiring further evaluations in the future. We first aimed to dissect disease outcomes with distinct genetic variants. *EPM2A* individuals may showearlier disease onset and faster disease progression with more severe deterioration of ambulation and speech. Conversely, later disease onset and slower progression can be observed in*EPM2B* patients, in line with previously reported cases in the literature. Moreover, specific gene variants in *EPM2B*, such as the p.(D146N),appear to be associated with even slower onset of disabling symptoms. The effort towards the collection of large case series will play a relevant role in providing insights into genotype-phenotype correlations in different countries, increasing our knowledge on the pathogenic mechanisms underlying LD and allowing the development of targeted therapies.

(19, 26, 33, 34)

Moreover regarding ophthalmological examination our results bring further evidence to data suggestion early retinal alterations in LD patients, regardless to the disease stage. However, the dysfunction grade is possibly related to disease duration and the ERG may be an important tool to detect stages of LD, allowing to evaluate either natural or treatment-related disease progression in a minimally invasive way.

REFERENCES:

1. Orsini A, Valetto A, Bertini V, Esposito M, Carli N, Minassian BA, et al. The best evidence for progressive myoclonic epilepsy: A pathway to precision therapy. Seizure. 2019;71:247-57.
2. Chan EM, Young EJ, Ianzano L, Munteanu I, Zhao X, Christopoulos CC, et al. Mutations in NHLRC1 cause progressive myoclonus epilepsy. Nature genetics. 2003;35(2):125-7.
3. Chan EM, Bulman DE, Paterson AD, Turnbull J, Andermann E, Andermann F, et al. Genetic mapping of a new Lafora progressive myoclonus epilepsy locus (EPM2B) on 6p22. Journal of medical genetics. 2003;40(9):671-5.
4. Minassian BA, Lee JR, Herbrick JA, Huizenga J, Soder S, Mungall AJ, et al. Mutations in a gene encoding a novel protein tyrosine phosphatase cause progressive myoclonus epilepsy. Nature genetics. 1998;20(2):171-4.
5. Minassian BA. Lafora's disease: towards a clinical, pathologic, and molecular synthesis. Pediatric neurology. 2001;25(1):21-9.

6. Striano BAMaP. Lafora Body Disease. Symptomatic Epilepsies of Genetic or Developmental Origin. 2018:5.
7. Turnbull J, Girard JM, Lohi H, Chan EM, Wang P, Tiberia E, et al. Early-onset Lafora body disease. Brain : a journal of neurology. 2012;135(Pt 9):2684-98.
8. Minassian BA, Striano P, Avanzini G. Progressive Myoclonus Epilepsy: The Gene-Empowered Era. Epileptic disorders : international epilepsy journal with videotape. 2016;18(S2):1-2.
9. C A Tassinari MB-P, B Dalla Bernardina, I Picornell-Darder, M C Mouren, C Dravet, J Roger. [Lafora disease. Rev Electroencephalogr Neurophysiol Clin. 1978:15.
10. Julie Turnbull 1 ET, Pasquale Striano 2, Pierre Genton 3, Stirling Carpenter 4, Cameron A. Ackerley 5, Berge A. Minassian. Lafora disease. Epileptic Disord 2016; 18 (Suppl 2): S38-S62. 2016.
11. Felix Nitschke1 SJA, Silvia Nitschke1, Sharmistha Mitra2, and Berge A. Minassian1,2. Lafora disease — from pathogenesis to treatment strategies. Nat Rev Neurol 2018:11.
12. Roberto MICHELUCCI1 JMS, Antonio V. DELGADO ESCUETA3, Pierre GENTON4, Patrizia RIGUZZI1, Carlo Alberto TASSINARI1, Michelle BUREAU4. PROGRESSIVE MYOCLONUS EPILEPSIES. Epileptic Syndromes in Infancy, Childhood and Adolescence. 2019.
13. Berkovic SF, Cochius J, Andermann E, Andermann F. Progressive myoclonus epilepsies: clinical and genetic aspects. Epilepsia. 1993;34 Suppl 3:S19-30.
14. 5.Serratosa MJ D-eV, Posada I, et al. The gene for progressive myoclonus epilepsy of the lafora type maps to chromosome 6q

Hum Mol Genet. 1995.
15. Striano P, Zara F, Turnbull J, Girard JM, Ackerley CA, Cervasio M, et al. Typical progression of myoclonic epilepsy of the Lafora type: a case report. Nature clinical practice Neurology. 2008;4(2):106-11.
16. García-Gimeno MA ea. Lafora Disease: A Ubiquitination-Related Pathology. cell. 2018.
17. Gentry MS DR, Worby CA, Mattoo S, Ecker JR, Dixon JE. The phosphatase laforin crosses evolutionary boundaries and links carbohydrate metabolism to neuronal disease. J Cell Biol 2007.
18. Wang W PG, Skurat A V., Raben N, DePaoli-Roach AA, Roach PJ. Relationship between glycogen accumulation and the laforin dual specificity phosphatase. Biochem Biophys Res Commun 2006.
19. Gomez-Abad C G-GP, Gutierrez-Delicado E, Saygi S, Michelucci R, Tassinari CA. Lafora disease due to EPM2B mutations: a clinical and genetic study. . Neurology 2005.
20. Dirani M NW, Abdulla F, Beydoun A. Seizure control and improvement of neurological dysfunction in Lafora disease with perampanel. (in eng). Epilepsy Behav Case Rep 2014. 2014.
21. Michelucci R PE, Riguzzi P, Andermann E, Kälviäinen R, Genton P. Myoclonus and seizures in progressive myoclonus epilepsies: pharmacology and therapeutic trials. Epileptic Disord 2016; 18 (Suppl 2): S38-S62. 2016.
22. Lori Israelian M, Peixiang Wang, PhD, Shoghig Gabrielian, BSc, Xiaochu Zhao, BSc, and Berge A. Minassian, MD. Ketogenic diet reduces Lafora bodies in murine Lafora disease. Neurol Genet. 2020.
23. Brenner D BT, von Spiczak S. Genotypes and phenotypes of patients with Lafora disease living in Germany. Neurol Res Pract. 2019.
24. Gentry MS AZ, Armstrong DD, et al. The 5th International Lafora Epilepsy Workshop: Basic science elucidating therapeutic options and preparing for therapy in the clinic. . Epilepsy & behavior : E&B. 2020.
25. 15. Franceschetti S MR, Canafoglia L, et al. Progressive myoclonic epilepsies: definitive and still undetermined causes.

. Neurology. 2014.
26. Franceschetti S GA, Canafoglia L,. Clinical and genetic findings in 26 Italian patients with Lafora disease. Epilepsia. 2006.
27. Yzer S, Fishman GA, Racine J, Al-Zuhaibi S, Chakor H, Dorfman A, et al. CRB1 heterozygotes with regional retinal dysfunction: implications for genetic testing of leber congenital amaurosis. Invest Ophthalmol Vis Sci. 2006;47(9):3736-44.

28. Kretschmann U, Seeliger MW, Ruether K, Usui T, Apfelstedt-Sylla E, Zrenner E. Multifocal electroretinography in patients with Stargardt's macular dystrophy. Br J Ophthalmol. 1998;82(3):267-75.
29. Holopigian K, Seiple W, Greenstein VC, Hood DC, Carr RE. Local cone and rod system function in patients with retinitis pigmentosa. Invest Ophthalmol Vis Sci. 2001;42(3):779-88.
30. Newman EA, Odette LL. Model of electroretinogram b-wave generation: a test of the K+ hypothesis. J Neurophysiol. 1984;51(1):164-82.
31. Fishman GA, Jacobson SG, Alexander KR, Cideciyan AV, Birch DG, Weleber RG, et al. Outcome measures and their application in clinical trials for retinal degenerative diseases: outline, review, and perspective. Retina. 2005;25(6):772-7.
32. Vincent A MA, Tumber A, Koukas N, Ahonen S, Striano P,. Ocular phenotype and electroretinogram abnormalities in Lafora disease: A "window to the brain". Neurology. 2018.
33. Baykan B SP, Gianotti S, . Late-onset and slow-progressing Lafora disease in four siblings with EOM2B mutation

Epilepsia. 2005.
34. Zutt R DG, et al. Unusual Course of Lafora Disease. Epilepsia Open. 2016.

Family	ID/Sex	Onset(y)	Follow-up duration (y)	Ambulation	Speech ability	Spontaneous myoclonus: severity	Action myoclonus: severity	Disease stage at last FU	Mutation
1	AMG/F	13	4.4	+	+	4	4	3	EPM2B: c.205C>G (p.Pro69Ala)
2a	BV/F	11	1.2	+	+	0	0	0	EPM2A:c.323G>T (p.Arg108Leu)
2b	BV/F	11	7.6	+	+	4	3	3	EPM2A:c.323G>T (p.Arg108Leu)
3	BG/F	14	8.8	+	+	2	2	2	EPM2B: c.205C>G (p.Pro69Ala)
4	CE/F	18	26.3	-	-	NA	NA	4	EPM2A: c.712C>T (p.Arg241Ter); c.835G>A(p.Gly279Ser)
5	DGB/M	14	10.0	-	-	5	5	4	EPM2A: c.712C>T (p.Arg241Ter)
6	DLM/M	13	4.0	+	+	3	2	2	EPM2B: c.436G>A (p.Asp146Asn); c.838G>A (p.Glu280Lys)
7	DPA/F	13	12.3	-	-	4	4	4	EPM2A: c.243_246del (p.Asp82ArgfsTer7)
8	DDG/M	16	5.3	+	+	4	4	2	EPM2B: c.436G>A (p.Asp146Asn); c.1133T>C (p.Leu378Pro)
9	DNM/M	10	13.0	-	-	5	5	4	EPM2A: c.712C>T (p.Arg241Ter)
10	FC/F	14	8.9	-	+	5	4	4	EPM2B: c.205C>G (p.Pro69Ala)
11	FE/F	15	10.0	-	-	4	4	4	EPM2A: deletionexon 2
12	FA/M	17	12.6	-	+	5	5	4	EPM2A: deletionexon 2
13	FM/F	12	19.1	-	-	NA	NA	4	EPM2B: c.612del (p.Phe204LeufsTer28)
14	GB/F	11	8.1	-	-	4	5	4	EPM2B: c.205C>G (p.Pro69Ala); c.826-829dup (p.Ala277AspfsTer23)
15	HA/M	14	2.6	+	+	NA	NA	2	EPM2B: c.992del (p.Gly331GlufsTer3); c.1049-1050del (p.Glu350GlyfsTer41)
16	IN/F*	11	19.1	-	-	3	5	4	EPM2B: c.199G>T (p.Glu67Ter)
17a	LC/M	14	3.6	+	+	3	3	2	EPM2B: c.992del (p.Gly331GlufsTer3)
17b	LM/F	13	8.9	-	-	5	5	4	EPM2B: c.992del (p.Gly331GlufsTer3)

18	LF/F	13	13.9	-	-	5	5	4	EPM2B: c.205C>G (p.Pro69Ala)
19	MI/F	13	4.6 (died)	-	-	NA	NA	4	EPM2A: c.491T>G (p.Ile164Ser); c.539T>C (p.Leu180Pro)
20	PL/F*	15	29.3	+	+	2	3	3	EPM2B: c.436G>A (p.Asp146Asn)
21	PV/M	16	3.1	+	+	3	3	2	EPM2B: c.468_469delAG (p.Gly158Argfs); c.205C>G (p.Pro69Ala)
22	RC/F*	15	21.3	-	-	NA	NA	4	EPM2B: c.332C>T (p.Pro111Leu); c.838G>A (p.Glu280Lys)
23	RE/F	22	24.2	+	+	1	3	2	EPM2B: c.436G>A, (p.Asp146Asn)
24	RF/M*	16	17.6	+	-	3	3	3	EPM2B: c.923A>T (p.Asp308Val)

Table 1. Table reporting retention of walk and speech capabilities, together with scores at the spontaneous and action myoclonus severity scale for each patient. Disease stage is referred to as the last follow-up.

	EPM2A mutations (N=9)	EPM2B mutations (N=17)	P
Gender: Male	3 (33.3%)	6 (35.3%)	1.00[a]
Female	6 (66.7%)	11 (64.7%)	
Onset: Epilepsy	3/9 (50.0%)	6/9 (42.8%)	
Myoclonus	1/5 (16.7%)	4/5 (28.6%)	1.00[a]
Epilepsy and myoclonus	2/6 (33.3%)	4/6 (26.6%)	
	Median [1st-3rd q]	**Median [1st-3rd q]**	
Age at disease onset (y)	13 (11-13)	14.5 (13-16) [n=16]	0.039[b]
Age at last evaluation (y)	23 (18.6-24)	22.8 (19.1-31.1)	0.83[b]
Follow-up duration (y)	10 (7.6-12.6)	8.9 (4.8-19.1) [n=16]	0.89[b]

Table 2. Summary of the patients' cohort and relationship between *EPM2A* and *EPM2B*mutated patients. Figures in round parentheses represent column percentages.

PtID	Sex (M;F)	Age at last evaluation(y)	Age at onset (y)	Disease duration (y)	ERG Results	Amplitude deviation (SD) RE/LE	Disease stage at last FU	Mutation
P1	M	18; 01/09/2001	13	5	3	ERG flicker: -7.0/-7.1 ; PHOT: -10/-8.3 ; SCOT: -9.4/-9.4	3	EPM2B: c.436G>A (p.Asp146Asn); c.838G>A (p.Glu280Lys)
P2	F	13; 14/03/2006	11	2	1	ERG flicker: -2.8/-3; PHOT: -3.3/-3.5; SCOT: -4/-3.9	0	EPM2A: c.323G>T (p.Arg108Leu)
P3	F	20; 16/10/1999	11	9	2	ERG flicker: -5.8/-6.9; PHOT: -6.5/-5.6; SCOT: -4.8/-4.3	3	EPM2A: c.323G>T (p.Arg108Leu)
P4	F	26; 22/03/1993	13	13	3	ERG flicker: -3.9/-2.9; PHOT: -10/-8.2; SCOT: -7.4/-9.5	4	EPM2A: c.243_246del (p.Asp82ArgfsTer7)

P5	F	20; 12/05/1999	11	9	2	ERG flicker: -4.6/-2.8; PHOT: -4.5/-5.6; SCOT: -3.6/-3.9	4	EPM2B: c.205C>G (p.Pro69Ala); c.826-829dup (p.Ala277AspfsTer23)
P6	M	20; 16/04/1999	16	4	3	ERG flicker: -5.4/-5.9; PHOT: -7.6/-9.4; SCOT: -8.1/-7.7	2	EPM2B: c.468_469del; c.205C>G

Table3: Demographics, clinical and genetic data, and ERG results of our LD patients cohort.

	Pearson's correlation	**p value**
Full field ERG	0,597	0,211
ERG-LE	-0,407	0,424
ERG-RE	-0,021	0,968
PHOT-LE	-0,525	0,285
PHOT-RE	-0,418	0,410
SCOT-LE	-0,184	0,727
SCOT-RE	-0,336	0,515

Table 4: Pearson's correlation coefficient and P value of cones' and rods' disfunction correlated with disease stage.

Legend: ERG=electroretinogram; LE=left eye; PHOT= photopic; RE=right eye; SCOT=scotopic.

	EPM2A	**EPM2B**
Full Field ERG	2,67	2,00
ERG-LE	-5,667	-4,167
ERG-RE	-5,267	-4,267
PHOT-LE	-7,367	-6,6
PHOT-RE	-7,67	-5,567
SCOT-LE	-7,033	-5,4
SCOT-RE	-7	-5,9

Table 5: Student's t-test results comparing the type of mutation and the cones' and rods'disfunction.

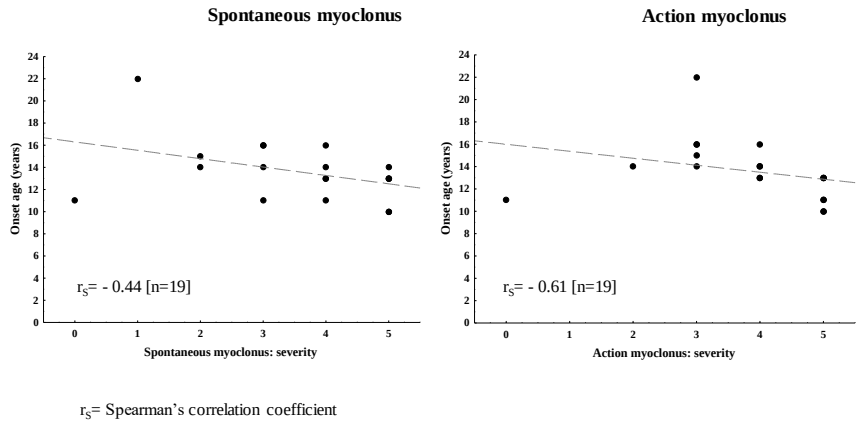

Figure 3: Correlation between age at disease onset and spontaneous or action myoclonus severities

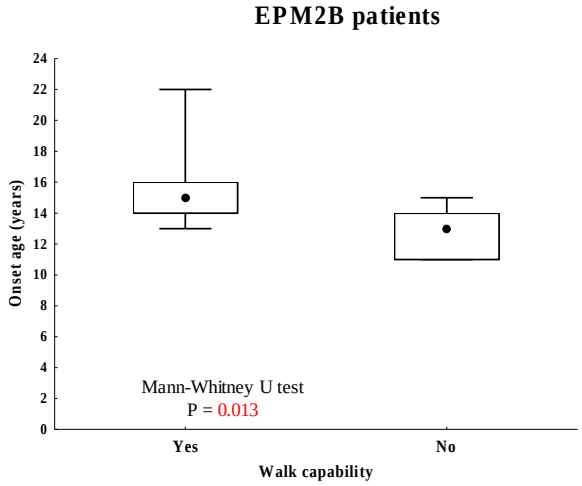

Figure 4. Relationship between onset ages and walk capability in *EPM2B* patients.

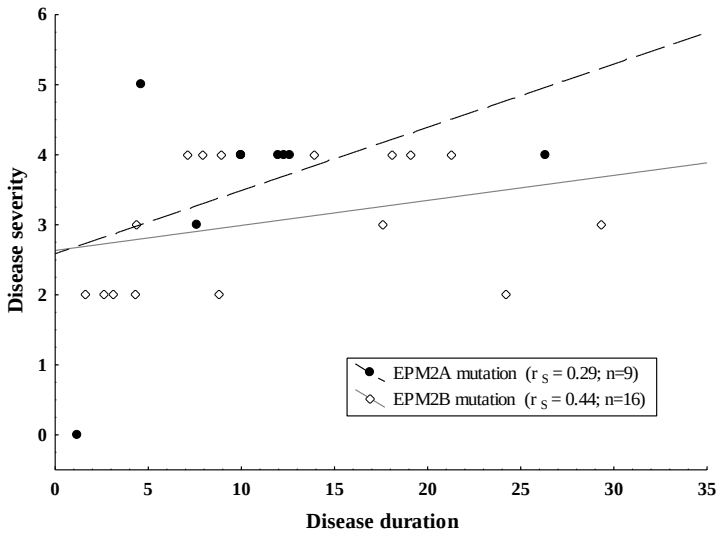

Figure 5. Relationship between disease severity and disease duration in the *EPM2A* and *EPM2B* patients. **Legend:** r_S= Spearman's correlation coefficient; n= number

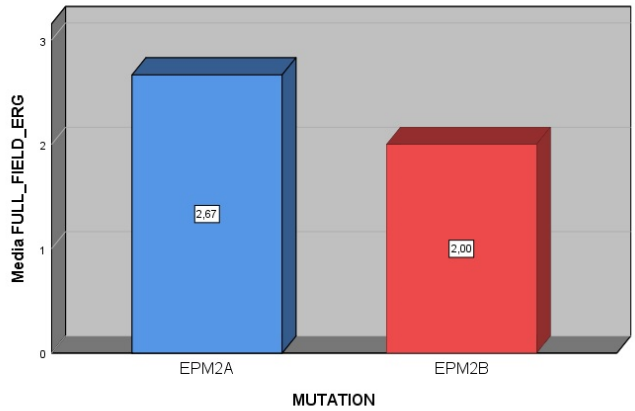

Figure 6. Mean full-field ERG values in the *EPM2A* and *EPM2B* mutations subgroups.

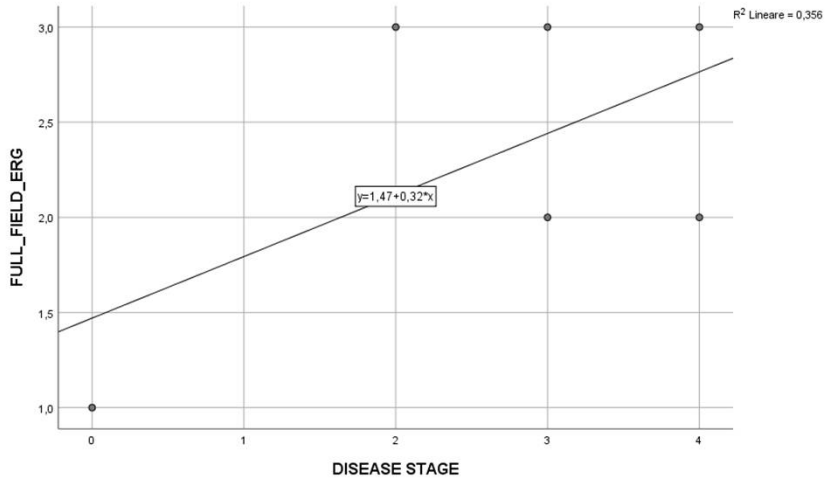

Figure 7. Graph showing positive linear correlation between LD stage and the grade of cones' and rodes' dysfunction at the ERG analysis.

Legend: R^2 Linear=

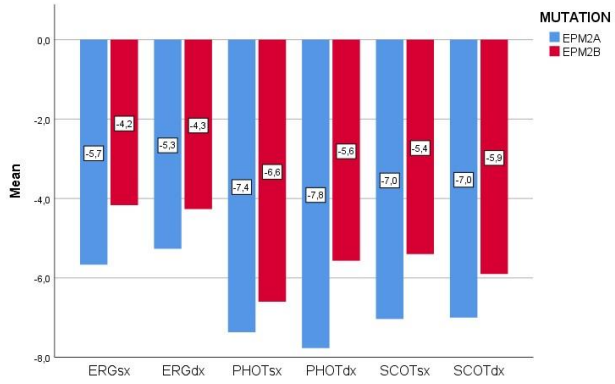

Figure 8: Mean full-field ERG results for the right and left eye each in the EPM2A and EPM2B subgroups.

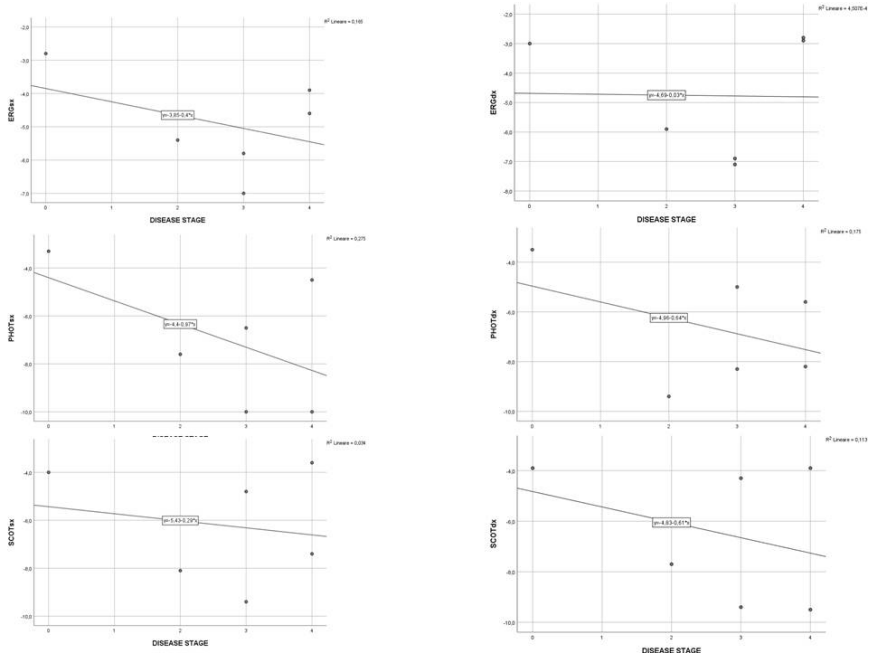
ERGtx
DISEASE STAGE
PHOTtx
DISEASE STAGE
SCOTtx
DISEASE STAGE
ERGdx
DISEASE STAGE
PHOTdx
SCOTdx
DISEASE STAGE

Figure 9: