TITLE: CLINICAL AND MOLECULAR CHARACTERIZATION OF INFANTILE NEUROAXONAL DYSTROPHY PATIENTS

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ABSTRACT

Neuroaxonal dystrophies are a group of clinically and genetically hetrerogeneous neurodegenerative disorders. In classic infantile neuroaxonal dystrophy (INAD) patients present with psychomotor regression, neurological deterioration and progressive optic atrophy. Mutations in the PLA2G6 gene are responsible for this phenotype. Case series report of this very rare condition. We describe the clinical findings of 3 unrelated portuguese INAD patients. Standard complete clinical and ophthalmological characterization were undertaken, ompleted with automated sequencing of the PLA2G6 gene.. We present the molecular findings and try to establish potential genotype-phenotype correlation.

RESUMO

As distrofias neuroaxonais englobam um grupo de patologias neurodegenerativas clinicamente e geneticamente heterogéneas. Doentes com distrofia neuroaxonal infantil clássica (INAD) apresentam uma clínica de regressão psicomotora, deterioração neurológica e atrofia ótica progressiva. Mutações no gene PLA2G6 são responsáveis por este fenótipo. Apresentamos neste trabalho achados clínicos em 3 doentes portugueses com INAD, sem relação de parentesco. Foi realizada uma caracterização oftalmológica completa e análise molecular por sequenciação automática do gene PLA2G6. Descrevemos os achados clínicos e moleculares destes doentes e analisamos potenciais relações genótipo-fenótipo

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KEYWORDS: Neurodegeneration with brain accumulation, Infantile Neuroaxonal Dystrophy, Atypical Neuroaxonal Dystrophy, PLA2G6, Seitelberger's Disease, Optic Atrophy.

ABBREVIATIONS:

Atypical NAD: Atypical Neuroaxonal Dystrophy

NBIA: neurodegeneration with iron accumulation

CNS: central nervous system

EEG: electroencephalogram

EMG: electromyogram

INAD: Infantile Neuroaxonal Dystrophy

iPLA2-VIA: group 6 phospholipase A2

MRI: magnetic resonance imaging

OMIM: Online Mendelian Inheritance in Man

PKAN: Phantothenate Kinase-Associated Neurodegeneration

PLA2G6: group 6 phospholipase A2 gene

PLAN: *PLA2G6*-Associated Neurodegeneration

INTRODUCTION

Neurodegeneration with Brain Iron Accumulation disorders (NBIA) overlie a homogenous group of inherited diseases that compound a common physiopathologic basis, despite the different genetic etiology and clinical outcomes. By definition, these disorders are characterized by the abnormal accumulation of iron in the basal ganglia The exact

relationship between iron accumulation and the symptoms of NBIA is not fully understood For most of these conditions, we cannot find a significantly large body of knowledge according to each complete metabolic and transcriptional pathway that lead to the gradual degeneration process. (Essay, 2013) For this reason, at this time, there is no cure.

Currently, there are nine identified forms of NBIA, although the majority of cases may be included in two sub-types: Phantothenate Kinase-Associated Neurodegeneration (PKAN) or Hallervorden–Spatz syndrome secondary to mutations in the *PANK2* gene, which is the most common of all NBIAs, appearing in a context of extra-pyramidal symptoms; and *PLA2G6*-Associated Neurodegeneration (PLAN), which may present two different clinical phenotypes, namely Infantile Neuroaxonal Dystrophy (INAD, #no. 256600 in OMIM), as the early-onset form, and Atypical Neuroaxonal Dystrophy, the late onset form. (Khateeb et al., 2006). The incidence of NBIAs is estimated to have a frequency of 3 per 1 million individuals and equally affects males and females. Because rare conditions like NBIA often go unrecognized, these disorders may be underdiagnosed or misdiagnosed, making it difficult to determine the accuracy of these estimates. (Kurian *et al* 2011)

INAD, also known as Seitelberger's disease, is an autosomal dominant condition which results from mutations in the *PLA2G6* gene; to date 44 distinct mutations have been described. The *PLA2G6* gene maps on the long arm of chromosome 22 (22q13.1), and encodes cytosolic Ca^{2+} -independent phospholipase A2, group VIA (iPLA2-VIA), which is a critical enzyme crucial for maintenance of cell membrane homeostasis. As previously stated, researchers are yet to find a direct relationship between this malfunctioning enzyme and iron deposition in axons, leading to the formation of spheroids. (Strokin *et al*, 2012)

iPLA2-VIA participates in a complex pathway of homeostasis upholding. As a Ca^{2+}-independent enzyme, it regulates the finalization of a yet incompletely understood mechanism. It is known that VIA iPLA2 has been shown to be essential to activate Orai1, a

pore-forming subunit that undertakes the Ca^{2+} release-activated Ca^{2+} channels. Though it has been demonstrated that Orai1 needs to be modified in order to launch a Ca^{2+} compensation. This signaling is established indirectly by the stromal interaction molecule 1, when a lack of Ca^{2+} is sensed in the Endoplasmic Reticulum storage. The reason why these storages run out of Ca^{2+} is explained by a mechanism of hydrolysis of Phosphatidyl-4,5-biphosphate, a specific phospholipid component of the cell membrane. This hydrolysis is performed by Phospholipase C, forming InsP3 (Inositol 1,4,5-Trisphosphate), which engages a second-messaging process to Gq protein-coupled metabolotropic receptor on the ER storages, inducing the aforementioned Ca^{2+}storage deflation (Strokin *et al*., 2012)

Mutations in the *PLA2G6* gene may trigger a lack of this Ca^{2+} compensatory mechanism which, in turn, may lead to a breach in cellular homeostasis, fatty acid accumulation, lysophospholipid accumulation, and then, a modification of the membrane composition and, ultimately, a change in cellular structure. This feature will somehow trigger a process that results in the deposition of iron, causing the appearance of symptoms that will lead to either phenotype: INAD or Atypical NAD. (Strokin *et al*., 2012)

We present a group of 3 unrelated INAD patients who present some distinct features from those expected in typical INAD.

INAD usually includes early-onset of disrupted development symptoms, starting before the age of 3; later presentations are quite rare. Affected children usually display progressive psychomotor delay or regression, associated with symmetric pyramidal signs (Babinsky sign is usually present). These signs comprise hypotonia, spastic tetraplegia and lower limb hyporreflexia. Convulsive frames may be also associated with INAD patients, which can be in part explained emphasizing the fact we are discussing neurologic features of young children, to whom these expressions are frequently associated. For these reason, this peculiarity is not fully considered as INAD parameter, along with lower limb hyporreflexia.

Ophthalmological signs and symptoms comprise insidious optic atrophy (noticed between 10 and 24 months of age) underlying the decrease of visual acuity and appearance of manifest sensory nystagmus (which may represent the first manifestation). Disease evolves rapidly with continuous motor and cognitive deterioration and most patients die before age 10. (Kurian *et al*, 2011)

Atypical NAD, as a juvenile presentation form, is a rare condition among the NBIA disorders. However, as a *PLA2G6*-associated disease, has a typical set of manifestations, including seizures, myoclonus, progressive ataxia and dementia. Prenatal or neonatal forms have also been described. (Zhang *et al.*, 2013)

INAD should be considered in a child with psychomotor regression or arrest, associated with a progression of locomotive deficit, and, mainly, an insidious increase of hypotonia and muscular weakness from 6 to 12 months old. (Carrilho *et al*., 2008)

Among the supplementary diagnostic exams, EEG.should be underscored. It should be performed after the identification of the first disease manifestations, and periodically to follow-up the degenerative process. INAD patients usually show records of fast rhythms with high amplitude, both in vigil and non-vigil states. Nevertheless, these characteristics are not pathognomonic of INAD, and diagnosis should always be clinically supported. Furthermore, these fast rhythms are hardly ever observed before age 2; recent studies, however, have documented some exceptions. Hence, we should investigate these fast rhythms, particularly those of early-onset, which can be confused for muscle artifacts, sleep spindles or drug-induced beta activity. Background activity is frequently normal, though occasionally slow. Also slow waves and epileptiform activity can be found on EEG. (Carrilho *et al*., 2008)

Even in patients who have confirmed INAD by molecular testing, EMG may fail to evidence degeneration. (Carrilho *et al*., 2008)

INAD patients present some distinctive MRI findings, such as insidious atrophy of the inferior cerebellar vermix, presence of T2-weighted hypersignal of the cerebellar cortex (these changes may be identified at disease presentation) and hypointensity of the globus pallidus and substantia nigra (detected in later stages and explained by iron deposition). Differential diagnosis may be MRI-assisted: patients with PKAN evidence the "tiger eye signal", which helps the exclusion of any NBIA by PLAN. (Rossi *et al*., 2012)

MRI spectroscopy also provides some important clues to diagnosis; some studies have identified N-acetylaspartate reduction and the presence of lactate in the basal ganglia. (Mader I et al, 2001). In order to avoid peripheral biopsy, a neuropediatrician/geneticist suspect a INAD diagnosis upon observing, comparing and interrelating neurophysiological studies and cerebral MRI, with subsequent molecular confirmation derived from mutation screening of the *PLA2G6* gene. (Carrilho *et al*., 2008)

Prior to molecular testing and in the rare cases where molecular testing fails to identify the genetic cause, INAD may be highly suspected from skin and peripheral nerve biopsy findings. As a pathologic signature, these biopsies may identify axonal edema, and the presence of neuroaxonal spheroids, present along the CNS and unmyelinated peripheral nerve endings of skin and conjunctiva. Electron microscopy (EM) examination may assist in clearly distinguishing these structures. Although the recognition of these spheroids is difficult, their evidence in a biopsy can be extremely important, especially in those patients in which no *PLA2G6* mutations are found, despite strong clinical suspicion. (Carrilho *et al*., 2008)

Prenatal diagnosis is essential in this devastating entity. However, some *in utero* studies have been reflecting peculiar and characteristic signs of a Fetal Neuroaxonal Dystrophy, not regarding, this way, a mutation that is *PLA2G6* related. This different genetic profile may show an even earlier onset and, therefore, a different pathophysiological and clinical approach.

Schindler disease is a differential diagnosis, which is caused by mutations in the α-N-acetilgalactosaminidasis (*NAGA)* gene and may be difficult to differentiate from INAD, both in clinical and histological terms. This lysosomal storage disease presents with severe psychomotor retardation, myoclonic seizures, optic atrophy, blindness, severe pyramidal signs and total loss of verbal communication around age 3-4. Spheroids are also observed in the terminal axons of grey matter and secondary dimetric demyelination of white matter. The diagnosis may be reached through the determination of oligosaccharides in urine and NAGA enzymatic activity assays in leucocytes or fibroblasts. (Khateeb *et al*., 2006) Many patients have been diagnosed with INAD in association with Schindler disease, showing that these conditions are not always established independently from each other. (Carpenter *et al*., 2012)

MRI, EEG, enzymatic assays results and clinical features allow the differentiation of INAD from a few conditions that may be considered at the time of the diagnosis, such as metachromatic leukodystrophy, GM1 gangliosidosis, ceroid neuronal lipofuscinosis (infantile) and Leigh syndrome. (Gordon N *et al*., 2002)

Forty percent of INAD patients in a study by Morgan *et al.* presented high iron composition in the globus pallidus. According to this, we should consider INAD as a strong differential diagnosis, despite the inexistence of reports of *PANK2* mutations in INAD patients. Moreover, we should highlight the aforementioned update of 44 *PLA2G6* independent mutations reported by Morgan *et al*.

Currently, there is limited information regarding *PLA2G6* mutations in Portugal. There seems to be a trend towards a specific *PLA2G6* nonsense mutation. Also, it is possible to establish a diagnosis of INAD by prenatal diagnosis in mutation positive families.

In 2008, a study from the University of Porto proposes a flowchart to assist on the establishment of INAD diagnosis, adapted to the portuguese reality. The study proposes an approach to all patients who present arrest or regression in psychomotor development

between age six months and three years, bilateral pyramidal tract signs, marked hypotonia and nystagmus. Hence, the patient only proceeds with genetic tests comprising *PLA2G6* mutation detection, if EEG and/or brain MRI are found with typical INAD findings. If those show an atypical presentation, the physician should consider another diagnosis and repeat the EEG 3 months later.

If one moves forward onto genetic testing, one should consider that not identifying a causative mutation is not an exclusion for the diagnosis. Thus one may get a diagnosis confirmation of INAD when a *PLA2G6* mutation is identified (redirecting the patient to genetic counseling) and still account a fair probability of INAD if mutation detection is negative. In order to ascertain this probability, we shall perform a skin biopsy, in which one should find the aforesaid findings. If the pathologist reports those as absent, one should, therefore, consider another diagnosis. On the other hand, upon diagnosis confirmation, more extensive molecular studies are required. (Carrilho *et al*., 2008)

POPULATION AND METHODS

We present a case series of 3 unrelated patients carrying the diagnosis of INAD. Detailed phenotypic characterization was performed, including family history, best-corrected visual acuity (BCVA), slit-lamp examination, fundus examination and fundus photography.

Colour fundus photographies (35º) of both eyes (centered to the macula and to the optic nerve) were taken in order to document the features of these structures, with a Topcon TRC-50 IA Retinal Ophthalmic Camera.

Genomic DNA was prepared from venous leukocytes and for genetic testing, coding exons and flanking intronic regions of the *PLA2G6* gene was PCR-amplified, purified, and sequenced.

This study was approved by the local ethics committee and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the patient prior to collection of clinical data and genomic samples.

RESULTS

Clinical and molecular results are summarized in tables 1, 2 and 3. It is important to underscore that all patients present *PLA2G6* nonsense mutations in either homozygosity or double heterozygosity.

Table 1- Clinical Features

Patient Sex and Birth date	Family History	Age at onset	Initial symptoms	First observation (age)	Seizures (age) medication	Ophthalmic Examination (age)	Other Findings
MBN Male 15/03/2010	N	15 M	Loss of acquired skills (lost ability to walk)	18 M Hypotonia, reflexes++, extensor plantar response	Absent	37m Variable visual contact, esotropia, abolished pupilary reflexes, optic pallor	Cerebellar atrophy
AFLC Female 13/12/2005	N	30 M	Loss of acquired skills (lost ability to walk with developmental regression)	33 M	Focal, generalized (7 years) Levetiracetam	28m Normal visual contact, esotropia, limitation of abduction, temporal optic Pallor	EMG: severe chronic motor neuronopathy. Muscle and nerve biopsy confirm diagnosis.

AJAS Female 10/09/2005	N	6 M	Loss of acquired skills (lost ability to sit, with developmental regression)	9 M Hypotonia, reflexes+, extensor plantar response	Absent	28m Normal visual contact, esotropia, limitation of abduction, temporal optic pallor	Muscle and nerve biopsy confirm diagnosis

Table 2 – Ophthalmological follow-up

Patient Sex and Birth date	Family History	Age at onset	First observation (age)	First Ophthalmic Examination (age)	Final Ophthalmic observation (age)
MBN Male 15/03/2010	N	15 M	18 M Hypotonia, reflexes++, extensor plantar response	37 M Variable visual contact, ET, abolished pupillary reflexes, optic pallor	48 M Poor visual contact (looks through) Abolished papillary reflexes. Variable ET. Optic atrophy with drop out NFL
AFLC Female 13/12/2005	N	30 M	33 M	28 M Normal visual contact, esotropia, limitation of abduction, temporal optic pallor	8 Y 8 M Poor visual contact. Alternating esotropia. Limitation of vertical motility. Optic atrophy with considerable NFL drop-out
AJAS Female 10/09/2005	N	6 M	9 M Hypotonia, reflexes+, extensor plantar response	28 M Normal Visual Contact, ET, Limitation of Abduction, Temporal optic pallor	6 Y. Age appropriate visual contact. Manifest latent nystagmus. Alternating esotropia. Temporal optic atrophy, worsening of NFL drop-out.

Legend: Y: Years; M: months; ET: esotropia; NFL: nerve fiber layer

Table 3- Genetic Findings

Patient Sex and Birth date	Family History	Gene *PLA2G6*	Polypeptide PLA2G6	Mutation Type	References
MBN Male 15/03/2010	No consanguinity	c.1442T>A c.2370T>G	p.Leu481Gln p.Tyr790X	Missense Nonsense	Morgan et al 2006. Nat Genet.38(7): 752-4
AFLC Female 13/12/2005	No consanguinity	c.2370T>G Partial deletion involving exons 3 and 4	p.Tyr790X p.251_G253del	Nonsense Nonsense	Morgan et al 2006. Nat Genet.38(7): 752-4
AJAS Female 10/09/2005	Consanguinity ?	c.2370T>G c.2370T>G	p.Tyr790X p.Tyr790X	Nonsense Nonsense	Morgan et al 2006. Nat Genet.38(7): 752-4

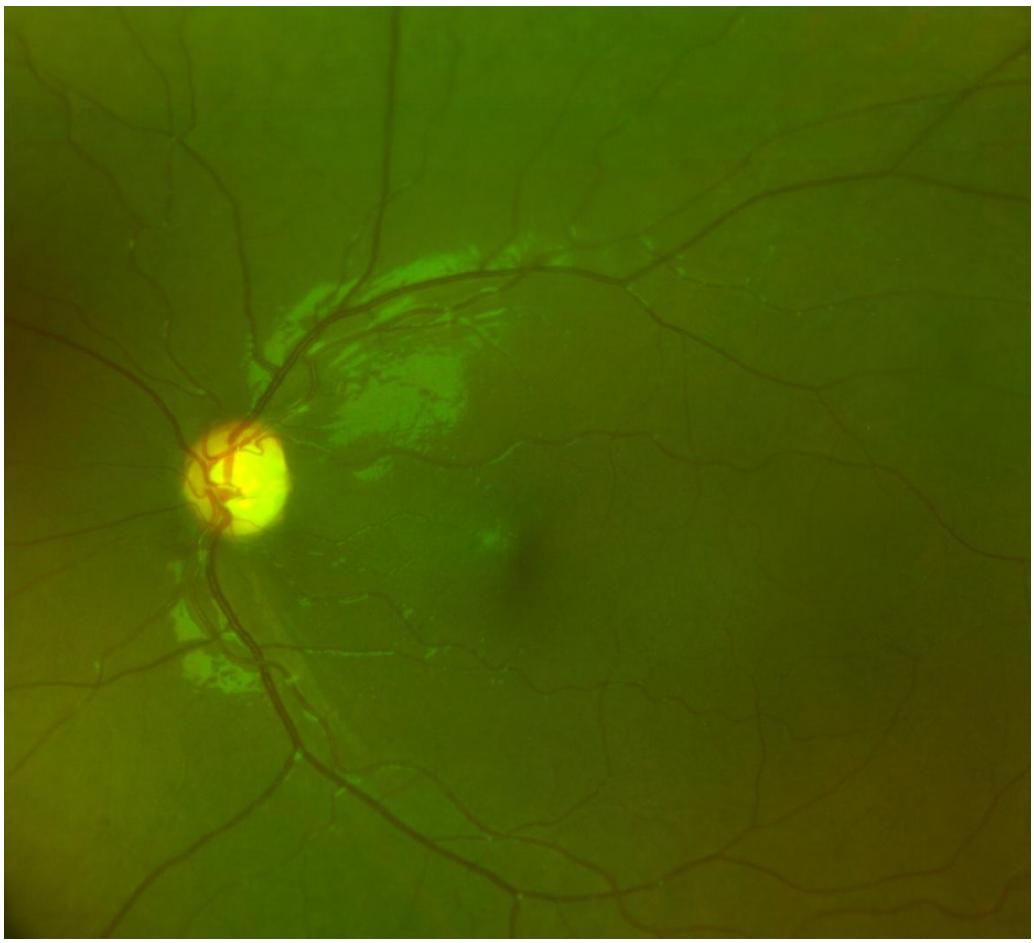

Figure 1: Left eye fundus image depicting optic disc pallor in the temporal half, vessels of normal caliber and normal retina.

DISCUSSION

Carrilho *et al* reported a group of 10 caucasian portuguese patients (seven girls and three boys), all with very similar clinical features; ophthalmic findings also included those which we highlight: nystagmus, hypotonia, optic atrophy, and, less frequently (only 2), strabismus. All of them presented loss or arrest of acquired skills (to sit, to walk, to talk, depending on the age). (Carrilho *et al*., 2008)

In our series, there is no history of pregnancy or perinatal complications. We report an early onset of the condition (from 6 to 30 months old). All patients presented with an evident development arrest/regression, reflected by loss of acquired skills according to the age (ability to sit/walk). This progressive motricity impairment remained, along with the appearance of other symptoms. The Porto's series reveals similar age of onset (ranging from 6 to 18 months) with the same initial features of developmental arrest, and with 4 patients showing regression in acquired skills.

In association with the developmental deficit, we highlight the presence of severe hypotonia in all patients. Visual behavior is usually maintained within normal limits during the initial phases of disease, however in our series one patient presented variable visual contact; this child also presented absence of pupillary reflexes, different from the clinical findings of the other two. There is no record of nystagmus in any of them, as well as any significant refractive error. Despite the absence of hyperopic refractive error,, all patients presented with esotropia.

Summarizing the clinical presentation, we report some relevant and distinct features and ophthalmologic findings, such as the typical loss of acquired skills (stressing mainly motricity skills), progressive impairment of visual contact, presence of esotropia and absence of significant refractive error.

With regards to the fundus appearance, we observed a rapid progressive impairment of the optic pallor with a significant dropout of the nerve fibers layer, corresponding to the ganglion cells apoptosis. We were able to obtain autofluorescence imaging in one case (data not shown). No changes were observed in a normal autofluorescence pattern, which allows inferring that no significant disturbances are found in the external layers of the retina (retinal pigment epithelium and photoreceptors layers).

Hence, fundus findings in combination with knowledge of the physiopathology of generalized NBIAs, allows the suggestion that the rapidly progressive optic atrophy may be related to iron deposition in the ganglion cells. Such finding is also observed in other neurodegenerations secondary to cerebral iron accumulation, like PKAN, in which identical fundi are commonly observed.

Regarding the genetic findings, all patients present a common *PLA2G6* mutations, reported by others (Carrilho et al, 2008; Morgan et al, 2006) which is also the most frequent mutation nonsense p2370>G in the other Portuguese series (Carrilho et al, 2008) (there are 4/10 patients with this mutation - 1 heterozygous and 3 homozygous). Curiously, the genetic change only truncates the C-terminal region of the protein, but apparently has significant function impact to the protein. This may be the result of abnormal folding of the protein which may compromise the appropriate position of the active motifs. The Y790X mutation may represent a hotspot and should be considered in the initial molecular diagnosis of every Portuguese INAD suspect. This mutation has been identified in cases of classic INAD and neurodegeneration with brain iron accumulation (Morgan et al, 2006). We should also underscore the fact that homozygous or double heterozygous missense mutations in the *PLA2G6* gene have been associated with a juvenile onset autosomal recessive Parkinson variant in chinese and japonese populations (Shi et al, 2012)..

Unfortunately, one can only offer these patients supportive measures (physical and psychological support to relatives) in addition to medically controlling seizures, when they are present. Researches are trying to pursue a cure for this type of NBIA. Until now, no relevant findings were reported regarding INAD definitive treatment.

Concerning the resmainder of NBIAs, a recent study (Ge et al, 2011) presents some preliminary results with Deep Brain Stimulation of the subthalamic nuclei as a possible cure for some NBIA type 1 (PKAN- Hallervorden–Spatz syndrome). Although, this is a single case report, the authors refer this therapy with DBS of *globus pallidus internus* or thalamus as a method with partial improvement. Because improvement was seen, the authors assume an advantage sub-thalamic nuclei DBS over the traditionally intervened areas. (Ge et al., 2011)

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