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TRABALHO FINAL DO 6ºANO MÉDICO COM VISTA À ATRIBUIÇÃO DO GRAU DE MESTRE NO ÂMBITO DO CICLO DE ESTUDOS DE MESTRADO INTEGRADO EM MEDICINA

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PHENOTYPICAL AND MOLECULAR CHARACTERIZATION OF PORTUGUESE LEBER CONGENITAL AMAUROSIS PATIENTS

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List of abbreviations

AIPL1 [604392]: *Arylhydrocarbon-interactiong receptor protein-like 1*

AR: Autosomic recessive

BCVA: Best corrected visual acuity

CABP4 [610427]: *Calcium binding protein 4*

CEP290/NPHP6 [610142]: *Centrosomal protein 290 kDa/Nephrocystin 6*

CNS: Central nervous system

CRB1 [604210]: *Crumbs homolog 1*

CRX [602225]: *Cone-rod otx-like photoreceptor homeobox transcription factor*

ERG: Electroretinogram

RPE: Retinal pigment epithelium

GUCY2D [600179]: *Retinal-specific guanylate cyclase*

IMPDH1 [146690]: *Inosine monophosphate dehydrogenase 1*

IQCB1/NPHP5 [609237]: *IQ motif-containing protein B1/ Nephrocystin 5*

KCNJ13 [603208]: *Inwardly rectifying potassium channel Kir7.1*

LCA: Leber Congenital Amaurosis

LCA5 [611408]: *Lebercilin*

LCA9 [608553]: *Leber congenital amaurosis 9*

LE: Left eye

LP: Light perception

LRAT [604863]: *Lecithin retinol acyltransferase*

NFNF: No fix no follow

NLP: No light perception

NV: Navigational vision

OTX2 [610125]: *Orthodenticle protein homolog 2*

OU: Both eyes

QRX [610362]: *Q50-Type Retinal Homeobox*

RD3 [180040]: *Retinal Degeneration 3 (Mouse Homolog of)*

RDH12 [608830]: *Retinol dehydrogenase 12*

RE: Right eye

RPE65 [180069]: *Retinal pigment epithelium-specific 65kD protein*

RPGRIP1 [605446]: *Retinitis pigmentosa GTPase regulator-interacting protein 1*

SLSN: Senior-Loken Syndrome

SPATA7 [609868]: *Spermatogensis-associated protein 7*

TULP1 [602280]: *Tubby-like protein 1*

Abstract

Introduction: Leber Congenital Amaurosis encompasses a group of early onset retinal dystrophies causing severe visual impairment, nystagmus and retinal dysfunction. It is mostly an autosomal recessive condition and to date 19 genes have been identified as potential culprits. Our aim is to characterize in a molecular and phenotypical standpoint, 28 affected Portuguese patients, determine if they carry mutations in the known genes and establish potential genotype-phenotype correlations both with respect to retinal structural and functional changes.

Methods: Twenty eight individuals from 26 unrelated families (twelve males, sixteen females) were characterized by clinical examination, electrophysiology (ERG), mutation analysis, optical coherence tomography (OCT), autofluorescence, head MRI and renal function testing.

Results: LCA was demonstrated in all patients. Consanguinity could be documented in 25% of families. Clinically, patients complained of nyctalopia in 21% of cases and the typical oculo-digital sign of Franceschetti was observed in only 18% of cases. High hyperopia was the most prevalent refractive error. In our cohort the fundus appearance varied from anatomically normal (4%), non-specific changes/atrophy of the retinal pigment epithelium (RPE) (25%), peripheral pigmented changes (64%) and macular coloboma-like defects (25%). We observed 29% of cases with some degree of developmental delay and 21% with clear signs that fit criteria of the autism/autistic behaviour spectrum. Molecular testing is still an ongoing process; thus far, causative mutations in the known LCA genes have been identified in 5 independent cases with the NPHP6 gene being mutated in 3 patients, the RPGRIP1 gene in one patient and the NPHP5 in another patient. The NPHP5

patient was later reclassified as Senior-Loken syndrome. Our results fit those found in international literature.

Conclusion: We characterize from a clinical and genetic standpoint, the largest series of Portuguese patients with LCA. In-depth knowledge of this group of conditions is invaluable for appropriate counselling and possibly treatment, in the near future.

Resumo

Introdução: A Amaurose Congénita de Leber abrange um grupo de distrofias retinianas de aparecimento precoce que causam baixa de visão grave e disfunção retiniana. É uma condição maioritariamente autossómica recessiva e, até à data, 19 genes foram identificados como possíveis causadores desta doença. O nosso objectivo neste trabalho é caracterizar molecular e fenotipicamente 28 doentes Portugueses, determinar se são portadores de mutações nos genes conhecidos e estabelecer potenciais correlações genotípicas-fenotípicas, tanto no que respeita à estrutura retiniana como às alterações funcionais.

Métodos: 28 doentes de 26 famílias não relacionadas (12 homens, 16 mulheres) foram caracterizados do ponto de vista clínico, electrofisiológico (ERG), análise de mutações, tomografia de coerência óptica (OCT), autofluorescência, ressonância magnética nuclear craniana e testes de função renal.

Resultados: O diagnóstico de Amaurose Congénita de Leber foi demonstrado em todos os doentes. A consanguinidade foi documentada em 25% das famílias. Clinicamente os doentes apresentavam-se com nictalopia em 21% dos casos e com o típico sinal oculo-digital de Franceschetti em apenas 18% dos casos. A hiperopia foi o erro refractivo mais prevalente. Neste estudo, a aparência do fundo ocular variou entre o anatomicamente normal (4%), sem alterações específicas/atrofia do epitélio pigmentado da retina (25%), alterações pigmentares na periferia (64%) e defeitos maculares *coloboma-like* (25%). Observámos 29% dos casos com algum grau de atraso de desenvolvimento e 21% com sinais claros de autismo/comportamento autista. Apesar de a análise genética ainda estar em curso, até agora foram identificadas mutações causais em 5 doentes, dos quais 3 se

localizam no gene NPHP6, 1 no gene NPHP5 e outra no RPGRIP1. O doente com NPHP5 mutado foi posteriormente reclassificado como Síndroma de Senior-Loken. Os nossos resultados são compatíveis com os encontrados na literatura internacional.

Conclusão: Caracterizamos, de um ponto de vista clínico e genético, a maior série de doentes Portugueses com LCA. O conhecimento aprofundado sobre esta condição é imprescindível para o aconselhamento e possível tratamento destes doentes, num futuro próximo.

Introduction

Leber Congenital Amaurosis (LCA) encompasses a group of early-onset retinal dystrophies caused by mutations in genes that are essential for the normal development and/or function of the retina. It was first described by Gustav von Leber in 1869 after noticing that 25% of the students in a school for the visually handicapped were descendants of consanguineous parents (1).

It is the most severe form of inherited retinal dystrophies causing blindness or severe visual impairment in childhood and accounts for approximately 5% of all inherited retinopathies (2), with a frequency between 1:30000 (2) and 1:81000 (3).

The clinical hallmarks of LCA are abolished or severely reduced electrical rod and cone signals on the electroretinogram, early and severe visual impairment, early-onset nystagmus (pendular or roving eye movements) and sluggish or near absent pupillary responses (1, 4). The visual function of LCA patients is highly variable, ranging from 20/50 to no light perception. These acuities usually remain stable, although some patients lose visual function with disease progression while, in rare cases, some patients improve (this pattern has been described in association with mutations in the *CRX* gene) (2, 5). Other clinical features sometimes present include keratoconus, cataracts, macular "coloboma", high refractive errors (mostly hyperopia), photophobia or nyctalopia, enophthalmos and the oculo-digital sign of Franceschetti. The association between LCA and mental retardation remains unclear with some studies reporting no cases (5, 6) while others describe several families with mental retardation when harbouring *CEP290* mutations (7). The same controversy applies to the potential association between LCA and autism/autistic behaviour. Some reports of olfactory dysfunction are found in the literature in patients with *CEP290* mutations (8).

Phenotypical heterogeneity is the hallmark of LCA. This applies to the fundus appearance, which can range from an almost anatomical intact retina to a variety of different appearances, such as salt-and-pepper pigmentation, whitish deposits, bone spicules (indistinct from retinitis pigmentosa), vessel attenuation, maculopathy or macular coloboma-like defects. There is a correlation between the genotype and observed phenotype, both in retinal appearance and visual function.(2, 5, 9)

To correctly diagnose this retinal dystrophy, an adequate ophthalmic history as well as a family history, a complete clinical evaluation and an electrophysiological testing are needed. Other exams may be necessary to exclude the differential diagnosis such as colour vision testing, visual fields, autofluorescence and ocular coherence tomography (OCT) scans (10, 11). Systemic assessment should also include head MRI and renal function testing.

LCA is mostly an autosomal recessive condition and to date more than 640 mutations in 19 genes have been identified as potential culprits. However, these genes only account for approximately 70% of LCA cases. A molecular diagnosis is extremely important to support the clinical diagnosis and provide a more accurate visual prognosis based on genotype-phenotype correlations that have been established for several LCA genes (10, 12). The genes discovered thus far are implicated in phototransduction (*AIPL1, GUCY2D*) (13, 14), in the retinoid cycle (*RDH12*, *LRAT*, *RPE65*) (15-17), in photoreceptor development and structure (*CRX*, *OTX2* and *CRB1*) (18-20), transport across the photoreceptor connecting cilium (*TULP1*, *RPGRIP1*, *CEP290*, *LCA5/Lebercilin*) (21-24), and guanine synthesis (*IMPDH1*) (25). The precise role of *RD3* and *SPATA7* in the pathogenesis of LCA remains unknown (12, 26, 27). Recently, *ICQB1/NPHP5*, *CABP4* and *KCNJ13* have been proposed as causative genes (28-30). *LCA9* has been mapped but the gene is yet to be cloned (31).

This condition was the first amenable for retinal gene therapy (*RPE65*) with positive results both in efficacy and safety (32-34). Thus, it became even more crucial to characterize every patient from a molecular standpoint.

The purpose of this paper is to characterize from a phenotypical and genotypical standpoint 28 unrelated Portuguese patients with LCA, determine if they carry mutations in the known genes, and establish potential genotype-phenotype correlations both with respect to retinal structural and functional changes.

Material and Methods

Patient and control population

Twenty eight individuals from 26 unrelated families (twelve males, sixteen females) were included in this study.

Affected individuals are followed at the Centre for Hereditary Eye Diseases of the Department of Ophthalmology, University Hospital of Coimbra (CHUC). Probands and affected family members presented at our clinic mostly due to early onset nystagmus associated with severe visual impairment. Controls were obtained from the general population and did not fit the diagnostic criteria of LCA.

All individuals, their parents or legal guardians included in the study were informed about its objectives and volunteered to participate. Informed consent was obtained from all subjects, their parents or legal guardians in accordance with the tenets of the declaration of Helsinki. The study was approved by the Ethics Committee of the University Hospital of Coimbra.

Clinical Examination

Ophthalmic examination included assessment of best corrected visual acuity (BCVA) after manifest or cycloplegic refraction, ocular alignment and motility, slit-lamp examination and fundus examination using a non-contact 78-diopter lens. Fundus images were acquired in accordance with the internationally accepted guidelines using a TOPCON TRC 50X (Topcon Optical, Tokyo, Japan) and/or a Pan-retinal camera (Optomap R)

(Optos PLC, Dunfermline, Scotland, UK). Very young probands were examined under anesthesia, whenever necessary.

All affected individuals were screened for systemic findings and, in selected cases, head MRI and renal function work-up was obtained. Special attention was given to developmental characteristics, obesity, finger/toes abnormalities and other phenotypical aspects commonly found in association with other ciliopathies (e.g. Senior-Loken syndrome, Bardet-Biedl syndrome and Joubert syndrome).

Electrophysiology (ERG)

Ganzfeld ERG was performed in accordance with the ISCEV (International Society for Clinical Electrophysiology of Vision) guidelines. In brief, patients were dark-adapted for a period of 30 minutes followed by scotopic assessment. The ERG was then completed with recordings obtained in photopic conditions.

Optical Coherence Tomography (OCT) and Autofluorescence

OCT was performed using an OCT device (Stratus OCT; Carl Zeiss Meditec, Dublin, CA; or Spectral domain OCT, Heidelberg Engineering, Dossenheim, Germany) to obtain cross-sectional images centered in the macula, 26 with axial resolution of 10µm or less, transversal resolution of 20µm, and longitudinal scan range of 2 mm. With this OCT device (Stratus OCT; Carl Zeiss Meditec), six radial line scans 6 mm in length and 128 A-scans 30° apart were scanned in 1.92 seconds, and a nine-region retinal thickness map was obtained by segmenting the retina from other layers with an algorithm detecting the edge of the RPE and the photoreceptor layer.

Autofluorescence was performed in selected cases using the HRAII device (Heidelberg Engineering, Dossenheim, Germany) in accordance with the instructions from the manufacturer.

Molecular genetic testing

Peripheral blood samples with EDTA anticoagulant were collected from each patient and close relatives (for segregation analysis). Genomic DNA was extracted using an automated DNA extractor (BioRobot EZ1, Qiagen, Hilden, Germany).

Molecular testing was performed in the Genetics Department, Institut de Recherche IFR94, Hopital des Enfants Malades, Paris (supervisor: Prof. Josseline Kaplan). This is part of a collaborative effort aiming for the identification of new genes and mutations associated with the LCA phenotype.

Genomic DNA is PCR-amplified using previously described primers and conditions. All exons and intron-exon boundaries for each published LCA gene are being tested for each proband of our Portuguese cohort.
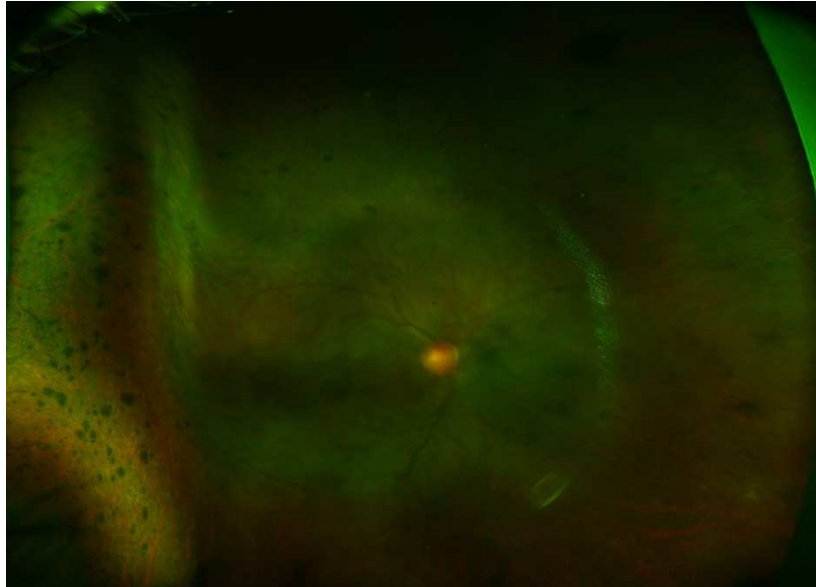
Results

Twenty eight individuals from 26 unrelated families (twelve males, fourteen females) met the diagnostic criteria for LCA and were included in this study. The clinical findings (ocular and systemic) are summarized in Tables I and II. All patients presented with various degrees of manifest nystagmus or roving eye movements (100%). ERG testing was undetectable in all probands and the typical oculo-digital sign of Franceschetti was observed in only 18% of cases. Nyctalopia was observed as an initial complaint in 21% of cases.

Two families had 2 affected individuals in the same generation (one case of homozygous twins and a sister-brother sibship) while the remainder were single affected. Consanguinity could be documented in 25% of families; however, in the majority of the remainder same geographic provenance was seen. We could not document a geographic preponderance in our cohort, with families widespread from north to south of Portugal and the Azores islands (one isolated case from an apparently non-consanguineous family). We had no cases coming from Madeira island.

Enophthalmos was present in 46%, being extremely marked in a few patients. Early onset cataracts were diagnosed in 14% and keratoconus was also present in 14%. As observed in other population studies, high hyperopia was the most prevalent refractive error, ranging from +2.00 to +10.00.

In our cohort the fundus appearance varied from anatomically normal (4%), non-specific changes/atrophy of the retinal pigment epithelium (RPE) (25%), peripheral pigmented changes (64%) (Fig.1) and macular coloboma-like defects (25%) (Fig.2).

Figure 1. Pan retinal fundus photo displaying severe pigmentary changes in the mid- and far-periphery. Shadow artefacts result from significant nystagmus.

Figure 2. Macular "coloboma-like" defect. Optic pallor, thin vessels and widespread changes in the RPE are present.

Special care was taken in obtaining a comprehensive systemic clinical evaluation. We observed 29% of cases with some degree of developmental delay and 21% with clear signs that fit criteria of the autism/autistic behaviour spectrum. Head MRI was performed in all cases with autism or developmental delay; however, significant structural abnormalities were not observed in our patients.

Molecular testing is still an ongoing process; thus far, causative mutations in the known LCA genes have been identified in 5 independent cases (Table III). These mutations severely affect protein function and represent null alleles. The *NPHP6* gene was mutated in 3 patients and 4 different alleles were identified: c.2991+1655A>G, c.2052+1delGT, c.2717C>T and a complete deletion of exon 20.

A homozygous *NPHP5* null mutation was identified in patient 3, c.1518-1519delCA. Finally, a homozygous mutation in the *RPGRIP1* gene, c.2759insT, was identified in another patient. In all cases, segregation analysis was performed and parents were found to be carriers of one disease allele.

In order to discuss potential genotype-phenotype correlations and to understand whether our results fit those found in the international literature we provide a clinical summary of every case for which the causative mutations have been found.

MFS is a 9 year-old female, second child born to non-consanguineous parents and no past family history of retinal disease. She presented at age 6 months with a typical LCA phenotype (roving eye movements, sluggish pupillary reflexes, high hyperopia, and flat ERG). She had no evidence of nyctalopia although she was always attracted to light sources. Until age 5 her retinas remained anatomically normal and she further developed whitish deep retinal deposits in the mid periphery with the sparing of the macular area (Fig.3).

Figure 3. Whitish pigment clumps in the mid-periphery. The macular area is relatively preserved.

Cortical cataracts were first noted ate age 6 evolving to total RE cataract. Systemic assessment was negative until age 8 and her developmental milestones were always reached before the average age. From a cognitive standpoint she always proved to be extremely intelligent. At age 8 renal assessment revealed moderate dysfunction that rapidly evolved to terminal renal failure and in the past five months she has been in peritoneal dialysis.

MHPAS is a 7 year old female, second child born to non-consanguineous parents and no family history retinopathies. She presented at age 9 months with a typical LCA phenotype (roving eye movements, sluggish pupillary reflexes, high hyperopia, and flat ERG). She had evidence of nyctalopia. Her bilateral enophtalmos has progressively worsened with age. Until age 4 her retinas remained anatomically normal and she further

developed whitish deep retinal deposits in the mid periphery with the sparing of the macular area (Fig.4). Systemic assessment was negative and her developmental milestones were within normal limits.

Figure 4. Fundus photo of an LCA child. Mid-periphery deep whitish deposits and slightly tortuous vessels are depicted.

FRNJN is a 9 year old female, first child born to non-consanguineous parents and no family history of retinal diseases. She presented at age 16 months with a typical LCA phenotype (extremely poor reaction to visual stimuli, manifest horizontal nystagmus, unreactive pupils, high hyperopia, and flat ERG). She had also evidence of nyctalopia. She was lost to follow-up for 6 years. At age 8 her binocular BCVA was 0.16 and her retinas disclosed whitish deep retinal deposits in the mid-periphery, sparing the maculas. Systemic assessment including head MRI was negative; however, she presented from the initial visit clinical traits highly suggestive of autistic behaviour.

MOL is a 33 year old male, born to non-consanguineous family with no past family history of retinopathies. He presented with a typical LCA phenotype (extremely poor reaction to visual stimuli, manifest horizontal nystagmus, arreactive pupils, and flat ERG). At his last ophthalmic assessment he presented with LP vision, cortical cataracts and RE keratoconus. His fundus examination disclosed macular and peripheral pigmentary changes, bilateral optic atrophy and extremely thin retinal vessels. He is otherwise normal both from systemic and developmental standpoints.

DSP is a 16 year old male, first child born to consanguineous parents and no family history of retinal diseases. He presented with a typical LCA phenotype (extremely poor reaction to visual stimuli, manifest horizontal nystagmus, paradoxical pupils, and flat ERG). At his last ophthalmic assessment he presented with NLP vision, cortical cataracts and his retinas disclosed coloboma-like central macular defects, pigment clumping in the mid and far-periphery (bone spicules), optic atrophy and extremely thin retinal vessels. He is otherwise normal from a systemic and developmental standpoint.

Table I. Clinical features and basic eye examination.

ID/family	Sex	Current Age (years)	BCVA*	Refractive errors (RE/LE)	Fundus appearance
LTR/1	Female	37	0,02	+7.00 / +6.00	Bull's eye maculopathy, peripheral whitish dots, RPE changes, narrow vessels, pale discs
MFS/2	Female	9	LP	+6.00 / +6.00	Mid peripheral whitish flecks, narrow vessels, pale discs
CJC/3	Female	16	LP, NFNF	+8.00 / +7.50	Bilateral macular coloboma, peripheral pigment, severe optic atrophy
JDC/3	Male	10	LP	+5.00 / +6.00	Macular coloboma, peripheral pigment
LCH/4	Male	15	LP	+7.00 / +7.00	Macular coloboma, peripheral pigment
FRNJN/5	Female	9	0.16	+6.50 / +6.50	Whitish dots mid periphery
MBN/6	Female	9	0.02	+2.00 / +2.00	Mid periphery whitish clumps, narrow vessels, pale discs
MOL/7	Male	33	LP	+7.00 / +7.00	Macular/periphery pigment clumps, extremely thin vessels, optic atrophy
IFB/8	Female	41	LP	+6.00 / +6.00	Macular/periphery pigment clumps, extremely thin vessels, optic atrophy
DSP/9	Male	16	NLP	+7.00 / +7.00	Coloboma-like, peripheral pigment, narrow vessels, optic atrophy
MHPAS/10	Female	7	LP	+5.50 / +5.50	Whitish alterations mid periphery
LJAA/11	Male	7	LP	+8.00 / +8.00	Narrow vessels, pale discs
MJP/12	Female	7	LP	+6.00 / +6.00	Macular coloboma, peripheral whitish dots
SARA/13	Female	11	0.02	+6.50 / +6.50	Macula/ periphery pigment clumps
MFRA/13	Female	11	0.02	+6.50 / +6.50	Macula/ periphery pigment clumps
MOSC/14	Female	42	0.05	+9.00 / +9.00	Macular coloboma, whitish dots, pigment clumps
RMARC/15	Female	34	0.1	+4.00 / +4.00	Peripheral whitish dots
MLPDM/16	Female	41	LP	+5.00 / +5.50	Macular pigment changes, salt-and-pepper retinopathy, RPE atrophy, narrow vessels, optic atrophy
TFVF/17	Female	16	0.1	+3.00 / -2.50	Heterogeneous centromacular changes
SMST/18	Female	29	0.02	+7.50 / +1.00	Whitish deposits, bone spicules, narrow vessels, pale discs
DLS/19	Male	16	0.02	-15.00 / -15.00	Macular coloboma, RPE changes, tilt disc
JCLA/20	Male	8	NV	+4.00 / +4.00	Without significant changes
MCM/21	Female	7	NFNF	+8.00 / +8.00	Peripheral pigment
PCM/22	Male	9	0.1	+3.00 / +3.00	Rod cone dystrophy, pale discs
NRPB/23	Male	32	0.002	+10.00 / +10.00	Centromacular changes, scarce vessels, pale discs
AJVG/24	Male	30	0.05	+4.00 / +5.00	Generalized RPE changes, narrow vessels, pale discs
DDPS/25	Male	4	NFNF	+5.00 / +5.00	Centromacular atrophy, generalized RPE changes, tortuous vessels, optic disc edema with pale discs
RFO/26	Male	4	NFNF	+7.50 / +7.00	Rod cone dystrophy, pale discs

* BCVA: Best corrected visual acuity; LP: Light perception; NFNF: No fix no follow; NLP: No light perception; NV: Navigational vision.

Table II. Additional clinical features and genetic results

ID/family	Inheritance	Other relevant ocular findings	Systemic findings	Gene Mutations
LTR/1	No			Pending
MFS/2	No	Severe enophthalmos; Cataract (total RE, cortical LE)	Very intelligent, renal involvement detected at age 8	NPHP5 HMz
CJC/3	No	Severe enophthalmos; Oculo-digital sign of Franceschetti	Autistic-like	NPHP6 neg
JDC/3	No	Severe enophthalmos	Autist	NPHP6 neg
LCH/4	Yes	Severe enophthalmos	Inteligent	Pending
FRNJN/5	No		Autistic behaviour	NPHP6
MBN/6	No	Enophthalmos	Development delay, ataxia	Pending
MOL/7	No	Severe enophthalmos; Keratoconus RE; Cataract OU		RPGRIP1
IFB/8	Yes			Pending
DSP/9	Yes	Severe enophthalmos		NPHP6
MHPAS/10	No	Enophthalmos; Nyctalopia		NPHP6
LJAA/11	No	Enophthalmos; Oculo-digital sign Franceschetti	Autistic behaviour	Pending
MJP/12	No			Pending
SARA/13	Yes	Enophthalmos		Pending
MFRA/13	Yes	Enophthalmos		Pending
MOSC/14	Yes	Enophthalmos; Nyctalopia		Pending
RMARC/15	No	Nyctalopia		Pending
MLPDM/16	Yes	Keratoconus		Pending
TFVF/17	Undetermined	Nyctalopia	Development delay	Pending
SMST/18	No			Pending
DLS/19	No	Nyctalopia		Pending
JCLA/20	No	Enophthalmos; Oculo-digital sign of Franceschetti; Photoatraction,	Autism, does not talk, hyperactive	Pending
MCM/21	No	Oculo-digital sign of Franceschetti		Pending
PCM/22	Undetermined	Nyctalopia		Pending
NRPB/23	No	Bilateral keratoconus; Cortical bilateral cataract		Pending
AJVG/24	No	Bilateral keratoconus; Esotropia; Cataract RE	Hearing and intelect above average	Pending
DDPS/25	No	Oculo-digital sign of Franceschetti	Very smart	Pending
RFO/26	No		Autistic-like	Pending

* *IQCB1/NPHP5* [609237]: *IQ motif-containing protein B1/ Nephrocystin 5; CEP290/NPHP6* [610142]: *Centrosomal protein 290 kDa/Nephrocystin 6; RPGRIP1* [605446]: *Retinitis pigmentosa GTPase regulator-interacting protein 1.*

Table III. Mutation analysis.

ID/family	Inheritance	VGene/Coding DNA/Allele 1/Allele 2	Predicted protein	Type
MFS/2	AR	NPHP5/c.1518-1519delCA/Homozygous	p.H506QfsX12	Frameshift null
MHAPS/10		NPHP6/c.2991+1655A>G/c.2052+1delGT/Double Heterozygous	p.Cys998X	Null
DSP/3		NPHNP6/c.2717C>T/Del exon 20	p.R908X	Null
FRNN/5	AR	NPHP6/c.2991+1655A>G/Homozygous	p.Cys998X	Null
MOL/7	AR	RPGRIP1/c.2759insT/Homozygous	p.Q920HfsX13	Null

* AR: Autosomic recessive; *IQCB1/NPHP5* [609237]: *IQ motif-containing protein B1/ Nephrocystin 5; CEP290/NPHP6* [610142]: *Centrosomal protein 290 kDa/Nephrocystin 6; RPGRIP1* [605446]: *Retinitis pigmentosa GTPase regulator-interacting protein 1.*

Discussion

We present the largest series of Portuguese LCA patients with an extensive follow-up. Each individual was extensively characterized from a clinical standpoint in an attempt to correlate the clinical findings with those derived from the molecular testing. However, to the present date, most of the genetics results are still pending. Molecular testing is still an ongoing process and is being performed in the Genetics Department, Institut de Recherche IFR94, Hopital des Enfants Malades, Paris (supervisor: Prof Josseline Kaplan). In our study, causative mutations in the known LCA genes have been identified in only 5 independent cases, which make it impossible to conclude about the frequency of each gene.

Molecular testing is essential to help in the identification of new genes and mutations associated with LCA. Having a molecular diagnosis can confirm preliminary clinical diagnosis, help predict the visual prognosis and is very important to couples who wish to have genetic counselling before pregnancy, to estimate the risk of LCA for future offspring. Finally, in recent years, gene therapy has become available for certain patients with a specific molecular diagnosis (RPE65 mutations), so it is crucial to help select those amenable for this type of treatment. However, the substantial allelic and genetic heterogeneity existent in LCA can cause severe technologic and economic difficulties therefore preventing the realization of more molecular diagnosis studies.

Phenotyical characterization of patients with LCA can be complicated by the presence of nystagmus. However, whenever OCT and autofluorescence studies can be done properly, they can be essential in differentiating patient's clinical characteristics (Fig.5 and 6).
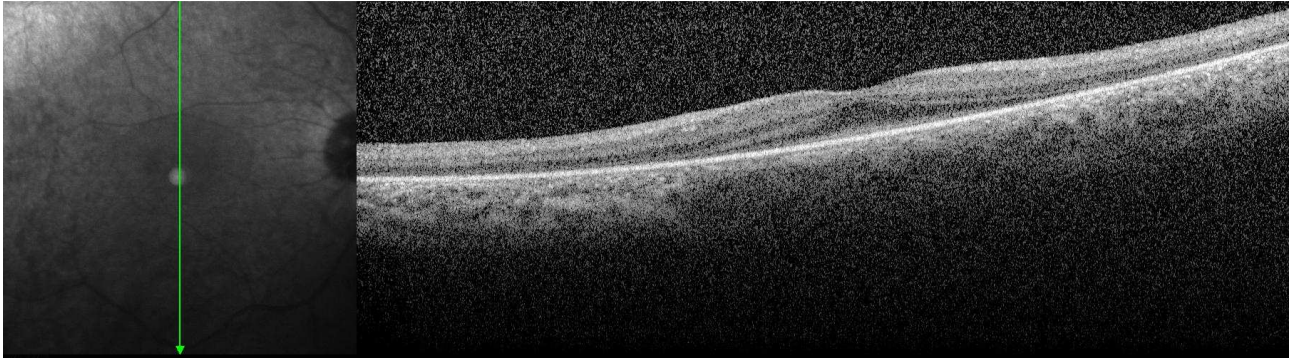
Figure 5. OCT spectralis from an adult LCA patient with nystagmus. Observe the very thin neurosensory retina without preservation of the normal retinal layers.

Figure 6. Fundus autofluorescence. Hyperautofluorescent macular ring.

To assist with potential genotype-phenotype correlations and to understand whether our results fit those found in the international literature we provide a summary of the knowledge of the function of the mutated genes as well as phenotype and genotype characteristics already described in actual literature.

IQCB1 (IQ motif-containing protein B1) or *NPHP5* (Nephrocystin 5) is a 69kDa protein in chromosome 3q13.33. It contains a central coiled-coil region and 2 IQ calmodulin binding regions and is down-regulated by p53 and DNA damage. Otto *et al.* (2005) identified 8 different mutations in the *IQCB1* gene in patients with Senior-Loken syndrome (which is characterized by nephronophtisis and retinitis pigmentosa). All individuals with *IQCB1* mutations had retinitis pigmentosa. It was subsequently concluded that mutations in *IQCB1* are the most frequent cause of SLSN. Stone *et al.* (2011) analysed the frequency of the *IQCB1* gene in 274 individuals with LCA and identified homozygosity or compound heterozygosity for frameshift or nonsense *IQCB1* mutations in 9 patients. Our patient carrying the *NPHP5/ICQB1* 2-bp deletion (1518delCA) follows the same phenotypical pattern described by Stone *et al.* (2011), who identified 5 patients initially diagnosed with LCA, one of whom later developed an elevated creatinine level that rapidly progressed to renal failure. However, in Stone *et al.* series, the 7 year old boy was homozygous for the 1516del CA mutation and presented no manifestations of renal disease, whereas the affected woman re-classified as Senior-Loken syndrome was a compound heterozygous for 2 null alleles, and the signs of renal involvement were detected later in life. This raises the important topic of nephrological testing in every patient with early-onset cone-rod dystrophy or LCA, as an early diagnosis of renal ailment can be crucial in the management of such patients, perhaps delaying the course of renal failure by imposing some dietary restriction measures.

CEP290 or *NPHP6* is a centrosomal protein with 290 kDa that is located in chromosome 12q21.3. It is involved in the transport across the photoreceptor connecting cilium and sustains the photoreceptors as well as the inner layer of the foveal cones architecture. Mutations in this gene have been associated with different ciliopathies, including LCA, Joubert syndrome, Senior Loken syndrome, Bardet-Biedl syndrome and Meckel-Gruber syndrome. All of them have important systemic features including renal (cystic disease) and SNC manifestations (molar tooth sign, encephalocele, etc) except LCA. No clear genotype-phenotype correlations have been identified thus the potential benefits from a clear molecular diagnosis are limited (35). Den Hollander *et al*. (2006) ascertained a consanguineous French canadian family with 4 affected sibs with LCA and linkage analysis assigned the gene to 12q21-q22. *CEP290/NPHP6* became the ideal gene candidate for this family, and the authors detected an A-to-G transition 5 bp downstream of a cryptic exon (2991+1655A-G; 610142.0005) as the cause of the disease. In most series, *NPHP6* is the most important gene with mutations associated with the LCA phenotype, with estimated prevalence between 8 and 20% (23) and the aforementioned became the most prevalent mutation found in LCA patients of European descent (23). Patients usually have extremely poor but stable vision loss. Several families with mental retardation when harbouring *CEP290* mutations have been described.

In our cohort, *NPHP6* gene was mutated in 3 patients and 4 different alleles were identified: c.2991+1655A>G, c.2052+1delGT, c.2717C>T and a complete deletion of exon 20. Not unsurprisingly, the most common *NPHP6* mutation is also the most frequent allele in our population. The phenotypical analysis of the affected *CEP290* patients reveals a significant heterogeneity both from a retinal and systemic standpoint; the homozygous c.2991+1655A>G girl presents an autistic-like trait while the double heterozygous with only one c.2991+1655A>G allele shows normal development and intellect. It will be

interesting to clearly demonstrate the consequences of this mutation in terms of CNS development and function. Since there is an ongoing effort to generate a strategy to "cure" this genetic variant, these individuals may soon be amenable for treatment.

The Retinitis Pigmentosa GTPase regulator-interacting protein 1 gene maps to 14q11.2 and the expressed protein is a direct binder of the GTPase gene regulator that is mutated in patients with X-linked retinitis pigmentosa. Its expression has been clearly demonstrated in the connecting cilium and the photoreceptor outer segment (22). Dryja *et al*. (2001) screened 57 unrelated patients with LCA for mutations in *RPGRIP1*. They found recessive mutations involving both alleles in 6% of their cohort. The identified mutations formed premature termination codons which are likely to represent null alleles. Gerber *et al*. (2001) found *RPGRIP1* mutations in 8 patients (5.6%) in a cohort of 142 unrelated LCA patients. In the 8 distinct mutations detected, 5 were truncating and 3 (2 missense and 1 in-frame deletion) concerned highly conserved amino acids in bovine and murine sequences. Roepman *et al*. (2005), identified the interaction between *RPGRIP1* and nephrocystin-4, encoded by *NPHP4*. This interaction can be disrupted by either mutations in *RPGRIP1*, found in patients with LCA, or by mutations in *NPHP4*, associated with patients with nephronophtisis or Senior-Loken syndrome (36). In our study, a homozygous mutation in the *RPGRIP1* gene, c.2759insT, was identified in one patient creating an alternate reading frame that results in premature truncation of the protein beyond aminoacid 933. This is likely a null allele. From a phenotypical standpoint our patient matches the typical severe cone-rod dystrophy phenotype with subsequent severe visual loss.

To date and to our surprise, no mutations have been found in *CRB1* (the most common mutation in the Spanish population) or in the *GUCY2D* gene which, in most series (mainly northern European) is the second most mutated gene.

As observed in the Spanish population, we also have yet to identify any individuals carrying mutations in the *RPE65* gene, already in gene therapy clinical trials. There is clearly a geographical distribution of LCA mutations across the different genetic backgrounds. Thus, the completion of the genetic analysis may clarify the number of individuals that have changes in the known genes or even assist in the identification of yet to be discovered genes, helping us understand the normal biology of the retina.

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