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Exome sequencing identifies *PEX6* mutations in three cases diagnosed with Retinitis Pigmentosa and hearing impairment

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Purpose: The aim of the present work is the molecular diagnosis of three patients with deafness and retinal degeneration.
Methods: Three patients from two unrelated families were initially analyzed with custom gene panels for Usher genes, non-syndromic hearing loss, or inherited syndromic retinopathies and further investigated by means of clinical or whole exome sequencing.
Results: The study allowed us to detect likely pathogenic variants in *PEX6*, a gene typically involved in peroxisomal biogenesis disorders (PBDs). Beside deaf–blindness, both families showed additional features: Siblings from Family 1 showed enamel alteration and abnormal peroxisome. In addition, the brother had mild neurodevelopmental delay and nephrolithiasis. The case II:1 from Family 2 showed intellectual disability, enamel alteration, and dysmorphism.
Conclusions: We have reported three new cases with pathogenic variants in *PEX6* presenting with milder forms of the Zellweger spectrum disorders (ZSD). The three cases showed distinct clinical features. Thus, expanding the phenotypic spectrum of PBDs and ascertaining exome sequencing is an effective strategy for an accurate diagnosis of clinically overlapping and genetically heterogeneous disorders such as deafness–blindness association.

Retinitis pigmentosa (RP) and deafness are clinical manifestations of several overlapping conditions. These disorders can be of genetic origin, such as Usher syndrome, Alström syndrome, Senior-Locken syndrome, mitochondrial disorders, and peroxisomal disorders. They can also be of non-genetic origin, such as intrauterine infections and perinatal complications. Moreover, both symptoms may be accompanied by additional features such as brain, cardiac, liver, renal or teeth abnormalities, and dysmorphism [1]. This heterogeneity, both in etiology and associated phenotype, leads to a difficult and, in many cases, delayed diagnosis in these patients [2].

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The most common genetic cause underlying the deafness and blindness combination is Usher syndrome (USH). USH is an autosomal recessive disorder characterized by hearing impairment, RP, and, in some cases, vestibular dysfunction. USH is a clinically and genetically heterogeneous systemic disorder classified in three different subtypes according to the age of onset and severity [3]. There are 12 known USH genes that encode proteins associated with the cilium of retinal photoreceptors and inner hair cells [4].

Another group of diseases presenting with RP and deafness are peroxisomal biogenesis disorders (PBDs). These are another heterogeneous group of pathologies caused by pathogenic variants in the *PEX* genes that encode proteins involved in the normal assembly and function of peroxisomes [5-7]. These pathologies compose the Zellweger spectrum disorders (ZSD), which include the congenital and lethal Zellweger syndrome (ZS), intermediate neonatal adrenoleukodystrophy (NALD), and the milder forms of

PBDs, Infantile Refsum disease (IRD) and Heimler syndrome (HS) [5-8].

Peroxisome disorders can be detected by means of biochemical screening because they usually show very long fatty acids and branched fatty acids alterations [5,9,10]. However, PBD patients with subclinical biochemical presentation have been reported [4,5,8,9]. The milder forms of PBDs (IRD and HS) share several clinical manifestations, such as retinal dystrophy, deafness, and enamel anomalies. Intellectual disability has been observed in both diseases, although it is more common in IRD [11] and is not a characteristic feature of HS [2].

Additionally, IRD usually presents other features, such as neurologic impairment (seizures, hypotonia, and cerebellar ataxia) or liver alteration, and HS patients are generally found to have nail anomalies. Pathogenic variants in *PEX1* and *PEX6* genes have been said to account for both IRD and HS [2,5,11-15].

Here we present three cases from two families presenting RP and hearing impairment, initially diagnosed as possible USH1 and previously screened by Next Generation Sequencing (NGS) custom gene panels. We have been able to molecularly characterize them as PBDs by exome sequencing, adding three new cases exhibiting previously undescribed features of the milder forms of the ZSD clinical continuum.

METHODS

Patients: Cases II-1 and II-2 from Family 1 were referred to University Hospital La Fe, and case II-1 from Family 2 was referred to University Hospital Fundación Jiménez Díaz to perform the genetic study of USH. Written informed consent was signed by the parents for patients who were under the consenting age. The institutional boards of the Ethics Committee of the University Hospital La Fe and the University Hospital Fundación Jiménez Díaz, approved the study according to the tenets of the Declaration of Helsinki and reviews. The individual II-1 from family 2 gave written informed consent to publish personal images in this manuscript.

Next generation sequencing: Genomic DNA was extracted from EDTA blood using an automated DNA extractor (Magna Pure, Roche). DNA samples were purified with the "QIAqick PCR Purification Kit" following the manufacturer's instructions. The concentration of the genomic DNA was determined with the "Qubit dsDNA BR Assay Kit" in the Qubit 2.0 fluorometer.

A custom HaloPlex panel was applied to the DNA sample II-1 of Family 1 to capture all exons and 25 bp of the intronic flanking regions of the genes associated with USH (*ADGVR1, CDH23, CIB2, CLRN1, DFNB31, HARS, MYO7A, PCDH15, USH1C, USH1G, USH2A*), *PDZD7,* which is a genetic modifier of USH genes [16], with the additional locus comprising the c.7595–2144A>G intronic mutation in *USH2A* and in two candidate genes (*MYO15A* and *VEZT*). Sequence capture was performed according to the "HaloPlex Target Enrichment System" (Protocol Version D.5, Agilent Technologies Inc., CA) and sequenced on a MiSeq with v2 chemistry (Illumina, Cambridge, UK) [17]). No pathologic variant in the index case was identified after this study. Subsequently, whole exome sequencing (WES) was applied to the four DNA samples of this family (father, mother, and the two affected siblings) to identify the gene responsible for the disease. WES was performed by CNAG (National Center of Genomic Analysis, Barcelona, Spain). For exome enrichment, the NimbleGen SeqCap EZ v3.0 system, following manufacturer's protocol version 4.2, was used and pre-capture multiplexing was applied. The sequencing was performed on an Illumina HiSeq 2000 instrument in a fraction of a sequencing lane, following the manufacturer's protocol, with a paired end run of 2×101bp. Several databases were used for filtering purposes: the 1000 genomes project, dbSNP, the NHLBI GO Exome Sequencing Project (ESP), the Exome Aggregation Consortium (ExAC), and the Genome Aggregation Database (gnomAD). All variants with a Minor Allele Frequency (MAF) >0.01 and a quality score <20 were removed. Variants consistent with autosomal recessive inheritance were considered. The impact of missense variants was analyzed using several predictor tools (DANN, FATHMN, GERP++, LR, LRT, M-CAP, CADD, MutationTaster, MutationAssessor, PhyloP, Polyphen2_HDIV, Polyphen2_HVAR, Provean, RadiaISVM, SIFT, SiPhy) [18]. The potential deleterious effects on splicing were assessed using several *in silico* splicing tools (Human Splicing finder and MaxEntScan).

The index case from Family 2 (II-1) had been previously studied using a custom Nextera gene panel for inherited syndromic retinopathies [19]. In that study, 104 genes associated with IRDs (reported in RetNet database, data accession in 2015), including the genes *ADGVR1, CDH23, CIB2, CLRN1, DFNB31, HARS, MYO7A, PCDH15, PDZD7, USH1C, USH1G* and *USH2A*, were discarded because it was determined they were not responsible for the disease. Then, a clinical exome sequencing (TruSightOne) was performed on a NextSeq500 with v1 chemistry.

Sequencing data were processed according to the National Center of Genomic Analysis (CNAG) for Family 1 and the Center for Applied Genomics' pipeline (Children's

Hospital of Philadelphia) for Family 2. Alignment and post-alignment BAM processing of paired-end reads (FASTQ files) were aligned to the GRCh37 human reference with decoy sequence using BWA (v0.7.12-r1039). PCR duplicate reads were marked using Picard Mark duplicate (v2.1.0). A Variant Call Format (VCF) file for each sample was generated using GATK Haplotype Caller (v3.4–46). For the identification of disease-causing variants, SnpEff and ANNOVAR were used to functionally annotate single nucleotide polymorphisms (SNPs), insertions, and deletions. Furthermore, ANNOVAR was used to identify variants that were previously reported in public databases, including the 1000 genomes project, dbSNP, the NHLBI GO Exome Sequencing Project (ESP), ExAC, and gnomAD. Potentially pathogenic variants were sequentially prioritized in a subpanel of 229 IRD-associated genes [20]. Rare variants were filtered out, as previously described [19], by a MAF less than or equal to 0.005 in the public databases mentioned above and on the CIBERER Spanish Variant Server (CSVS). The pathogenicity of missense variants was analyzed using the predictor tools mentioned above. The potential deleterious effects on splicing were assessed using several *in silico* splicing tools (Human Splicing finder, MaxEntScan, Splice Site Finder-like, NNSPLICE, GeneSplicer) implemented in Alamut software (Interactive Biosoftware, Rouen, France).

Validation of variants: In Family 1, the presence and status of the two variants were confirmed in all four samples by Sanger sequencing. The DNA fragments containing the variants were amplified by PCR with specific primers (PEX6_c.2807–2A>G_F: 5′- AGT GGG AGA CAA ACC TAG TCC −3′ and PEX6_c.2807–2A>G_R: 5′- CTA GCA GGC AGC AAA CTT GC −3′; PEX6_Gly437Asp_F: 5′- CCC ATA CCT CCT TGT ACA TGG −3′ and PEX6_Gly437Asp _R: 5′- CTT ACA GAA AGG AGT GGC CTG −3′). To amplify *PEX6* Exon1 for Family 2, the following primers were used: PEX6_ex1F: F 5′- CTA GGT TGG GCA CTG CTT GG −3′ and PEX6_ex1R: R 5′-GAC TCT GGA CAC AGT CTG GC −3′).

PCR products were sequenced on both strands using the Big Dye 3.1 Terminator Sequencing Kit. The purified sequence products were analyzed on a 3500xl ABI instrument (Applied Biosystems, Thermo Fisher Scientific, Inc. Whaltam, MA).

Biochemical analysis: Peroxisomal parameters were measured in plasma. Very-long-chain fatty acids (VLCFAs: C26:0/C24:0/C22:0) and branched-chain fatty acids (phytanic acid and pristanic acid) were detected and measured as described by Dacremont and collaborators [10].

RESULTS

Clinical presentations: Three patients belonging to two unrelated Spanish families are presented here (Appendix 1; Figure 1 and Figure 2). The clinical features of Family 1 are shown in Appendix 2. The two affected siblings, a girl and a boy, presented the following medical records:

The firstborn daughter was born at 41 weeks by Cesarean section. The Apgar score was seven at 1 min and nine at 5 min, birthweight was 2,825 g, birth length was 48 cm, and head circumference was 33 cm. She displayed no dysmorphic features and only had physiologic jaundice. The results of the test for the early detection of hearing loss were normal for both ears. At the age of 15 days, she was diagnosed with a right inguinal hernia that was removed by herniorrhaphy at the age of four months. At the age of 20 months, she displayed bilateral prelocutive progressive sensorineural hearing loss that evolved from severe to profound. Brain stem evoked response audiometry revealed a hearing level response loss (wave V) of 75 dB in the right ear and 85 dB in the left ear. She was diagnosed with severe sensorineural hearing loss and started speech therapy rehabilitation using hearing aids. At age 24 months, she was noted to have visual problems, and at age 36 months, she was found to suffer from RP: the electroretinogram (ERG) was non-recordable, visual evoked potential test results were normal, but the eye fundus examination showed abnormal retinal pigmentation including peripheral pigment accumulation and arterial narrowing (Figure 1A). At this time, she was clinically diagnosed as possible USH type 1. At the age of 4 years, her visual acuity (decimal) was 0.3 for the right eye and 0.4 for the left eye, and at 9 years, these were 0.3 and 0.1, respectively. An optic coherence tomography (OCT) examination showed abnormal macular thickness and profile (Figure 3).

The son was born at 39 weeks with labor dystocia. The Apgar score was 9 at both 1 and 5 min, birthweight was 2,920 g, birth length was 49 cm, and head circumference was 33 cm. He displayed no dysmorphic features, but presented with intrapartum febricula and had respiratory distress with Silverman 6 due to the prolonged delivery. At 4 h of life, he needed assisted breathing until 12 h later. The result of the test for the early detection of hearing loss was normal for both ears. However, at 3 months of age he displayed a negative response to auditory stimuli. Brain stem evoked response audiometry revealed a hearing level response loss (wave V) of 50 dB in the right ear and 60 dB in the left ear. He was diagnosed with moderate sensorineural hearing loss. At the age of 8 months, an ophthalmoscopic examination showed peripheral temporal pigment deposits suggesting RP. At the age of 6 years, visual acuity was 0.4 for the right eye and 0.1

for the left eye, and a fundus ophthalmoscopy confirmed RP (Figure 1B). Thus, he was diagnosed with USH and started speech therapy rehabilitation using hearing aids.

The third patient belongs to Family 2 (Appendix 1 and Appendix 2). She is a female born from consanguineous parents (second cousins). Pregnancy and delivery data are unknown. She was diagnosed with congenital sensorineural hearing loss and was first noticed to have visual impairment at the age of two, presenting with night blindness and visual field constriction. She also displayed intellectual disability and learning difficulties and had attended an adapted school since early childhood. The first complete medical assessment, including visual and auditory evaluation, was performed when she was 21 years of age at the Department of Clinical Genetics, University Hospital Fundacion Jiménez Díaz (FJD), Madrid, Spain. The patient had cochlear implants and the auditory test showed moderate–severe bilateral sensorineural hearing loss to low frequencies and profound bilateral sensorineural hearing loss to high frequencies with

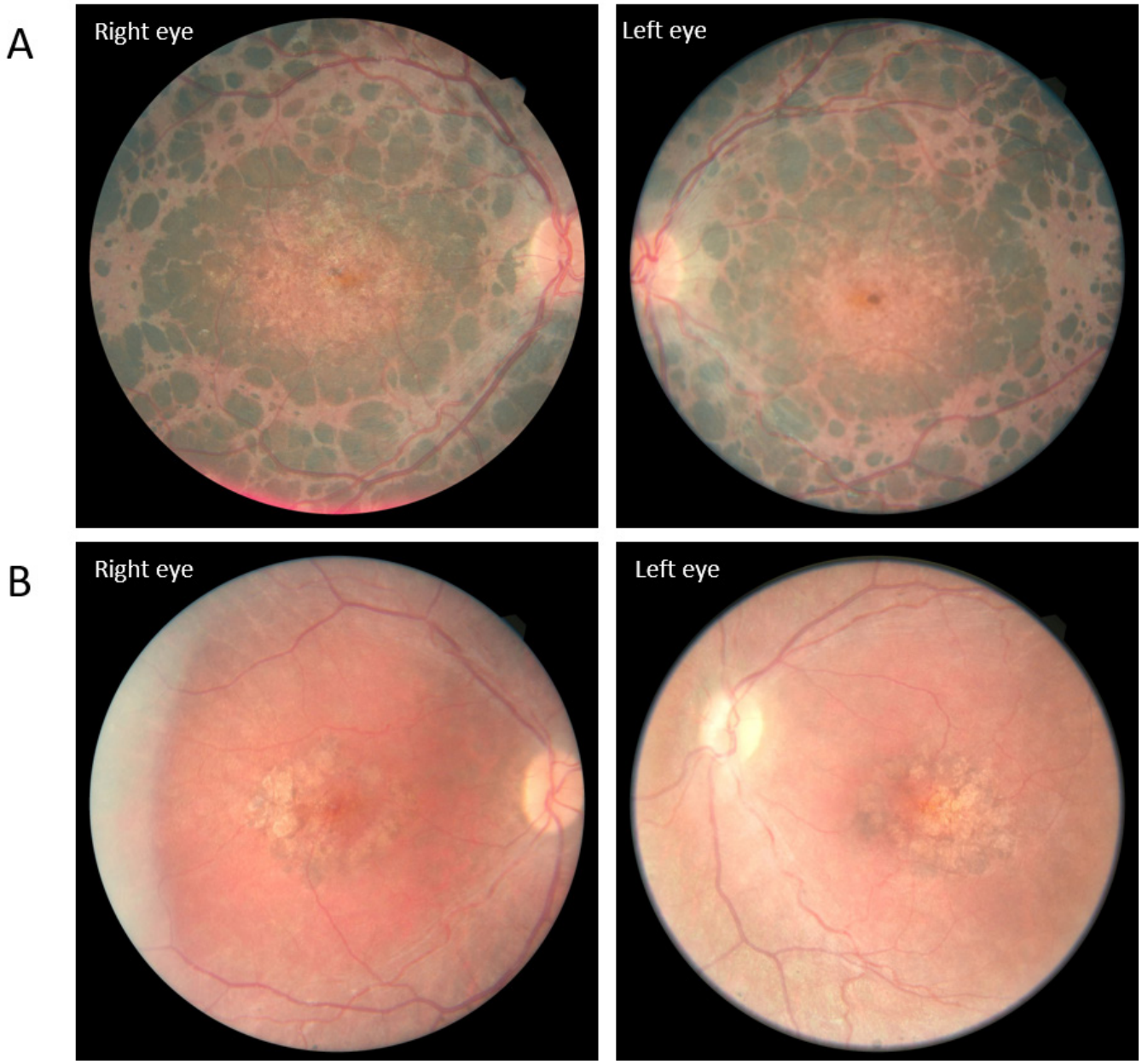

Figure 1. Fundus photographs of the individuals from Family 1 (II-1 and II-2). **A**: Ophthalmoscopic examination of case II-1 at 9 years of age. **B**: Ophthalmoscopic examination of case II-2 at 6 years of age.

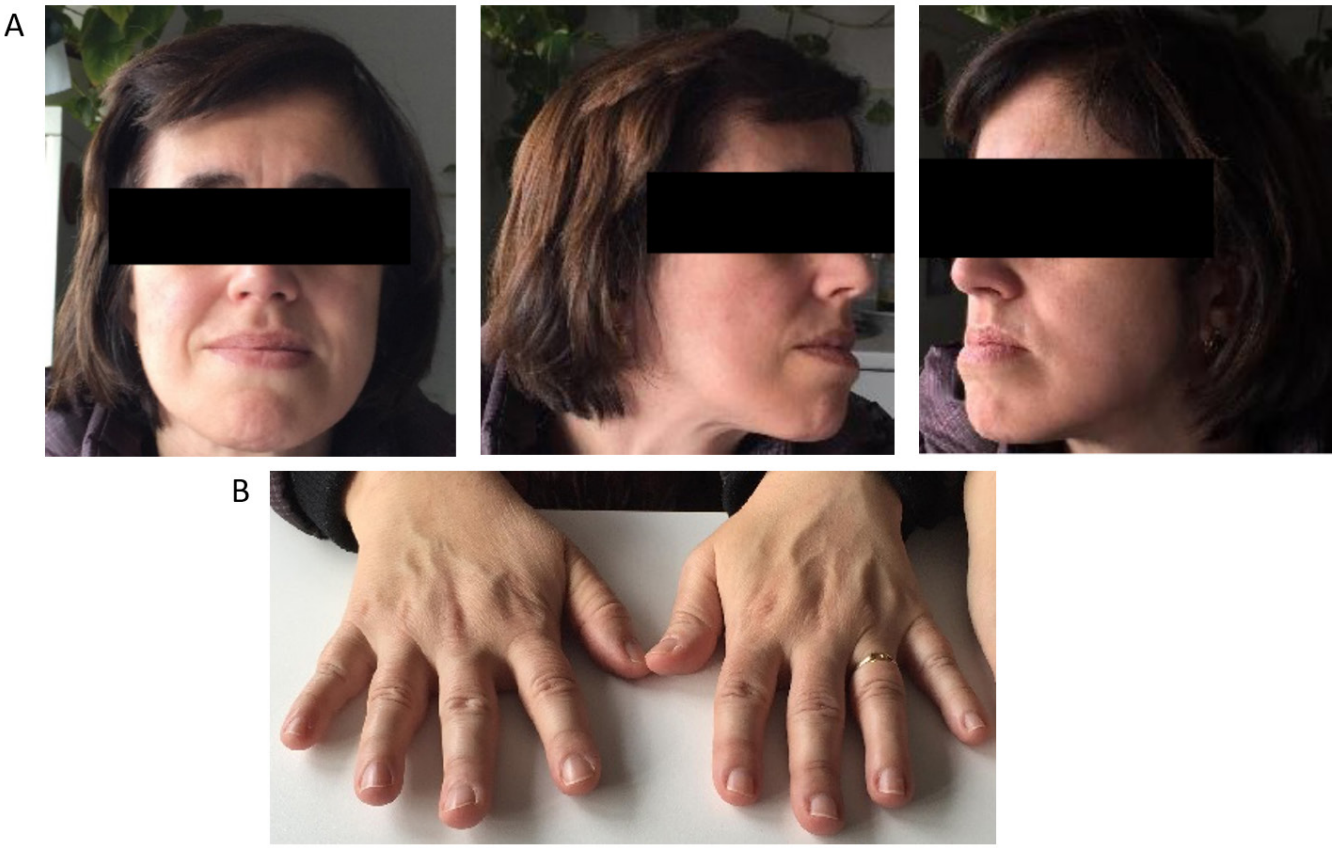

Figure 2. Clinical characteristics of the individual from Family 2 (II-1). **A:** Dysmorphic features: impression of "hypotonic face" (inexpressive), rectangular face, hypotelorism, epicanthic folds, down-slanting palpebral fissures and eyebrows, prominent philtrum, thick lips, prognatism. **B:** Brachydactyly.

preserved vestibular responses. She was then diagnosed with RP. Ophthalmoscopic evaluation was described as suggestive of RP with pre- and retro-equatorial bone spicules, atrophic lesion on the left macula, normal right macula, and normal papilla in both eyes. The ERG was abnormal in both eyes with non-recordable rods and much reduced amplitude for mixed and cones (by flash and flicker ERG recordings) recordings. The visual field was non-assessable. Clinical evaluation evidenced dysmorphic features (impression of "hypotonic face," rectangular face, hypotelorism, epicanthic folds, down-slanting palpebral fissures and dense eyebrows, low set ears, prominent philtrum, thick lips, and prognathism), brachydactyly, moderate intellectual disability, and enamel alteration (Figure 2).

Molecular findings: Both families were enrolled in an exome sequencing study. In Family 1, after filtering there only remained candidate variants in six genes. However, according to the function of the gene and the associated phenotypes, only two likely pathogenic variants in the *PEX6* gene remained: c.2807–2A>G [p.(Leu937fs)], previously described by Ebberink et al. [15], and another novel change, c.1310G>A [p.(Gly437Asp)] (NM_000287.3). In Family 2, we performed a clinical exome sequencing analysis and identified a homozygous variant c.424_427delinsTGGT [p.(Arg142_Pro143delinsTrpSer)] (NM_000287.3). No additional likely pathogenic variants segregated to the family were identified. All variants were confirmed by Sanger sequencing and co-segregated with the disease in the families (Figure 4).

Variant c.1310G>A [p.(Gly437Asp)] was only detected in the gnomAD database, but with a very low frequency (1/246238 alleles). Variant c.424_427delinsTGGT [p.(Arg142_Pro143delinsTrpSer)] and c.2807–2A>G were not described in any of the consulted databases (EVS, 1000 Genomes, ExAC, gnomAD, and CSVS) or in the literature. The variant c.1310G>A [p.(Gly437Asp)], detected in Family 1, was predicted to be pathogenic by 12 out of 16 methods (Appendix 3). Variant c.424_427delinsTGGT [p.(Arg142_Pro143delinsTrpSer)] found in Family 2 was predicted to two contiguous missense changes, affecting well conserved

residues predicted as damaging in 15 out of 16 in silico programs (Appendix 1).

Clinical re-evaluation of patients: Subsequently, a further clinical assessment based on the molecular findings was performed for Family 1. The additional information provided was as follows: At the age of 14 months, the son of Family 1 was noted to present mild neurodevelopment delay, coordination and balance issues, and possible attention deficit disorder. At that time, he started an early stimulation intervention for neurodevelopment delay with a positive evolution. Additionally, he presented nephrolithiasis since the age of ten months (bilateral at the age of 15 months), which required surgical removal of stones at the age of five years. He also presented long philtrum, thin upper lip, enamel anomalies, and wide thumbs. His sister demonstrated upper incisor agenesis and enamel anomalies. Peroxisomal parameters were measured and elevated levels of branched chain fatty acids and very long chain fatty acids were detected in both siblings (Table 1).

DISCUSSION

Re-analysis of the diagnosis will be done after a negative genetic testing which prolongs the process of diagnosis. Patients presenting with RP and deafness are usually quickly diagnosed as USH since USH is the most common association of both symptoms [20]. However, RP and deafness may appear along with other clinical manifestations like obesity, polydactyly, intellectual disability, renal dysfunction, etc., which may allow for the suspicion of a specific diagnosis. These additional manifestations generally do not appear at the same time, they might not appear at all, or they may be subtle, and their presence could be dependent upon the progression of the disease. As a result, the diagnosis of these patients might be even more complex. In this context, exome sequencing has demonstrated that it not only allows for the confirmation of a clinically suspected diagnosis, but it also makes re-classification and accurate diagnosis of patients presenting overlapping and pleiotropic conditions possible [21-24].

That is the case of the three patients presented in this work. They were each suspected of having USH, but the study of the more prevalent USH genes failed to reach a diagnosis. After exome sequencing, these patients were clinically re-classified as having mild–intermediate forms of ZSD. ZSD presents with a high phenotypic variability associated with the nature of the underlying genes and variants. Pathogenic variants in *PEX1* and *PEX6*, the two most commonly involved PEX genes, have been related to the full continuum of clinical phenotypes: lethal (ZS) forms, intermediate forms (NALD), and milder forms (IRD and HS). Biallelic frameshift and nonsense variants ("truncating variants" leading to a loss of protein function) result in ZS, while genotypes that include a missense variant (hypomorphic variants) probably lead to NALD or IRD. Similarly, HS has also been observed to be related to the presence of hypomorphic variants [2,12].

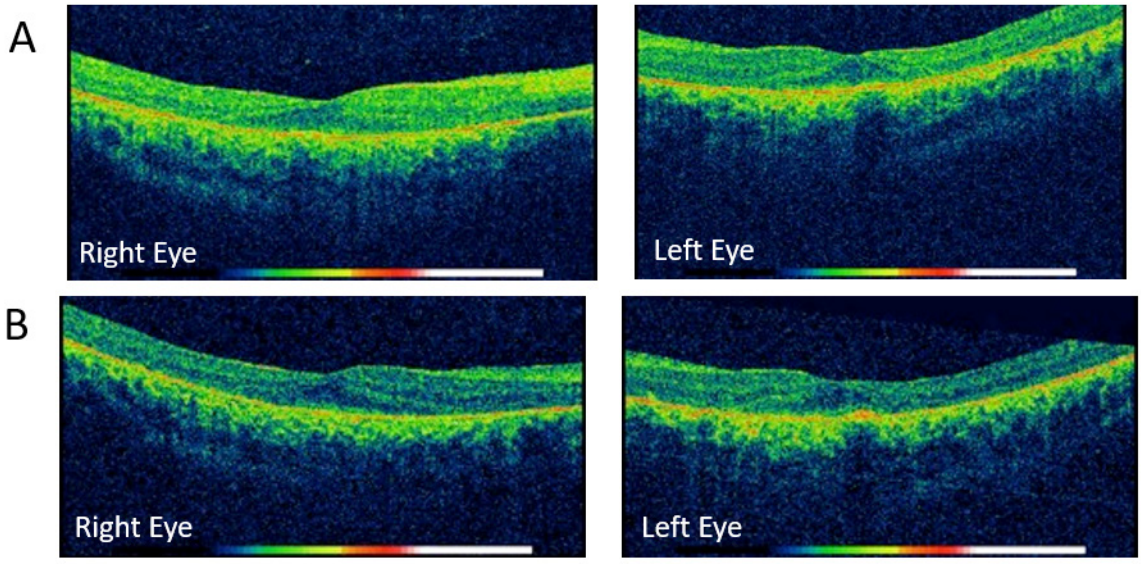

Figure 3. Optic coherence tomography of the individual from Family 1 (II-1). **A:** The OCT at 8 years of age presented a slight decrease in the macular thickness in both eyes (central macular thickness right eye: 238 microns/left eye 235 microns) and alteration of the photoreceptor layer. **B**: OCT at 9 years of age showed a progressive decrease in macular thickness in the left eye.

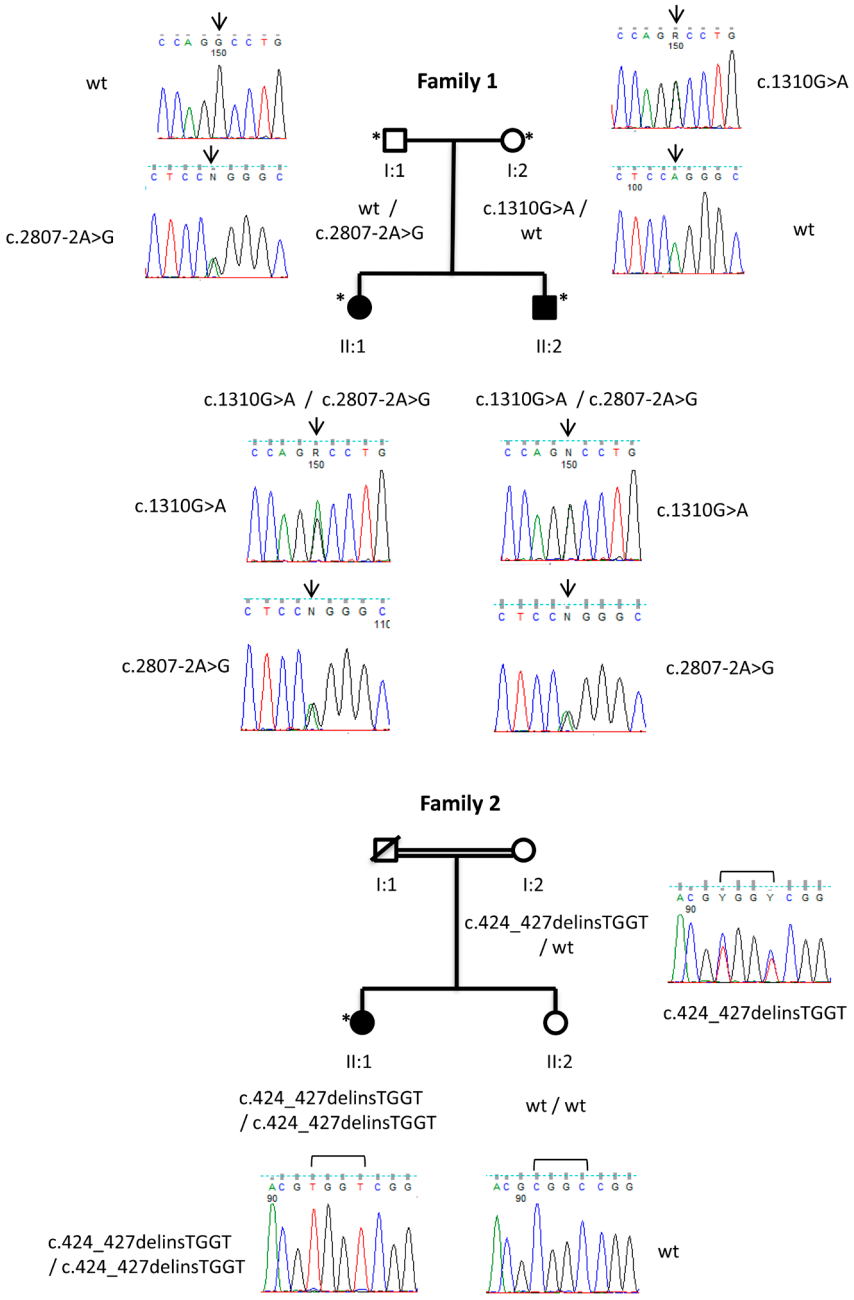

Figure 4. Pedigrees of the families and segregation analysis of the detected variants. Arrows indicate the nucleotide position where the variants are localized. Asterisks indicate individuals analyzed by whole exome sequencing in Family 1 and by clinical exome in Family 2. wt: wild-type sequence.

Therefore, considering that ZSD are progressive disorders with additional systemic features appearing from late childhood onwards, we encourage the study of *PEX1* and *PEX6* as potentially causative genes in patients presenting the RP–deafness combination at any age, but particularly in infants and children.

In this study, we have presented two families with heterogeneous clinical manifestations and at least one missense variant in the *PEX6* gene. In Case II:1 of Family 2, the patient showed RP and deafness when first assessed as a child and further enamel alteration and distinctive facial features when assessed at 21 years of age after molecular

diagnosis. This patient, although presenting deafness and retinal dystrophy, would not have initially fallen into a clinical diagnosis of ZSD. The other 2 cases from Family 1 presented enamel anomalies in addition to hearing and visual impairment. However, the presence of some degree of developmental delay and the nephrolithiasis in patient II:2 would have pointed in a different direction than a mild form (like HS) of a ZSD diagnosis.

It is possible that early onset nephrolithiasis is an additional feature not previously described in ZSD patients. However, we cannot discard the existence of mutations in other genes that could explain the presence of all the symptoms since WES is efficient in 50%–60% of genetically heterogeneous diseases.

Patients with alterations on peroxisome biogenesis disorders typically display alteration in very long fatty acids and branched fatty acid levels. However, cases with normal biochemical data for these metabolites have been reported [9]. Hence, this biochemical method for the diagnosis of peroxisomal disorders is known not to show enough sensibility, making exome sequencing a better tool to obtain an accurate and sometimes more straightforward diagnosis in these patients.

Exome sequencing technology is changing the diagnostic algorithms in clinical genetics departments, making genetic data a primary diagnostic tool [25]. Regularly, available clinical data does not completely match the expected phenotype associated to the molecular finding. This leads to the clinical re-evaluation of patients and quite often to the widening of the phenotype of the disease and the genotype-phenotype correlations. This is probably the case with ZSD, in which various specific sub-diagnoses are found but in which the lines between these sub-diagnoses may not always be clearly definable [12,26]. The lack of genotype–phenotype match may be due to different reasons, such as missing clinical information, the presence of mild or overlooked features, signs appearing later in the time continuum of findings that do not always follow the known line of the spectrum, intermediate phenotypes that do not fall into a specific reported subtype, and overlapping conditions with different genetic backgrounds. Thus, exome sequencing techniques are especially useful for the detection of genotype patients in complex clinical entities (such as PBDs) to establish a correct diagnosis, management, surveillance, and prognosis, all of which are crucial for patients' health care.

APPENDIX 1. PATHOGENICITY PREDICTIONS OF MISSENSE VARIANTS IDENTIFIED IN *PEX6*.

To access the data, click or select the words "Appendix 1." *PEX6*: NM_000287. Detailed information for all the databases are given below (ANNOVAR): SIFT - D: deleterious PolyPhen 2 HDIV - D: probably damaging, P: possibly damaging PolyPhen 2 Hvar - P: possibly damaging; B: benign LRT - D: deleterious; B: benign MutationTaster – D: disease_causing MutationAssessor - N: neutral FATHMM -D: deleterious RadialSVM - D: deleterious LR - D: deleterious DANN - D: deleterious PROVEAN – B: benign; D: deleterious CADD – P: pathogenic GERP++ higher scores are more deleterious, GERP++ RS Conservation score Score >4.4 PhyloP - higher scores are more deleterious, PhyloP Conservation score Score >1.6 SiPhy - higher scores are more deleterious, SiPhy Conservation score Score >12.17

APPENDIX 2. CLINICAL FEATURES OF THE PATIENTS AND THE DISORDER ASSOCIATED.

To access the data, click or select the words "Appendix 2."

Table 1. Biochemical parameters in blood of patients from Family 1.

VCFAs species		Family 1 - Case II:1	Family 1 - Case II:2	Reference values
VLCFA (µmol/l)	C22:0	26	24,8	51,1–113,4
	C24:0	24,8	30	44,3–92,4
	C26:0	0,695	1,32	0,220–0,880
VLCFA ratio	C24:0/C22:0	0,954	1,21	0,550–0,890
	C26:0/C22:0	0,027	0,053	0,004–0,021
Phytanic acid (µmol/l)		6,9	11	<2
Pristanic acid (µmol/l)		2,7	4,5	<1

VLCFA: very-long-chain fatty acids

APPENDIX 3. PATHOGENICITY PREDICTIONS OF MISSENSE VARIANTS IDENTIFIED IN *PEX6*.

To access the data, click or select the words "Appendix 3." NM_000287. Detailed information for all the databases are given below (ANNOVAR): SIFT - D: deleterious; PolyPhen 2 HDIV - D: probably damaging, P: possibly damaging; PolyPhen 2 Hvar - P: possibly damaging; B: benign; LRT - D: deleterious; B: benign; MutationTaster – D: disease_causing; MutationAssessor - N: neutral; FATHMM -D: deleterious; RadialSVM - D: deleterious; LR - D: deleterious; DANN - D: deleterious; PROVEAN – B: benign; D: deleterious; CADD – P: pathogenic; GERP++ higher scores are more deleterious, GERP++ RS Conservation score Score >4.4; PhyloP - higher scores are more deleterious, PhyloP Conservation score Score >1.6; SiPhy - higher scores are more deleterious, SiPhy Conservation score Score >12.17

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