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Spectral-Domain Optical Coherence Tomography Analysis in Syndromic and Nonsyndromic Forms of Retinitis Pigmentosa due to *USH2A* Genetic Variants

Leonardo Colombo[a] Paolo Enrico Maltese[b] Dario Romano[a] Paolo Fogagnolo[a] Marco Castori[c] Giuseppe Marceddu[d] Francesca Cristofoli[d] Marcella Percio[b] Barbora Piteková[e] Antonio Mattia Modarelli[a] Matteo Bertelli[b, d] Luca Rossetti[a]

[a]Department of Ophthalmology, ASST Santi Paolo e Carlo Hospital, University of Milan, Milan, Italy; [b]MAGI'S Lab s.r.l., Rovereto, Italy; [c]Division of Medical Genetics, Fondazione IRCCS-Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; [d]MAGI Euregio s.c.s., Bolzano, Italy; [e]Department of Pediatrics, Comenius University Faculty of Medicine, National Institute of Children's Diseases, Bratislava, Slovak Republic

Keywords
Spectral-domain optical coherence tomography · Autosomal recessive retinitis pigmentosa · Usher syndrome · Usher syndrome type 2A

Abstract

Introduction: This study aimed to analyze macular structure by using spectral-domain optical coherence tomography (SD-OCT) in a cohort of patients affected by autosomal recessive retinitis pigmentosa and Usher syndrome, due to genetic variants in *USH2A* gene, and to correlate optical coherence tomography (OCT) parameters with functional and genetic data. ***Methods:*** The subjects of this study were 92 patients, 46 syndromic (Usher syndrome type IIa [Ush2]) and 46 nonsyndromic (autosomal recessive RP [arRP]), with clinical and genetic diagnosis of *USH2A*-related retinal dystrophy, who underwent a complete ophthalmic examination and spectral-domain OCT analysis. The study focused on evaluating the differences between the 2 groups in the following parameters: best-corrected visual acuity (BCVA), ellipsoid zone (EZ) width, presence of epiretinal membrane (ERM), and cystic macular lesions (CMLs). Variants in *USH2A* gene were divided into 3 categories, according to the expected impact (low/high) at protein level of the different variants on each allele. ***Results:*** BCVA and EZ width were significantly lower in Ush2 than in arRP patients ($p < 0.0001$ and $p = 0.001$). ERM was detected in 34.8% (16/46) of arRP patients and in 65.2% (30/46) of Ush2 patients ($p = 0.003$). CML was detected in 17.4% (8/46) of arRP patients and 30.4% (14/46) of Ush2 patients ($p = 0.14$). The allelic distribution was statistically different ($p = 0.0003$) by dividing the 2 diseases: for Ush2 patients it was 45.7% (high/high), 39.1% (low/high) and 15.2% (low/low); for arRP patients it was 8.7% (high/high), 56.5% (low/high), and 34.8% (low/low). The severity class of the variants significantly affected visual acuity and EZ width parameters ($p = 0.004$ and $p = 0.002$, respectively). ***Conclusion:*** Retinal disease, as evaluated by means of SD-OCT, shows more advanced degeneration signs in the syndromic than the nonsyndromic form of retinal dystrophy related to *USH2A* gene. Variant types and allelic profiles are determining factors for the onset of syndromic features. However, since the 3 allelic profiles can be found in both Usher and RP patients, other factors must necessarily play a determining role.

karger@karger.com
www.karger.com/ore

Correspondence to:
Paolo Enrico Maltese, paolo.maltese@assomagi.org
Matteo Bertelli, matteo.bertelli@assomagi.org

Introduction

In the last decade, the advent of next-generation sequencing (NGS) technology allowed the identification of many disease-causing genes, thus increasing the understanding of genetic basis of retinitis pigmentosa (RP) [1, 2]. To date, >90 genes are known in association with RP (https://sph.uth.edu/retnet/sum-dis.htm#A-genes), and among these, the variants occurring in *USH2A* gene are the one most prevalently causing both autosomal recessive RP (arRP) or Usher syndrome type IIa (Ush2) [3–5].

The *USH2A* gene is located on chromosome 1q41a-bd and encodes for usherin, a basement membrane protein of many – but not all – tissues [6]. Two isoforms of this protein have been described, both expressed in the cells of photoreceptors and cochlear hairs. Isoform 1 (comprising 5,202 amino acids) is the longer one, translated by 72 exons, while the shorter isoform 2 (1,546 amino acids), is translated by the first 21 exons [7]. Usherin is a member of the USH2 complex involved in the maturation of the stereocilia of cochlear hair cells in the inner ear and in the long-term maintenance of photoreceptors in the retina [8], thus justifying its role in both arRP and Ush2. However, the relationship between the variants and the resulting phenotypes is still unclear [5].

New therapies – such as gene replacement or silencing, optogenetics, stem cells, retinal implants, and neuroprotective approaches – raised the need of increasingly accurate genotype-phenotype correlations and natural history studies [9–11]. Likewise, universally accepted guidelines are still required to objectively monitor the deterioration of retinal functions and structures, even in the short period.

Outer retinal evaluation by means of spectral-domain optical coherence tomography (SD-OCT) became part of clinical routine evaluation of RP patients. Ellipsoid zone (EZ) width is a well-accepted parameter to follow disease progression, also correlated with functional aspects like visual acuity (VA) and visual field (VF) [12–15]. The aim of our study was to provide new insights in the genotype-phenotype correlation of *USH2A*-related retinal dystrophies: for this purpose, we evaluated macular structure by means of SD-OCT in a large and well-defined cohort of patients affected by arRP and Ush2 due to *USH2A* genetic variants and we correlated the parameters with VA and the type of variants.

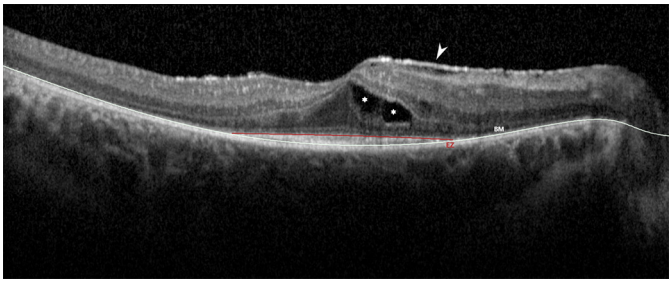

Fig. 1. SD-OCT scan of a patient affected by *USH2A*-related retinitis pigmentosa. The red line shows the EZ width; to measure it, the nasal and temporal boundaries were set where the EZ line meets Bruch's membrane line (as shown by the white line – BM). CML (asterisks) and ERM (arrow) were also evaluated. SD-OCT, spectral-domain optical coherence tomography; EZ, ellipsoid zone; ERM, epiretinal membrane; CML, cystic macular lesions; VA, visual acuity.

Materials and Methods

Study Population

We recruited subjects with *USH2A*-related retinal dystrophies from outpatients of the Retinal Dystrophies department at the University Eye Clinic of ASST Santi Paolo e Carlo Hospital (Milan). RP diagnosis was based on the typical clinical signs (optic disc pallor, bone spicule pigmentation, and retinal vessel attenuation); the characteristic full-field electoretinographic patterns, which vary from reduced amplitudes of A and B waves in the early stages to nondetectable EGR in the advanced stages (as established by the International Society for Clinical Electrophysiology of Vision); VF constriction; and finally by the results of genetic analysis. We reviewed 101 electronic health records of patients with *USH2A*-related retinal dystrophies and we divided the sample into 2 groups: patients with sensorineural bilateral hearing loss were classified as Ush2, while the others were classified as nonsyndromic RP. The exclusion criteria were the lack of the psychophysical requirements to adequately understand the aim of the study, complete the required examinations, and provide the informed consent; and the presence of any other systemic or ocular conditions – unrelated with *USH2A* variants – that could worsen their vision abilities.

The patients meeting the criteria prospectively underwent the following evaluations: best-corrected visual acuity; slit-lamp examination of the anterior segment and indirect fundus biomicroscopy after pupil dilatation; and spectral-domain optical coherence tomography (OCT). Only good quality OCT scans (over 25 dB) were used for the measurements: patients with cataract, other media opacities or any other condition (like nystagmus) affecting SD-OCT scan quality were excluded.

The final cohort consisted of 46 patients with Ush2 and 46 with nonsyndromic RP. We included 3 pairs of siblings in the Usher group and 1 in the nonsyndromic group: for each pair, both siblings presented the same genotype and phenotype.

The study was compliant to the tenets of the Declaration of Helsinki and it was approved by the Ethics Committee of ASST Santi Paolo e Carlo Hospital. All participants were required to sign a written informed consent to take part to the study.

Table 1. Demographic characteristics of Usher syndrome and RP patients

Group (92 patients)	Sex	Age, years	Onset	VA	EZ	ERM, *n*/*N* (%)	CML, *n*/*N* (%)
46 RP	24 M	50.33±12.83	35.4±11.7	0.653±0.281	2,166.38±1,268.09	9/24 (37.50)	5/24 (20.83)
	22 F	53.95±13.23	34.9±15.2	0.552±0.334	1,754.32±1,554.55	7/22 (31.82)	3/22 (13.64)
46 Usher IIa	18 M	41.78±13.96	18.4±9.6	0.431±0.287	1,078.33±1,099.80	14/18 (77.78)	5/18 (27.78)
	28 F	45.67±13.68	20.3±10.2	0.535±0.310	1,453.39±1,330.75	16/28 (57.14)	9/28 (32.14)

RP, retinitis pigmentosa; VA, visual acuity; EZ, ellipsoid zone; ERM, epiretinal membrane; CML, cystic macular lesion.

Optical Coherence Tomography

Structural retinal analysis was performed with SD-OCT and Heidelberg Eye Explorer (HEYEX) software (Heidelberg Engineering, Heidelberg, Germany). We analyzed single-line scans of 30° crossing the fovea horizontally and centered using the automatic eye tracking system, obtained with Automatic Real-time Tracking (ART 100); a volume scan, performed using a 20° × 20° scan field, with 49 B-scans (ART 12); and a radial layout, made of 6 scans of 20° centered on the fovea (ART 12).

The external limiting membrane is the innermost of the 4 hyper-reflective bands on SD-OCT that can be identified in the human outer retina. The EZ is the second one, separated from the external limiting membrane by a hypo-reflective region called myoid zone. EZ was manually segmented for every single 30° horizontal scan. Its nasal and temporal boundaries were defined to be the locations where this layer drops to the outer segment of photoreceptors and then its width was measured [12]. EZ width was manually measured by 2 experienced OCT-readers using the caliper of the HEYEX software; then the values obtained by the 2 physicians were averaged (shown in Fig. 1).

In addition, the presence/absence of cystic macular lesions (CMLs) and of the epiretinal membrane (ERM) was recorded analyzing volumetric scans. We defined CML as the presence of intraretinal hypo-reflective cystoid spaces in the central macula (1.5 mm diameter) that could be seen on at least 2 consecutive scans. CML was then divided – as proposed by [16] – into 3 categories: mild, moderate, and severe. ERM was defined as a hyper-reflective line above the internal limiting membrane and adherent to the inner retina, with or without distortion of the underlying retinal layers. OCT scans were obtained with dilated pupils (using 1% tropicamide eye drops). Patients with poor OCT scan quality or prior vitreoretinal surgeries were excluded.

Genetic Analysis

Genetic evaluations were performed in MAGI's laboratories (MAGI'S Lab s.r.l.; Rovereto, and MAGI Euregio, Bolzano, Italy) starting from whole blood samples. The DNAs of the study subjects were extracted using a commercial kit (E.Z.N.A. Blood DNA kit Omega; Bio-Tek, Norcross, GA, USA) and used for subsequent analyses. All probands' samples were tested via NGS on an Illumina MiSeq personal sequencer (Illumina, San Diego, California) and a custom-designed multigene panel. Sequence variant calling and annotation were performed using an in-house bioinformatics pipeline, as described elsewhere [17, 18]. Sanger sequencing was used to validate NGS results and to perform segregation studies of selected variants in probands' relatives. Copy number variants were analyzed by multiplex ligation-dependent probe amplification (www.mrc-holland.com), using the Beckman Coulter CEQ 8000 sequencer (Beckmann Coulter, Milan, Italy) following the manufacturer instructions. The identified variants were searched in databases such as dbSNP (https://www.ncbi.nlm.nih.gov/snp/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), and Human Gene Mutation Database, professional version 2017.2 (https://www.portal.biobase-international.com/hgmd/pro/). The pathogenicity of the variants was assessed following the American College of Medical Genetics and Genomics (ACMG) standard and guidelines [19] through the VarSome on-line software [20]. Genetic data were already available at the beginning of the present study and were retrospectively reviewed, to select only those patients who tested positive for *USH2A* gene from a larger sample of RP and Usher patients [3].

Variants in *USH2A* gene were divided into 3 groups based on the expected impact (low/high) at protein level of variants on each allele (maternal/paternal), according to the following classification: low/low, low/high, and high/high. Only missense variants were considered as having a low-impact. Nonsense, frameshift, in frame, splice site, and large deletions/insertions – such as copy number variants – were considered high impact variants.

Statistical Analysis

Analyses were performed on 1 eye per patient. Worst eye was defined as the one with lower VA or (in case of identical VA) larger EZ width. Data normality was tested according to Kolmogorov-Smirnov. Continuous variables were described as mean and standard deviations (±), while categorical variables by using frequency. Covariance analysis was applied to the dataset by appointing age, sex, and allele status as covariates; in case of statistically significant results, the differences between groups were inspected using 2-ways unpaired *t* test (continuous data) or χ^2 (categorical data). We also tested Pearson correlation between variables of interest. The analyses were performed using IBM SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0.; IBM Corp., Armonk, NY, USA).

Results

We considered data from 92 patients affected by *USH2A*-related retinal dystrophy: 46 of them were classified as arRP and 46 as Ush2. In the arRP group, 24 sub-

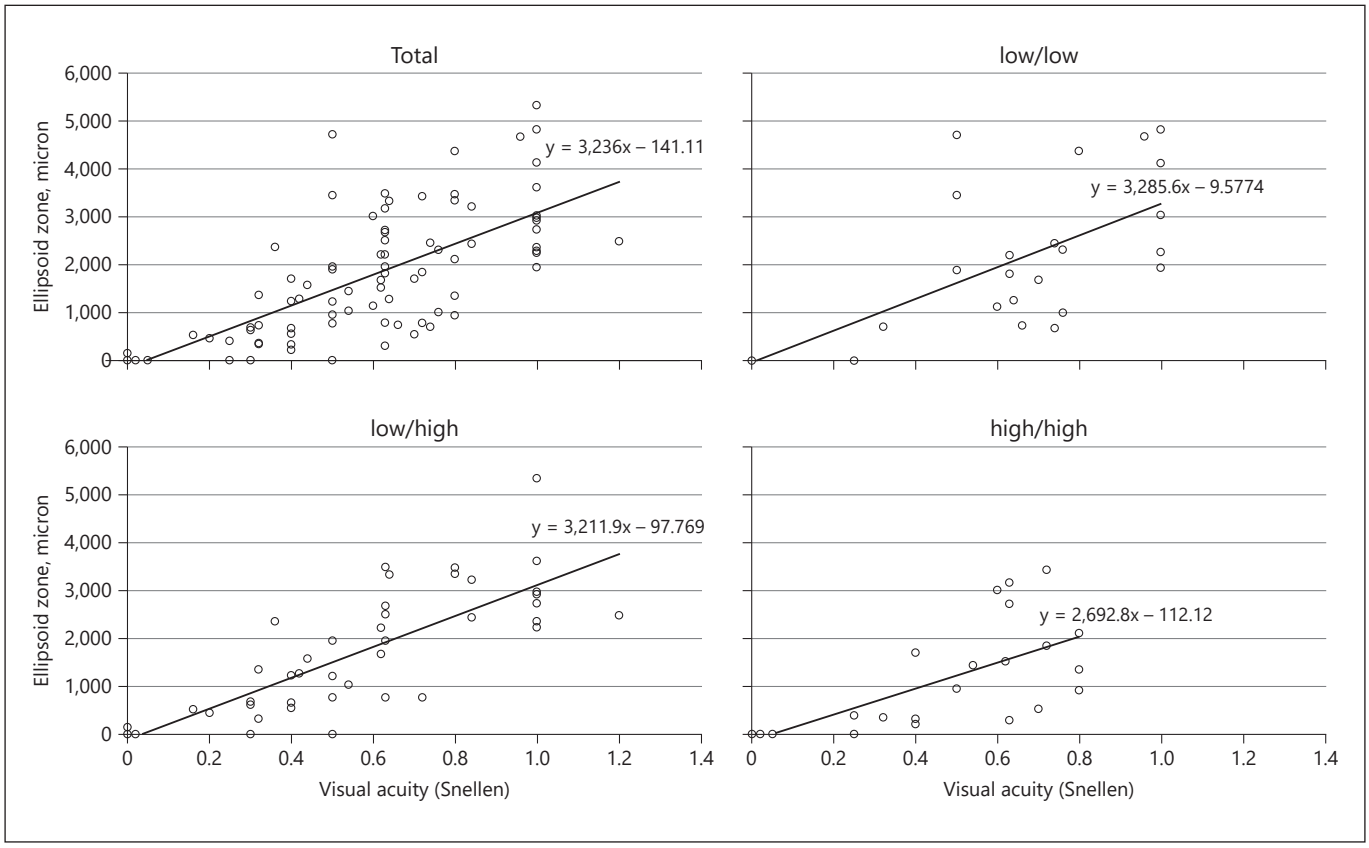

Fig. 2. Correlation between VA and EZ width in the whole cohort (total) or divided according to the genotype distribution by the expected variant impact (low/high) on each allele. VA, visual acuity; EZ, ellipsoid zone

jects were males and 22 were females, while in Ush2 group they were 18 and 28, respectively. Table 1 summarizes the clinical data: the mean age of the whole cohort was 48.16 ± 13.67 (52.32 ± 12.94 in the arRP group and 44.08 ± 13.24 in the Ush2 group). The disease onset was earlier in Usher patients as compared to those affected by nonsyndromic RP, irrespective of sex. Mean VA was 0.61 ± 0.31 and mean EZ width was 1,969.30 ± 1,411.73 μm in nonsyndromic patients, while mean VA was 0.49 ± 0.30 and mean EZ width was 1,306.63 ± 1,246.49 μm in syndromic ones.

This shows that VA and EZ width were significantly lower in Ush2 than in arRP patients ($p < 0.0001$ and $p = 0.001$, respectively). This difference was statistically significant also when a model of analysis of covariance was applied to the dataset, using the criteria stated above.

ERM was detected in 34.8% (16/46) of arRP patients and in 65.2% (30/46) of Ush2 patients ($p = 0.003$). A gender-related difference of ERM incidence was also detected: ERM was more frequent in males than in females, but it was not statistically significant. In the arRP group, the incidence of ERM was 37.5% (9/24) in males and 31.8% (7/22) in females ($p = 0.78$), while in Ush2 group it was 77.8% (14/18) in males and 57.1% (16/28) in females ($p = 0.52$).

CML was detected in 17.4% (8/46) of arRP patients and 30.4% (14/46) of Ush2 patients ($p = 0.14$). CML incidence in the arRP group was 20.8% (5/24) in males versus 13.6% (3/22) in females ($p = 0.59$), while in the Ush2 group it was 27.8% (5/18) in males versus 32.1% (9/28) in females ($p = 0.82$). We classified CML according to the grading system proposed by [16]: in the arRP group 12.5% (1/8) of patients had mild CML, 50% (4/8) moderate, and 37.5% (3/8) severe. In the Ush2 group, 42.9% (6/14) of patients had mild CML, 50% (7/14) moderate, and 7.1% (1/14) severe.

Overall, the genotype distribution by the expected variant impact on each allele was 27.2% high/high, 47.8% low/high, and 25% low/low. The genotype distribution was statistically different ($p = 0.0003$) by dividing the 2

Table 2. Demographic, clinical, and genetic characteristics of Usher syndrome patients carrying *USH2A* (NM_206933) variants

Patient ID	Sex	Age, years	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
USHER-1	M	33	UNK	0.8	924	1	0	ex13	c.2209C>T	p.(Arg737*)	HET	rs111033334	High	[21]
								ex18	c.3920C>G	p.(Ser1307*)	HET	rs756623509	High	[22]
USHER-2	F	18	15	0.63	2,720	1	1	ex50	c.9811del	p.(Met3271Cysfs*30)	HET	rs767328784	High	[23]
								int29	c.5776+1G>A	p.(?)	HET	rs876657731	High	[24]
USHER-3	F	37	16	0.6	3,011	1	0	ex47	c.9270C>A	p.(Cys3090*)	HET	rs779572631	High	[25]
								int40	c.7595-2144A>G	p.(?)	HET	rs786200928	High	[26]
USHER-4	M	31	7	0.4	1,697	1	0	ex69	c.14977_14978del	p.(Phe4993Profs*7)	HET	rs747160949	High	[27]
								int31	c.6164-3C>G	p.(?)	HET	rs755593389	High	[3]
USHER-5	F	64	30	0.25	388	0	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
USHER-6	M	50	19	0.4	205	0	0	ex12	c.1841-2A>G	p.(?)	HOM	rs397518003	High/High	[29]
USHER-7	M	35	19	0.25	0	1	0	ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
								int69	c.15053-2A>T	p.(?)	HET	NA	High	[30]
USHER-8	M	56	15	0.62	1,515	1	1	ex17	c.3684T>A	p.(Cys1228*)	HOM	NA	High/High	[31]
USHER-9 (F1)	F	46	UNK	0.5	944	0	1	int28	c.5776+1G>A	p.(?)	HET	s876657731	High	[24]
								ex23-32	c.4758+3787_c.6325+9314del	p.(?)	HET	NA	High	[27]
USHER-10 (F1)	F	38	UNK	0.63	296	0	0	int28	c.5776+1G>A	p.(?)	HET	rs876657731	High	[24]
								ex23-32	c.4758+3787_c.6325+9314del	p.(?)	HET	NA	High	[27]
USHER-11	F	48	24	0.05	0	1	0	ex5	c.785-?_1840+?del	p.(?)	HET	NA	High	[3]
								ex2	c.194del	p.(Thr65Ilefs*80)	HET	NA	High	[3]
USHER-12	F	74	20	0.4	320	0	0	ex27	c.5418_5424del	p.(Lys1807Alafs*8)	HOM	rs756486771	High/High	[32]
USHER-13	F	42	38	0.54	1,435	1	1	ex30	c.6029del	p.(Val2010Alafs*10)	HET	rs762482370	High	[32]
								ex51	c.10182G>A	p.(Lys3394 =)	HET	rs751903445	High	[33]
USHER-14	M	43	UNK	0	0	1	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
USHER-15	F	48	15	0.32	345	1	0	ex26	c.5189_5199del	p.(Tyr1730Trpfs*6)	HOM	NA	High/High	[34]
USHER-16	M	55	16	0	0	1	0	ex5-10	c.784+?_1841-?del	p.(?)	HOM	NA	High/High	[3]
USHER-17	M	67	30	0	0	1	0	ex48	c.9424G>T	p.(Gly3142*)	HET	rs397518048	High	[35]
								int12	c.2167+5G>A	p.(?)	HET	rs771583281	High	[36]
USHER-18	M	28	20	0.72	3,430	1	1	ex18	c.3932C>A	p.(Ser1311*)	HET	rs79279902	High	[24]
								ex48	c.9424G>T	p.(Gly3142*)	HET	rs397518048	High	[35]
USHER-19	M	28	14	0.8	1,347	1	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex51	c.10182G>A	p.(Lys3394 =)	HET	rs751903445	High	[33]

Table 2 (continued)

Patient ID	Sex	Age, years	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
USHER-20	F	53	8	0.02	0	0	0	ex11	c.1876C>T	p.(Arg626*)	HET	rs534534437	High	[37]
								ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
USHER-21	M	45	15	0.7	531	1	0	ex2	c.187C>T	p.(Arg63*)	HET	rs781223647	High	[38]
								ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
USHER-22	F	46	UNK	0.72	770	1	0	ex69	c.14977_14978del	p.(Phe4993Profs*7)	HET	rs747160949	High	[27]
								ex8	c.1547G>T	p.(Gly516Val)	HET	rs1415484067	Low	[39]
USHER-23	M	24	UNK	0.5	766	1	0	ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
								ex12	c.2071T>C	p.(Cys691Arg)	HET	rs770293341	Low	[40]
USHER-24	F	26	1	0.3	681	1	0	ex54	c.10712C>T	p.(Thr3571Met)	HET	rs202175091	High	[41]
								ex48	c.9424G>T	p.(Gly3142*)	HET	rs397518048	Low	[35]
USHER-25	M	52	40	0.44	1,570	1	0	ex7	c.1256G>T	p.(Cys419Phe)	HET	rs121912600	High	[37]
								ex63	c.13335_13347delinsCTTG	p.(Glu4445_Ser4449delinsAspLeu)	HET	rs1553252388	Low	[25]
USHER-26	F	63	20	0.54	1,037	1	1	ex32	c.6317_6318del	p.(Ile2106Serfs*51)	HET	NA	High	[3]
								ex13	c.2435C>T	p.(Thr812Ile)	HET	rs768560709	Low	[3]
USHER-27	F	41	30	0.5	1,213	0	0	ex10	c.1760_1762del	p.(Asn587del)	HET	NA	High	[39]
								ex51	c.10182G>A	p.(Lys3394 =)	HET	rs751903445	Low	[33]
USHER-28	F	59	15	0.2	449	1	0	ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
								ex41	c.7939C>T	p.(Pro2647Ser)	HET	NA	Low	[3]
								ex65	c.14204C>G	p.(Pro4735Arg)	HET	rs1333608275	Low	[31]
USHER-29 (F2)	F	24	UNK	1	2,969	1	1	ex10	c.1663C>G	p.(Leu555Val)	HET	rs35818432	High	[42]
								int10	c.1841-2A>G	p.(?)	HET	rs397518003	Low	[29]
USHER-30 (F2)	F	30	UNK	0.5	1,952	0	1	ex10	c.1663C>G	p.(Leu555Val)	HET	rs35818432	High	[42]
								int10	c.1841-2A>G	p.(?)	HET	rs397518003	Low	[29]
USHER-31 (F3)	F	61	14	0.5	0	1	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex8	c.1531G>A	p.(Glu511Lys)	HET	rs767209934	Low	[27]
USHER-32 (F3)	F	57	14	0	0	0	1	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex8	c.1531G>A	p.(Glu511Lys)	HET	rs767209934	Low	[27]
USHER-33	F	35	29	0.8	3,467	1	0	ex54	c.10699del	p.(Leu3567*)	HET	NA	High	[43]
								ex6	c.908G>A	p.(Arg303His)	HET	rs371777049	Low	[44]
USHER-34	F	49	42	1	2,230	1	0	ex65	c.14174G>A	p.(Trp4725*)	HET	NA	High	[30]
								ex14	c.2953T>C	p.(Cys985Arg)	HET	rs1171264735	Low	[3]
USHER-35	M	32	UNK	0.63	2,498	0	1	ex6	c.1000C>T	p.(Arg334Trp)	HET	rs397517963	High	[45]
								ex70	c.15208G>T	p.(Glu5070*)	HET	rs1426135314	Low	[46]
USHER-36	F	33	12	0.63	1,945	1	0	ex12	c.2071T>C	p.(Cys691Arg)	HET	rs770293341	High	[40]
								ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	Low	[28]

Table 2 (continued)

Patient ID	Sex	Age, years	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
USHER-37	F	22	18	0.8	3,345	1	1	ex27	c.5418_5424del	p.(Lys1807Alafs*8)	HET	rs756486771	High	[32]
								ex12	c.2071T>C	p.(Cys691Arg)	HET	rs770293341	Low	[40]
USHER-38	F	44	30	0.84	3,214	1	1	ex12	c.2071T>C	p.(Cys691Arg)	HET	rs770293341	High	[40]
								ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	Low	[7]
USHER-39	M	63	7	0	0	1	0	ex13	c.2710_2720dup	p.(Leu908Profs*63)	HET	rs755218835	High	[30]
								ex35	c.6713A>C	p.(Glu2238Ala)	HET	rs41277212	Low	[33]
USHER-40	M	50	UNK	0.25	0	1	0	ex8	c.1515A>T	p.(Arg505Ser)	HET	NA	Low	New [47]
								ex63	c.12575G>A	p.(Arg4192His)	HET	rs199605265	Low	
USHER-41	F	54	28	0.6	1,132	0	0	ex42	c.8395G>C	p.(Gly2799Arg)	HOM	NA	Low/Low	[3]
USHER-42	F	39	UNK	1	4,827	0	0	ex61	c.11713C>T	p.(Arg3905Cys)	HOM	rs368675850	Low/Low	[48]
USHER-43	M	42	UNK	0.63	2,207	1	1	ex62	c.12112A>G	p.(Thr4038Ala)	HET	rs754979740	Low	New [27]
								ex10	c.1729T>C	p.(Cys577Arg)	HET	rs1553327470	Low	
USHER-44	F	46	6	0.74	2,448	1	1	ex12	c.2071T>C	p.(Cys691Arg)	HET	rs770293341	Low	[40]
								ex42	c.8254G>A	p.(Gly2752Arg)	HET	rs201863550	Low	[49]
USHER-45	F	68	27	0	0	1	0	ex9	c.1606T>C	p.(Cys536Arg)	HET	rs111033273	Low	[38]
								ex25	c.5153A>C	p.(Gln1718Pro)	HET	NA	Low	[48]
USHER-46	F	29	15	1	2,277	1	0	ex6	c.1055C>T	p.(Thr352Ile)	HOM	rs780308389	Low/Low	[50]

In bold: biallelic variants that were confirmed via segregation study. *F* (*n*), number of the diseased subjects from the same family; ex, exon; int, intron; HOM, homozygous; HET, heterozygous; NA, not available; VA, visual acuity; EZ, ellipsoid zone; ERM, epiretinal membrane; CML, cystic macular lesions.

Table 3. Demographic, clinical and genetic characteristics of retinitis pigmentosa patients carrying *USH2A* (NM_206933) variants

Patient ID	Sex	Age	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
RP-1	M	67	47	0	0	0	0	int28	c.5776+1G>C	p.(?)	HOM	NA	High/High	[3]
RP-2	M	67	45	0.63	3,170	0	1	int11	c.1841-2A>G	p.(?)	HET	rs397518003	High	[29]
								ex63	c.13335_13347delinsCTTG	p.(Glu4445_Ser4449delinsAspLeu)	HET	rs1553252388	High	[25]
RP-3	F	46	24	0.72	1,841	0	0	ex63	c.13335_13347delinsCTTG	p.(Glu4445_Ser4449delinsAspLeu)	HET	rs1553252388	High	[25]
								int10	c.1841-2A>G	p.(?)	HET	rs397518003	High	[29]
RP-4	M	36	26	0.8	2,106	0	0	ex63	c.13335_13347delinsCTTG	p.(Glu4445_Ser4449delinsAspLeu)	HET	rs1553252388	High	[25]
								int43	c.8682-9A>G	p.(?)	HET	rs372347027	High	[33]
RP-5	F	52	30	0.4	549	1	0	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	High	[51]
								ex54	c.10699del	p.(Leu3567*)	HET	NA	Low	[43]
RP-6	F	58	53	0.84	2,435	0	1	int40	c.7595-2144A>G	p.(?)	HET	rs786200928	High	[26]
								ex35	c.6713A>C	p.(Glu2238Ala)	HET	rs41277212	Low	[33]
RP-7	F	25	22	1	3,615	1	0	ex18	c.4046del	p.(Ser1349Phefs*17	HET	NA	High	[23]
								ex55	c.10817T>C	p.(Leu3606Pro)	HET	rs1402464909	Low	[25]
RP-8	F	72	43	0.02	0	1	0	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	High	[51]
								ex17	c.3332T>G	p.(Leu1111*)	HET	NA	Low	[3]
RP-9	F	55	15	0	0	1	0	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	High	[51]
								ex49	c.9676C>T	p.(Arg3226*)	HET	rs760858249	Low	[52]
RP-10	F	46	30	1	2,914	0	0	ex2	c.187C>T	p.(Arg63*)	HET	rs781223647	High	[38]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
								ex27	c.5330G>A	p.(Arg1777Gln)	HET	rs541275063	Low	[3]
RP-11	F	63	54	0	144	0	0	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	High	[51]
								int11	c.1841-2A>G	p.(?)	HET	rs397518003	Low	[29]
RP-12	M	64	39	0.63	3,483	1	1	ex27	c.5418_5424del	p.(Lys1807Alafs*8)	HET	rs756486771	High	[32]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-13	M	80	UNK	0.63	769	1	0	ex13	c.2209C>T	p.(Arg737*)	HET	rs111033334	High	[21]
								ex65	c.14219C>A	p.(Ala4740Asp)	HET	rs539192853	Low	[53]
RP-14	M	49	40	1.2	2,480	0	0	ex30	c.5933_5940del	p.(Pro1978Glnfs*5)	HET	NA	High	[25]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-15	M	41	44	0.32	1,359	0	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-16	F	52	26	0.63	2,672	1	0	ex53	c.10450C>T	p.(Arg3484*)	HET	rs111033379	High	[24]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-17	F	61	54	0.42	1,269	1	0	int8	c.1551-5T>G	p.(?)	HET	rs770011395	High	[3]
								ex60	c.11660G>C	p.(Trp3887Ser)	HET	NA	Low	[3]
RP-18	M	49	UNK	0.64	3,333	1	0	ex61	c.11864G>A	p.(Trp3955*)	HET	rs111033364	High	[7]

Table 3 (continued)

Patient ID	Sex	Age	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
								ex63	c.12574C>T	p.(Arg4192Cys)	HET	rs750396156	Low	[54]
RP-19 (F1)	M	54	UNK	1	2,731	0	0	ex8	c.1412_1415dup	p.(Asn472Lysfs*2)	HET	NA	High	New
								ex19	c.4124C>T	p.(Ser1375Leu)	HET	rs751479180	Low	New
RP-20 (F1)	F	50	UNK	1	5,332	0	0	ex8	c.1412_1415dup	p.(Asn472Lysfs*2)	HET	NA	High	New
								ex19	c.4124C>T	p.(Ser1375Leu)	HET	rs751479180	Low	New
RP-21	F	52	47	0.62	2,212	0	1	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	High	[51]
								int10	c.1841-2A>G	p.(?)	HET	rs397518003	Low	[29]
RP-22	M	66	35	0.32	319	1	0	ex27	c.5418_5424del	p.(Lys1807Alafs*8)	HET	rs756486771	High	[32]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-23	F	34	22	0.4	1,231	1	0	ex18	c.3890_3891	p.(Phe1297Sfs*17)	HET	NA	High	[31]
								ex36	c.6929C>T	p.(Thr2310Met)	HET	rs151057466	Low	[3]
RP-24	F	53	UNK	0.3	0	0	0	ex54	c.10699del	p.(Leu3567*)	HET	NA	High	[43]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-25	M	40	20	0.36	2,359	0	1	ex61	c.12006C>A	p.(Tyr4002*)	HET	NA	High	[25]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
								ex27	c.5330G>A	p.(Arg1777Gln)	HET	rs541275063	Low	[3]
RP-26	M	44	7	1	2,362	1	0	ex63	c.13374del	p.(Glu4458Aspfs*3	HET	rs727503715	High	[35]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-27	M	50	38	0.4	657	1	0	ex45	c.8906C>G	p.(Ser2969*)	HET	NA	High	[23]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-28	F	45	UNK	0.62	1,667	0	0	ex24	c.4957C>T	p.(Arg1653*)	HET	rs754768875	High	[24]
								ex65	c.14219C>A	p.(Ala4740Asp)	HET	rs539192853	Low	[53]
RP-29	F	39	18	0.16	519	0	0	ex44	c.8834G>A	p.(Trp2945*)	HET	rs760302201	High	[25]
								ex13	c.2296T>C	p.(Cys766Arg)	HET	rs368687374	Low	[33]
RP-30	F	80	33	0.3	620	0	0	ex13	c.2299del	p.(Glu767Serfs*21)	HET	rs80338903	High	[28]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-31	M	38	UNK	0.5	3,454	0	1	ex11	c.1891G>C	p.(Asp631His)	HET	rs552400144	Low	[3]
								ex30	c.5858C>G	p.(Ala1953Gly)	HET	rs41302239	Low	[55]
RP-32	M	55	45	0.64	1,266	1	0	ex53	c.10429T>C	p.(Ser3477Pro)	HET	NA	Low	[3]
								ex61	c.11713C>T	p.(Arg3905Cys)	HET	rs368675850	Low	[48]
RP-33	M	48	44	0.7	1,693	0	0	ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
								ex53	c.10437G>T	p.(Trp3479Cys)	HET	rs1308924086	Low	[43]
RP-34	M	33	22	1	1,934	0	0	ex6	c.908G>A	p.(Arg303His)	HET	rs371777049	Low	[44]
								ex65	c.10817T>C	p.(Leu3606Pro)	HET	rs1402464909	Low	[25]
RP-35	F	27	18	1	4,131	0	0	ex13	c.2276G>T	p.(Cys759Phe)	HOM	rs80338902	Low/Low	[51]

Table 3 (continued)

Patient ID	Sex	Age	Onset	VA	EZ	MER	CML	Exon/intron	Nucleotide change	Amino acid change	Allele state	dbSNP rs	Severity	Ref.
RP-36	F	64	57	0.8	4,370	0	0	ex9	c.1571C>A	p.(Ala524Asp)	HET	rs772624410	Low	[3]
								ex63	c.12575G>A	p.(Arg4192His)	HET	rs199605265	Low	[47]
RP-37	M	39	38	0.96	4,680	0	0	ex19	c.4106C>T	p.(Ser1369Leu)	HET	rs201709513	Low	[50]
								ex23	c.4862T>A	p.(Ile1621Asn)	HET	NA	Low	[3]
RP-38	M	46	30	1	3,035	0	0	ex10	c.1751G>T	p.(Cys584Phe)	HET	NA	Low	[56]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-39	M	42	28	0.76	2,310	0	0	ex12	c.2071T>C	p.(Cys691Arg)	HOM	rs770293341	Low/Low	[40]
RP-40	M	67	56	0.5	4,717	1	0	ex41	c.7915T>C	p.(Ser2639Pro)	HET	rs398124620	Low	[31]
								ex50	c.9815C>T	p.(Pro3272Leu)	HET	rs764182950	Low	[57]
RP-41	F	66	43	0.5	1,895	0	0	ex41	c.7915T>C	p.(Ser2639Pro)	HET	rs398124620	Low	[31]
								ex50	c.9815C>T	p.(Pro3272Leu)	HET	rs764182950	Low	[57]
RP-42	F	53	62	0.32	715	1	0	ex19	c.4106C>T	p.(Ser1369Leu)	HET	rs201709513	Low	[50]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-43	M	61	33	0.63	1,811	1	1	ex8	c.1434G>C	p.(Glu478Asp)	HET	rs35730265	Low	[39]
								ex19	c.4106C>T	p.(Ser1369Leu)	HET	rs201709513	Low	[50]
								ex71	c.15373C>T	p.(Arg5125Cys)	HET	rs771243585	Low	[3]
RP-44	F	69	24	0.74	689	0	0	ex12	c.1991G>T	p.(Cys664Phe)	HET	NA	Low	[3]
								ex13	c.2296T>C	p.(Cys766Arg)	HET	rs368687374	Low	[33]
RP-45	F	59	22	0.76	1,006	1	1	ex6	c.1055C>T	p.(Thr352Ile)	HET	rs780308389	Low	[50]
								ex13	c.2276G>T	p.(Cys759Phe)	HET	rs80338902	Low	[51]
RP-46	M	46	UNK	0.66	734	0	0	ex50	c.9815C>T	p.(Pro3272Leu)	HOM	rs764182950	Low/Low	[57]

In bold: biallelic variants that were confirmed via segregation study. RP, retinitis pigmentosa; *F* (*n*), diseased subjects of the same family; ex, exon; int, intron; HOM, homozygous; HET, heterozygous; NA, not available; VA, visual acuity; EZ, ellipsoid zone; ERM, epiretinal membrane; CML, cystic macular lesions.

diseases: it was 45.7% high/high, 39.1% low/high, and 15.2% low/low for Usher patients (Table 2) and 8.7% high/high, 56.5% low/high, and 34.8% low/low for RP patients (Table 3). Considering the whole cohort of 92 subjects (46 arRP + 46 Ush2 patients), the genotype severity class significantly affected the parameters of VA and EZ width ($p = 0.004$ and $p = 0.002$, respectively), while ERM and CML incidences were not affected ($p = 0.26$ and $p = 0.86$, respectively).

The correlation between VA and EZ was substantial in the whole cohort ($r = 0.76$, $p < 0.0001$) and in the high/high ($r = 0.68$, $p = 0.01$) and low/high groups ($r = 0.74$, $p < 0.0001$); while it was moderate in the low/low group ($r = 0.50$, $p = 0.02$) (shown in Fig. 2). About the onsets, the average ages of onset were distributed among the 3 groups (from earliest to latest) as follows: 22 ± 10.8 years in high/high patients, 28.5 ± 14.4 years in high/low patients, and 33.2 ± 15.4 years in low/low patients. The same statistics performed on the arRP and Ush2 groups, taken individually, did not reach significance in any of the considered parameters (data not shown).

Discussion/Conclusion

In our study, we evaluated macular structure using SD-OCT in a large cohort of *USH2A*-related arRP and Ush2 patients and found that syndromic cases show more advanced signs of retinal degeneration. As described in Materials and Methods section, we divided the patients into 3 categories (according to the severity of their variants) and found a significant correlation between their anatomical data and genotypes.

The differences in the disease course in syndromic versus nonsyndromic forms of *USH2A*-related retinal dystrophy were previously investigated: Sandberg et al. [58] compared VA, VF, and cone electroretinogram amplitudes in a large cohort of *USH2A*-related arRP and Ush2 and they did not find any statistically significant difference between the 2 groups for all the considered parameters. It is worth noting that the research compared only RP patients with the p.(Cys759Phe) – *USH2A* variant most frequently associated with the nonsyndromic disease – with Usher patients carrying the p.(Glu767Serfs*21) – the most common *USH2A* variant associated with the syndrome. About half of our RP patients (21/46, 45.6%) carried the p.(Cys759Phe) and 13% of our Usher syndrome patients carried the p.(Glu767Serfs*21); however, in our population the most frequent Usher variant (8/46, 17.4%) was the p.(Trp3955*). Despite it could eliminate some confusion, the choice made in the study of Sandberg et al. [58] of comparing only 2 types of variants does not allow to generalize the result to other variants and/or populations.

Earlier impairment of retinal function in syndromic versus nonsyndromic *USH2A* patients was described by Pierrache et al. [30]; they found that Ush2 patients become visually impaired earlier, both according to VF and VA (13 and 18 years earlier, respectively). We analyzed the structural data in a different way: EZ width, as evaluated via SD-OCT, has become one of the most important markers to evaluate retinal involvement and to monitor the disease in IRDs services. EZ hyper-reflective band corresponds to the photoreceptor inner/outer segment junction: published literature shows the reproducibility and repeatability of EZ measurements and its correlation with functional data, like VA and VF [12–15, 59]. In our study, EZ width was significantly smaller in the Ush2 group than the arRP group, thus confirming that outer retina atrophy occurs at earlier age in the former rather than in the latter.

Consistently with EZ data, VA was found to be significantly lower in Ush2 than in arRP patients. A substantial correlation was also found between EZ width and VA ($p < 0.0001$), which has also been previously reported in RP patients by many authors [60–62]. Given the extreme variability of VF or ERG data, the wide spread of SD-OCT machines in ophthalmology clinics, and the straightforward acquisition and analysis of tomographic images, EZ width and integrity can be considered as the objective choice parameter to evaluate photoreceptors functionality.

Sengillo et al. [63] found in a small cohort of patients affected by *USH2A*-related retinal dystrophy that 30 Hz flicker responses were significantly higher in arRP patients than in Ush2 patients, thus confirming that Usher patients experience a faster decline of their visual functions. They also measured the EZ width in the 2 groups but, unlike in our study, the comparison did not reach statistical significance. We guess that the small number of patients and the younger age of their cohort could explain the differences with our findings.

We then assessed the presence of other macular abnormalities in our cohort of patients affected by *USH2A*-related dystrophies: specifically, ERM and CML. In our dataset ERM was more prevalent than CML (50% vs. 23.9%, $p = 0.0004$); most importantly, we found an increased incidence of ERM in Ush2 patients compared to arRP (65.2% vs. 34.8%, $p = 0.0068$). Many clinical studies investigated the prevalence of ERM in RP and Usher population, with results ranging from 0.6 to

80.5% [64–69]. This great variability could be due to at least 2 different factors. First of all, there is no universal consensus on the interpretation and classification of ERMs in SD-OCT scans: some authors include VMA and VMT as part of the ERMs group [70], but in other cases the presence of focal cells proliferation on the inner surface of the retina could be seen as the presence of ERMs by some OCT-readers, thus overestimating the incidence of such condition. Second, other factors such as age, inflammation, trauma, and previous ocular surgeries or laser treatments can play a role in ERM growth. Interestingly, in our cohort ERM was more prevalent in males rather than in females in both groups, but the difference was statistically significant only in the Ush2 group. Our results are in line with Testa et al. [66] in Ush2 but differ from another report by the same group in RP patients [67] and from literature about ERM incidence that indicates that for females the risk of developing ERM is higher [71].

On our daily practice experience, a small percentage of patients affected by arRP or Ush2 complicated by ERM requires vitreoretinal surgical procedures. Nevertheless, considering therapeutic surgical approaches such as gene therapy and artificial retina implant, ERM assessment plays a significant role in the preoperative evaluation of the patient.

As described above, we classified CML in both groups according to the grading system proposed by Sliesoraityte et al. [16]. Similarly to ERM, incidence of CML was higher in Ush2 rather than in arRP patients (30.4% vs. 17.4%, $p = 0.2234$); moreover, patients with arRP usually have a moderate or severe grade of CML (50 and 37.5%, respectively, $p = 0.3181$), while Ush2 patients usually have a mild or moderate grade of CML (42.9 and 50%, respectively, $p = 0.6357$). Our data are in line with previous CML reports [66, 67, 72–75], and have a strong correspondence with the one by Sliesoraityte et al. [16] in terms of incidence of CML in *USH2A* syndromic patients (30.4% vs. 29%).

CML is a frequent issue for clinicians in IRDs services: there are no universally accepted guidelines available for the treatment of CML in both pediatric and adult population. Many strategies have been proposed – oral or topical administration of carbonic anhydrase inhibitors, anti-vegf agents, steroidal and anti-inflammatory treatments [76]. Recently published literature shows the efficacy of dexamethasone implants on CML reduction, but it is necessary to be cautious in considering this treatment: it is an invasive procedure that needs to be performed periodically and that can lead to possible complications, such as IOP increase or cataract formation [77, 78]. To the best of our knowledge, this is the first comparison of the incidence of ERM and CML in syndromic and nonsyndromic *USH2A* patients. As described in detail in the Materials and Methods section, we divided the patients into 3 groups, according to severity of the variants we identified in *USH2A* gene.

Another research [30] attempted to associate *USH2A* variant types with visual prognosis in RP and Usher syndrome patients. In their report, patients were grouped based on the presence of truncating variants in their alleles, thus considering more severe the variants leading to a shortened or absent protein, the latter resulting from nonsense-mediated mRNA decay. Despite this little difference, the distribution of highly pathogenic variants was very similar to our findings for both Usher syndrome (high/high: 44% truncating/truncating vs. 45.7%; low/high: 40% nontruncating/truncating vs. 39.17%; low/low: 16% nontruncating/nontruncating vs. 15.2%) and RP patients (high/high: 3% truncating/truncating vs. 8.7%; low/high: 62% nontruncating/truncating vs. 56.5%; and low/low: 36% nontruncating/nontruncating vs. 34.8%).

The results show that variant types and genotype classes are determining factors for the appearance of syndromic features. However, since the 3 genotypes can be found in both Usher and RP patients, other factors must necessarily play a determining role: among these could be the position of the variants at the protein level, which of the 2 usherin isoform is impacted by the variant, and the modifying influences of the genetic background in which the variant acts.

Considering the whole cohort, we found significant genotype-phenotype correlations: the 2 main clinical parameters evaluated, VA and EZ width, were substantially associated with the severity class of the variants. In particular, both EZ width and VA were lower in the high/high group than in the low/high and low/low ones and the association was stronger in the low/high and high/high groups ($p < 0.0001$ and $p = 0.01$, respectively) than in the low/low group ($p = 0.02$). While earlier VA decline in patients with truncating variants in *USH2A* gene has been previously reported [30], to the best of our knowledge, this is the first report of the association between EZ width and the severity of genetic variants in *USH2A*-related retinal dystrophies.

Additionally, we found that the disease onset occurs earlier in Usher patients, regardless of their genotype. Moreover, the genotype class was associated with both the age of onset – regardless of the disease – and the type

of disease (i.e., Usher or RP). We can therefore conclude that the genotype influences both the age of onset of the disease and the possible manifestation of deafness, which distinguishes Usher syndrome from nonsyndromic RP.

New emerging therapies renewed enthusiasm in the field of IRDs and, on the other hand, pointed out the necessity to deeper understand disease mechanisms and to establish clinical parameters to assess the efficacy of therapies and to identify the patients that could benefit more from them. From this perspective, our study highlighted that retinal diseases, as evaluated by means of SD-OCT, show more advanced signs of degeneration in the syndromic rather than in the nonsyndromic form of retinal dystrophy related to *USH2A* gene, although the relationship between genetic variants and the resulting phenotype needs to be further investigated.

Our study has some limitations: the main concern is that we prospectively performed a single SD-OCT analysis, so we do not present follow-up data for each patient. This choice was made in order to have homogeneous data, since SD-OCT was not available for all patients and since in the past different OCT machines were used for clinical assessments. We know that we did not evaluate inter-individual disease progression; however, the 2 large cohorts considered allowed us to represent the differences in inter-group disease progression. Another drawback is that EZ width cannot be used as a parameter to monitor disease progression, both in the early stages – when EZ boundaries exceed the posterior pole (even if the advent of wide-field imaging is helping to overcome this limit) – and in the late stages – when outer retina is completely atrophic and EZ band is not easily detectable.

Overall, considering the number of homozygous patients and families studied by segregation analysis, the vast majority of our studied subjects (73.9%) were confirmed to carry biallelic *USH2A* variants. However, we cannot exclude that at least some of the remaining patients included in this study carried in cis *USH2A* variants and they could not be considered genetically solved.

Furthermore, the severity classification of the variants, which considers only the missense as low-impact variants, is here extremely simplified. For example, lacking experimental evidence, we cannot be sure that noncanonical splice variants and in-frame variants could be considered to have the same high impact on protein function as truncating variants. However, these variants are very rare exceptions that do not affect the final result of this study.

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Statement of Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki. This study protocol was reviewed and approved by the Ethical Committee of Azienda Sanitaria dell'Alto Adige, Italy, approval number 94-2016. Written informed consent has been obtained from the patients to publish this article.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

L.C. and P.E.M. contributed to conceptualization; P.F., G.M., F.C., and M.P. contributed to methodology; G.M. and P.F. contributed to software; F.C. and M.P. contributed to validation; L.C., P.F., and P.E.M. contributed to formal analysis; L.C., D.R., A.M.M., and B.P. contributed to investigation; M.B. and L.R. contributed to resources; P.F., P.E.M., and B.P. contributed to data curation; L.C. and P.E.M. contributed to writing – original draft preparation; L.C., P.E.M., and M.C. contributed to writing – review and editing; M.C., M.B., and L.R. contributed to visualization; M.B., M.C., and L.R. contributed to supervision; L.C. contributed to project administration; M.B. contributed to funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Clinical data reported in this work are available upon request from the corresponding authors. Results of patients' genetic analysis were reported in Colombo et al. [3], *Invest Ophthalmol Vis Sci*. 2021;62:13.

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