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Identification of numerous novel disease-causing variants in patients with inherited retinal diseases, combining careful clinical-functional phenotyping with systematic, broad NGS panel-based genotyping

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Purpose: The widespread consensus is that genotyping is essential for patients with inherited retinal disease (IRD). Given the numerous ongoing gene therapy clinical trials for IRDs, identifying the pathogenic mutation in these patients has potential important therapeutic implications. In this study, we demonstrate how we identified with a high degree of confidence numerous novel disease-causing mutations, deletions, and duplications in a large consecutive IRD case series by using a judicious combination of careful, in-depth clinical-functional phenotyping to guide and integrate our genotyping approach.

Methods: We conducted a retrospective analysis of data between November 2016 and March 2018 from the Duke Center for Retinal Degenerations and Ophthalmic Genetic Diseases IRD patient database, which encompassed 378 IRD cases that had not yet been previously genotyped. With the exception of some patients who presented with classical clinical-functional phenotypes that allowed for targeted gene testing, all other subjects systematically underwent next-generation sequencing-based broad, IRD-focused panel testing. Most cases were also tested for parental allele phase. Results were reviewed vis-à-vis the clinical-functional phenotypes for reconciliation and potential addition of supplemental testing such as deletion/duplication microarrays or copy number variant (CNV) analysis. Supplemental testing was driven by an IRD specialist-laboratory consensus, and decisions were clinically or genetically driven or both.

Results: By judiciously using this two-way approach and leveraging to its full potential the benefits of careful, in-depth clinical-functional phenotyping by an experienced IRD specialist, more than 80% of the cases in this series were successfully genotyped. We also identified with a high degree of confidence 52 novel disease-causing mutations, deletions, and duplications.

Conclusions: The combination of meticulous, expert clinical-functional phenotyping studies with systematic next-generation sequencing panel-based genotyping and microarray deletion/duplication testing or CNV analysis as applicable in accordance with the above-mentioned consensus was extremely effective at the diagnostic end, reduced costs, and saved time. IRD specialist-laboratory two-way interactions and case discussions would augment the efficacy of this approach and improve the diagnostic yield in successfully solving and genotyping IRD cases.

Inherited retinal diseases (IRDs) are a group of disorders in which genetic abnormalities cause a wide spectrum of visual disorders. They span from congenital conditions that cause significant visual incapacitation from birth but with limited progression over time, such as achromatopsia, blue cone monochromacy, congenital stationary night blindness, and fundus albipunctatus, to other conditions that have variable ages of onset but are characterized by progressive photoreceptor degeneration that leads to significant progressive loss of vision and often to blindness. IRDs are a vastly heterogenous group of disorders, both in terms of genotype and clinical phenotype (National Organization for Rare Diseases) [1-4]. Its overall incidence is estimated to range from 1 in 3,500 to 1 in 4,000 population worldwide [2,4]. The widespread consensus is that genotyping is essential for patients with IRD [4-6]. Well over 300 causal genes have been discovered thus far (RetNet). Each gene has numerous variants associated with diseases that range from point mutations to large changes (e.g., deletions and duplications). The latter ones are often challenging to detect during initial panel-based sequencing and often require further testing ranging from copy number variant (CNV) determination to DNA microarray analysis.

Given the increasing number of retinal gene therapy clinical trials and other gene or mechanism-driven interventions with promising results [3,5] finding the disease-causing

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genetic etiologies of IRDs has not only critically important diagnostic, counseling, and prognostic implications for affected patients but also important potential therapeutic implications. However, the best approach for genotyping patients with IRD is still controversial in the ophthalmic community [7-9]. The American College of Medical Genetics (ACMG) has set guidelines and recommendations to standardize the process of identifying novel disease-causing variants [8]. These extremely valuable and helpful guidelines have greatly enhanced the accuracy and reproducibility of molecular genetic diagnostic testing. Among the criteria defined by the ACMG, the phenotype expressed by affected patients is considered no more than a supportive finding in establishing the pathogenicity of novel or otherwise uncertain genetic changes (the so-called variants of uncertain significance [VUS]). Phenotypes are certainly variable, but, at times, they are only partially expressed in affected patients, and their characterization relies heavily on the experience and meticulousness of the examining clinicians. Hence, their value from a strict laboratory perspective is understandably generally limited. For IRDs, however, standardized approaches to phenotyping have long been implemented. Owing to the technological advancements in retinal diagnostics and the relative accessibility of the human eye, a tremendous amount of information is available about IRD-specific features. Thus, by engaging actively in clinician-laboratory two-way interactions, the IRD specialist and ophthalmic geneticist have the unique opportunity to provide especially detailed, standardized, highly reproducible, and accurate ocular and systemic phenotyping information that can significantly augment the ability to reach correct diagnostic conclusions about variants that would otherwise be considered only VUS by pure laboratory criteria and other ambiguous cases.

This approach has led to the efficient identification of the genetic etiology in more than 80% of the cases in our database. Herein, we demonstrate how we identified with a high degree of confidence 52 novel disease-causing mutations, deletions, and duplications in consecutive IRD cases by using this judicious combination of careful, in-depth clinical-functional phenotyping to guide and integrate our genotyping approach and report the results.

METHODS

We retrospectively reviewed a consecutive case series of 378 patients with previously molecularly uncharacterized IRD who visited the Duke Center for Retinal Degenerations and Ophthalmic Genetic Diseases between November 2016 and March 2018. Institutional review board (IRB) approval was obtained before the initiation of this study, and informed consent was obtained from the patients in accordance with IRB policies. All research procedures were adherent to the tenets of the Declaration of Helsinki. In addition to pedigree collection, phenotyping included an in-depth history collection using a standardized vision and review-of-systems questionnaire, complete eye examinations, imaging studies, various types of visual field and electrophysiological studies, including, when appropriate, full-field flash electroretinography (ffERG), multifocal ERG (mfERG), and electrooculography (EOG).

Targeted/focused genotyping was undertaken only in highly selected cases where the findings from the aforementioned clinical evaluation by the IRD specialist were highly suggestive of a specific genetic etiology by virtue of clinical presentation, functional findings, family history, inheritance pattern, or a combination thereof (see Appendix 1 for a summary flowchart of the decision-making process). For the remaining majority of patients, next-generation sequencing (NGS)-based panel testing, inclusive of CNV detection approaches once this technology became available, was undertaken at baseline. The NGS-based testing included 942 genes and additional mitochondrial genome and mitochondrial nuclear gene testing. Exons, exon-intron boundary regions, and previously known relevant deep intronic regions were sequenced. Whole gene sequencing was not performed in any of the patients included in this retrospective study. Further details regarding the sequencing methods are available in the Appendix 2 section.

In some cases where the etiology remained elusive, panel-based sequencing was then followed by deletion/duplication (del/dup) microarray testing. One or both parental samples were included for case phase assessment to help determine if variants were present in the cis versus trans configuration whenever possible. In cases where parental samples could not be obtained, possible affected or unaffected children and/or siblings/relatives were tested as surrogate best-available alternatives to parental testing to establish the phase of any detected mutation and to confirm segregation among other affected individuals. All genotyped patients underwent Clinical Laboratory Improvement Amendments (CLIA)-certified testing. Each novel variant was then assessed for pathogenicity using a combination of ClinVar, VarSome, PolyPhen-2 missense variant predictions, and laboratory assessments. This information, combined with phase determination and phenotype data, if all concordant was then used to label a variant as pathogenic or "definitely disease causing." If there was any missing information or even slight ambiguity in the clinical picture, the variant was labeled as just "likely disease causing." Additional specifics of the phenotypic criteria

Table 1. Summary of novel disease-causing mutations identified in our case series.

Subject	Gene	Allele 1 (A1)	Allele 2 (A2)
PT1	*ABCA4*	**c.2603delC, p.Pro868HisfsTer33 (M)**	c.2894A>G, p.Asn965Ser
PT2	*ABCA4*	**c.6213C>G, p.Tyr2071Ter**	c.6319C>T, p.Arg2107Cys
PT3	*ABCA4*	**c.1304G>T, p.Gly435Val**	c.768+358C>T
PT4	*ABCA4*	**c.4861A>T p. Asn1621Tyr**	c.5461-10T>C
PT5	*AIPL1*	**c.146T>C, p.Ile49Thr (M)**	c.834G>A, p. Trp278Ter
PT6	*BBS2*	**c.535-2A>G (M)**	**c.535-2A>G (P)**
PT7	*BBS6/MKKS*	**c.29C>A, p. Ser10Ter (M)**	c.155G>A, p.Gly52Asp (P)
PT8	*BBS6/MKKS*	**c.875T>G, p.Leu292Arg (M)**	**c.875T>G, p.Leu292Arg (P)**
PT9	*BEST1*	**c.75C>A, p.Tyr25Ter (P)**	**Exon 1-2 deletion (M)**
PT10	*BEST1*	**c.72G>T, p.Trp24Cys (M)**	N/A
PT11	*CDH23*	**c.6254-3_6254CAGGinsT (p.?)**	**c.6254-3_6254CAGGinsT (p.?)**
PT12	*CDHR1/ PCDH21*	**c.863-1G>A (IVS9-1G>A) (M)**	**c.863-1G>A (IVS9-1G>A)**
PT13	*CHM*	**c.117-1G>A**	N/A
PT14	*CHM*	**c.875A>C, p.Lys292Thr (M)**	N/A
PT15	*CHM*	**c.875A>C, p.Lys292Thr (M)**	N/A
PT16	*CRB1*	**c.70+2T>C (P)**	**c.71-24005_71-24004delAA (M)**
PT17	*EYS*	**c.32dupT, p.Met12AspfsTer14**	**c.32dupT, p.Met12AspfsTer14**
PT18	*EYS*	**IVS19+1G>T (M/P)**	IVS19+1G>T (M/P)
	EYS	**c.4402G>C, p.Asp1468His (M/P)**	**c.4402G>C, p.Asp1468His (M/P)**
PT19	*GPR98/ USH2C*	**c.17314C>T, p.Arg5772Ter**	**c.5111-68C>T (M)**
PT20	*MERTK*	**c.1724delA, p.Asn575IlefsTer3 (M)**	**c.2009T>A, p.Ile670Asn (P)**
PT21	*MYO7A*	**c.133G>T, p.Glu45Ter**	**c.4225delC, p.Leu1409SerfsTer2 (M)**
PT22	*MYO7A*	**c.1091dupC, p.Asp365ArgfsTer8**	c.1563delC, p.Asp521GlufsTer8

(M) and (P) indicate confirmed maternal and paternal inherited allele variants by genetic testing, respectively. (M/P) indicates both parents are carriers of same variant. N/A stands for not applicable, for X-linked or autosomal dominant inheritance patterns of inheritance. The novel mutations are illustrated in bold. See Table 2 for remainder of novel variants. See Supplemental Table for further details

used to reach our conclusions are presented in Column M of Appendix 3 ("Phenotype Assessment – Salient Clinical Features") and 2.

RESULTS

The novel disease-causing genetic changes that were identified in our case series (shown in bold) are illustrated in Table 1 and Table 2. Table 3 and Table 4 outline the summarized phenotypic data of each solved case. In selected cases, the disease expression proved to be so uniquely typical that it permitted successful targeted testing. This strategy can be especially useful in monogenic disorders and when medical health insurance coverage is limited or not available to affected patients. Some of these cases are illustrated briefly and further underscore the potential importance of meticulous, in-depth phenotyping.

However, the phenotypic presentation of our patients required, in most of cases, a broad, non-targeted approach. Broad NGS-based panels often revealed many novel genetic changes that only met VUS attribution by strict adherence to the ACMG guidelines. In 10 cases (including some homozygous cases), the parental allele phase could not be fully established. However, even in these cases, careful reconciliation of genotypic results with the aforementioned phenotypic data allowed us to molecularly characterize 44 patients harboring 52 novel mutations, deletions, and duplications that we can define as pathogenic with a very high degree of confidence on the basis of the aggregate evaluation of both the genotypic and compelling phenotypic information. In addition, certain atypical and ambiguous phenotypes were clarified only after genotyping. Further details about each of the 44 patients, the novel mutations they harbored, and their detailed phenotypic

Table 2. Summary of novel disease-causing mutations identified in our case series-continued (M) and (P) indicate confirmed maternal and paternal inherited allele variants by genetic testing, respectively.

Subject	Gene	Allele 1 (A1)	Allele 2 (A2)
PT23	*PDE6B*	**c.2003A>T, p.Asp668Val**	c.2193+1G>A (M)
PT24	*PDE6B*	**c.1933_1938delTACCAG (in-frame), p.Tyr645_Gln646del**	**c.2038C>T, p.Q680X (M)**
PT25	*POC1B*	**c.114G>A, p.Trp38Ter (M)**	**c.114G>A, p.Trp38Ter**
PT26	*POC1B*	**c.934delA, p.Arg312GlufsTer32 (P)**	**c.631A>G, p.Lys211Glu (M)**
PT27	*PROM1*	**c.1234delT, p.Tyr412Metfs*34 (M)**	N/A
PT28	*PROM1*	**c.1234delT, p.Tyr412Metfs*34**	N/A
PT29	*PROM1*	**c.2198_2199delCT, p.Ser733CysfsTer4**	N/A
PT30	*PROM1*	**c.2198_2199delCT, p.Ser733CysfsTer4**	N/A
PT31	*RDH12*	**c.63_66delCATC, p.Ile22GlyfsTer19 (M)**	**c.63_66delCATC, p.Ile22GlyfsTer19**
PT32	*RDH12*	**c.749T>C, p.Leu250Pro (M)**	**c.189delA, p.Ala64ProfsTer11**
PT33	*RDH5*	**c.733+6T>C (M)**	**c.814_815delCT, p. p.Leu272AspfsTer63**
PT34	*RP1*	**c.4746C>A, p.Cys1582Ter (exon 4)**	c.2025dupA, p.Ser676Ilefs*22 (exon 4)
PT35	*RP1L1*	**c.4396G>A, p.Glu1466Lys**	N/A
PT36	*RP2*	**c.663delT, p.Pro222GlnfsTer16 (M)**	N/A
PT37	*RPGR*	**c.2945_2964delAAGAGGAGGAAGGAGAAGGG, p.Glu982GlyfsTer90 (M)**	N/A
PT38	*RS1*	**c.322_326+3delTTTGGGTAinsCT**	N/A
PT39	*RS1*	**c.511G>A p.Gly171Arg hemizygous (M)**	N/A
PT40	*SNRNP200*	**c.1662C>G, p.Ser554Arg (M)**	N/A
PT41	*TRPM1*	**c.2741 C>A, p.Ala914Glu (P)**	c.3004A>T, p. Ile1002Phe (M)
PT42	*USH2A*	**c.133335_13343del9 (in-frame), p.Glu4445_Met4447del and c.13346_13347CT>TG, p.Ser4449Phe (M)**	**c.14219C>A, p.Ala4740Asp**
PT43	*USH2A*	**c.1256G>T, p.Cys419Phe (P)**	**Large exon 27 deletion (M)**
PT44	*VPS13B*	**Exon 40-43 duplication (M/P)**	**Exon 40-43 duplication (M/P)**

(M/P) indicates both parents are carriers of same variant. N/A stands for not applicable, for X-linked or autosomal dominant inheritance patterns of inheritance. The novel mutations are illustrated in bold. See Table 1 for remainder of novel variants. See Supplemental Table for further details.

characteristics that allowed us to conclude that these novel changes were pathogenic are presented in the Appendix 3. Using this approach, we successfully identified the genetic etiology in >80% of the 378 patients with IRD. Only the novel mutations identified in the course of this retrospective investigation are presented herein.

Example cases in which targeted genotyping based on phenotypic presentation proved successful: The following cases demonstrate some of the few instances in which careful phenotyping pointed to either a monogenic disorder or to a condition for which only a few genes were known to be involved in causing the observed phenotype. In these cases, this approach was faster and cheaper than full panel-based sequencing.

Fundus albipunctatus associated with RDH5 mutations: PT33 (Figure 1, Table 2, and Appendix 3) was a 22-year-old white male patient who presented with a history of congenital night blindness and exhibited a typical fundus albipunctatus phenotype based on both clinical and ERG criteria (improved rod response after prolonged dark adaptation and actually normalized in this case). Previous evidence suggested a stationary nature of the condition. However, cases clinically suggestive of fundus albipunctatus could also be precursors to its progressive phenocopy, *RLBP1*-linked retinitis punctata albescens. Therefore, on the basis of this presentation, targeted testing with a limited panel inclusive of only *RDH5* (gene ID: 5959; OMIM 601617) and *RLBP1* (gene ID: 6017; OMIM 180090) was undertaken. No mutations were identified in the *RLBP1* gene, whereas two novel *RDH5* gene

Table 3. Phenotypic case characterization.

Subject	Gene	Inh.	Sex	Age	Phenotype/Comment
PT1	ABCA4	AR	M	10	CORDS
PT2	ABCA4	AR	F	29	STGD with Fundus Flavimaculatus
PT3	ABCA4	AR	M	33	STGD, also has an affected sister
PT4	ABCA4	AR	M	63	CORD, also has an *IFT140* c.2399+1G>T change (het)
PT5	AIPL1	AR	M	26	COD
PT6	BBS2	AR	F	15	BBS (RP), splice site mutation
PT7	BBS6/MKKS	AR	M	12	BBS (RP)
PT8	BBS6/MKKS	AR	M	16	BBS (RP)
PT9	BEST1	AR	F	49	Bestrophinopathy - Rod-cone dystrophy ERG with cystoid macular edema and Best's-like EOG, Asymptomatic mother carries the deletion
PT10	BEST1	AD	F	10	Best's vitelliform macular dystrophy, 4 consecutive generations affected including mother on pedigree
PT11	CDH23	AR	M	75	RP with profound congenital SNHL (USH1, cochlear implant)
PT12	CDHR1/ PCDH21	AR	F	62	STGD-like CORD, splice site mutation, mother is unaffected carrier, consanguinity
PT13	CHM	XL	M	72	Choroideremia, splice site mutation
PT14	CHM	XL	M	20	Choroideremia (mild), mother is clinically overt carrier
PT15	CHM	XL	M	27	Choroideremia (mild) - brother of PT14, mother is clinically overt carrier
PT16	CRB1	AR	F	14	PPRPE-RP, A1: splice site mutation
PT17	EYS	AR	M	33	RP
PT18	EYS	AR	F	45	RP, splice site mutation, homozygous for both changes (each parent carries both - consanguinity)
PT19	GPR98/ USH2C	AR	M	41	RP with sloping SNHL (USH2) A2: abnormal splicing, mother is unaffected carrier of A2 only
PT20	MERTK	AR	F	27	RP w/ monocular macular atrophy
PT21	MYO7A	AR	F	16	RP with profound cong SNHL (USH1, cochlear implant) , mother is unaffected carrier of A2 only
PT22	MYO7A	AR	F	53	RP with profound cong SNHL (USH1)

Phenotypic summary of solved novel variant cases, relevant corroborating phase determination data, and predicted splice site mutation information also included. Age refers to age at initial diagnosis. Inh: Inheritance, CORD: Cone-rod dystrophy, STGD: Stargardt disease, COD: Cone Dystrophy, BBS: Bardet-Biedl Syndrome, RP: Retinitis Pigmentosa, SNHL: sensorineural hearing loss, USH1: Usher Syndrome Type 1, USH2: Usher Syndrome Type 2, PPRPE: Preserved para-arteriolar retinal pigment epithelium.

mutations were identified (Table 2), segregating correctly in the parents, corroborating the clinical and functional diagnosis of fundus albipunctatus.

Enhanced S-cone syndrome associated with a homozygous NR2E3 mutation: This case was that of a 7-year-old white female patient who presented with congenital night blindness and displayed a typical enhanced S-cone syndrome (ESCS) clinical and ERG phenotype (Figure 2). This prompted targeted sequencing of the *NR2E3* (gene ID: 10002; OMIM 604485) gene, which revealed a previously reported homozygous c.119- 2A>C (IVS1–2A>C) mutation. No further testing was necessary, and the diagnosis was rapidly confirmed. This case was recently reported in an *NR2E3*-focused case series [10].

Dominant retinitis pigmentosa associated with the RHO P23H mutation: This case was that of a 33-year-old male patient of color who was minimally symptomatic and had a strong family history of retinitis pigmentosa (RP) consistent with autosomal dominant inheritance (histories of three generations affected and male-to-male transmission). Despite the fact that the P23H *RHO* (gene ID: 6010; OMIM 613731) mutation had never been previously reported outside of Caucasian families of Northern European descent, the degeneration showed a strictly altitudinal pattern (Figure 3) [11-13]. In addition, we have reported other black patients

with an altitudinal dominant RP phenotype linked to other *RHO* mutations. Thus, targeted *RHO* gene testing was undertaken, confirming the phenotypically driven conclusion that the patient's condition was due to a *RHO* mutation and identifying the well-known disease-causing P23H mutation. It was subsequently established that the patient had mixed African-American and Northern European ancestry.

Example cases in which broad NGS panel-based testing, and at times subsequent dup/del microarray testing, proved successful: In some cases, broad NGS panel-based testing and, at times, subsequent dup/del microarray testing successfully uncovered the genetic etiology of a patient's IRD. In several situations, phenotyping can be greatly aided and further directed in a "hypothesis testing" manner by genotyping results or at least refined to understand which genetic variants may truly be at play in a given patient or family. In some cases, family history led our investigations and initial interpretations in the incorrect direction and a second round of testing both at the clinical and molecular levels proved essential to resolve ambiguity and establish the genetic etiologies of the conditions exhibited by our patients. In an additional common scenario, initial broad NGS panel-based sequencing, inclusive of CNV detection approaches once this technology became available, did not yield a convincing

Table 4. Phenotypic case characterization-continued.

Subject	Gene	Inh.	Sex	Age	Phenotype, Comments
PT23	PDE6B	AR	M	12	RP, A1: de novo, not found in either parental sample
					A2: Splice site mutation, mother is unaffected carrier
PT24	PDE6B	AR	M	18	RP, Mother is unaffected carrier of A2 only
PT25	POC1B	AR	M	19	COD
PT26	POC1B	AR	F	35	COD
PT27	PROM1	AD	M	18	Stargardt-like Macular Dystrophy
PT28	PROM1	AD	F	45	Mild macular imaging and functional changes (mother of PT7), Likely incomplete penetrance or variable expressivity
PT29	PROM1	AD	F	58	CORD
PT30	PROM1	AD	M	61	CORD - brother of PT29
PT31	RDH12	AR	F	20	Early-onset RP (aka SECORD), Mother is unaffected carrier, consanguinity
PT32	RDH12	AR	F	7	RP, Mother is unaffected carrier of A1 only
PT33	RDH5	AR	M	22	Fundus albipunctatus, A1: ? abnormal splicing, mother is unaffected carrier of A1 only
PT34	RP1	AR	F	8	RP, Patient is adopted
PT35	RP1L1	AD	M	26	OMD, See case description
PT36	RP2	XL	M	7	RP, Mother is unaffected carrier
PT37	RPGR	XL	M	36	RP, No mutations by sequencing, affected male relatives carry A1
PT38	RS1	XL	M	16	XLRS
PT39	RS1	XL	M	3	XLRS, Two affected maternal uncles carry A1
PT40	SNRNP200	AD	F	62	RP, Affected brother/nephew carry A1
PT41	TRPM1	AR	M	9	CSNB1
PT42	USH2A	AR	M	53	Severe RP (no hearing loss) - Allele 1 is an in-frame deletion *and* a point mutation, Also has *TTLL5* p.Arg479Ter, c.1435C>T and *TTLL5* p.Ala164Val, c.491C>T, mom unaffected carrier of A1 but does NOT possess either TTL5 mutation
PT43	USH2A	AR	M	44	RP with sloping SNHL (USH2), No A2 mutation by sequencing
PT44	VPS13B	AR	M	22	Cohen Syndrome (RP), No mutations by sequencing, both parents carry same duplication, consanguinity

Phenotypic summary of solved novel variant cases, relevant corroborating phase determination data, and predicted splice site mutation information included (continued from Table 3). Inh.: Inheritance, RP: Retinitis Pigmentosa, COD: Cone Dystrophy, STGD: Stargardt disease, CORD: Cone-rod dystrophy, OMD: Occult macular dystrophy, XLRS:Xlinked Retinoschisis, CSNB1: Congenital Stationary Night Blindness Type 1, SNHL: sensorineural hearing loss, USH2: Usher Syndrome Type 2.

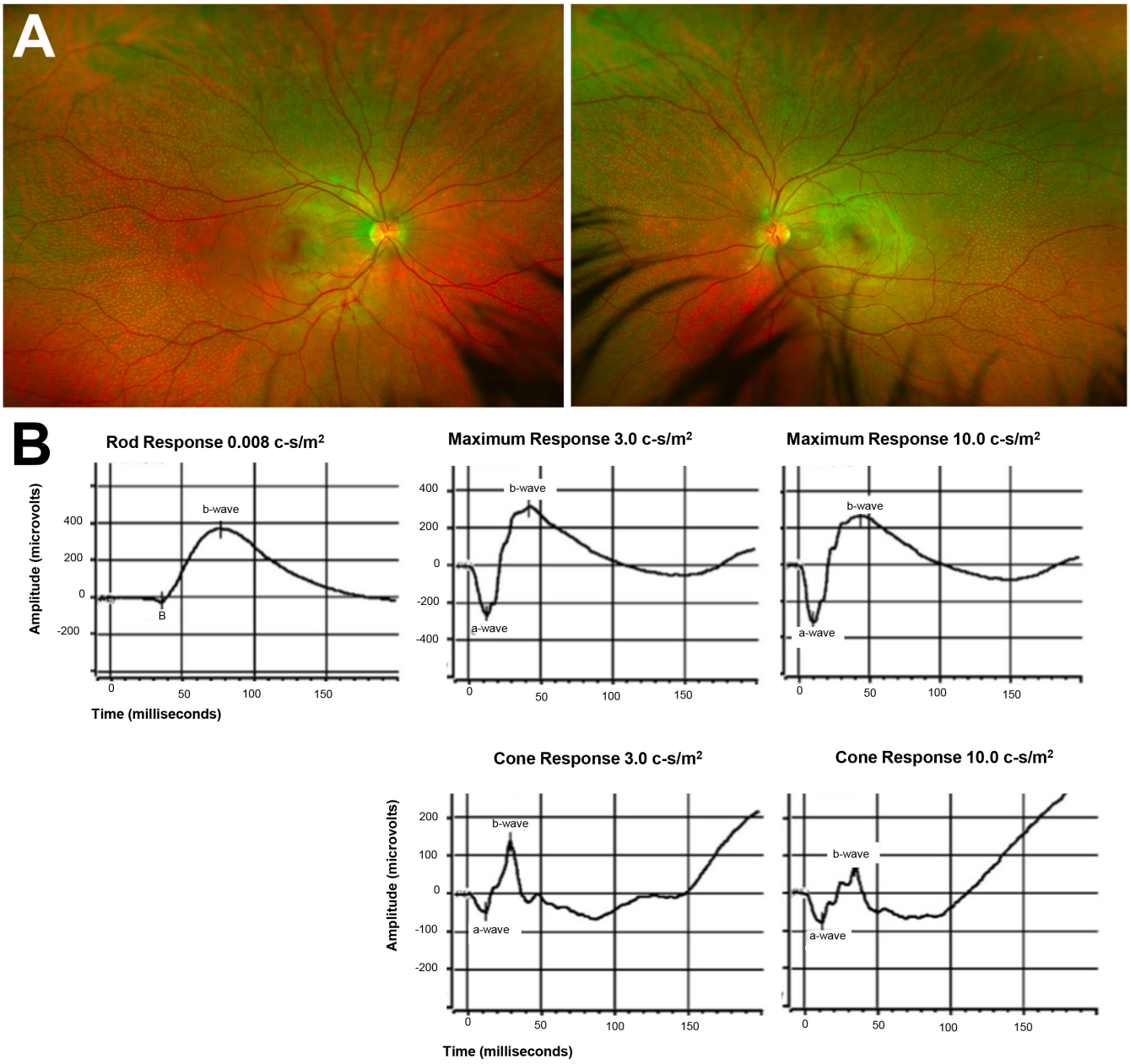

Figure 1. PT33, who had a fundus albipunctatus phenotype, underwent targeted genotyping, which revealed 2 novel *RDH5* mutations in trans. **A**: The fundus photograph shows bilateral disseminated white dots in the mid-periphery with macular sparing, demonstrating the classic findings of fundus albipunctatus. **B**: The rod and mixed full-field flash electroretinograms (ffERGs) after prolonged dark adaptation reached high normal values also under dark adapted conditions, whereas the cone responses remained unchanged (and normal), which is also typical of fundus albipunctatus.

pathogenic etiology, but subsequent del/dup microarray testing successfully elucidated novel pathogenic variants that escaped detection.

Autosomal recessive bestrophinopathy (ARB) associated with BEST1 changes: Autosomal recessive bestrophinopathy (ARB) associated with a *BEST1* (gene ID: 7439; OMIM 607854) mutation and an exon 1–2 deletion in the second allele was found in PT9 (Figure 4, Table 1, and Appendix 3). The patient was a 47-year-old white woman with an atypical clinical presentation and a complex family history, inclusive of a maternal uncle with a form of macular degeneration (MD) with vitelliform features. When first examined at the Duke

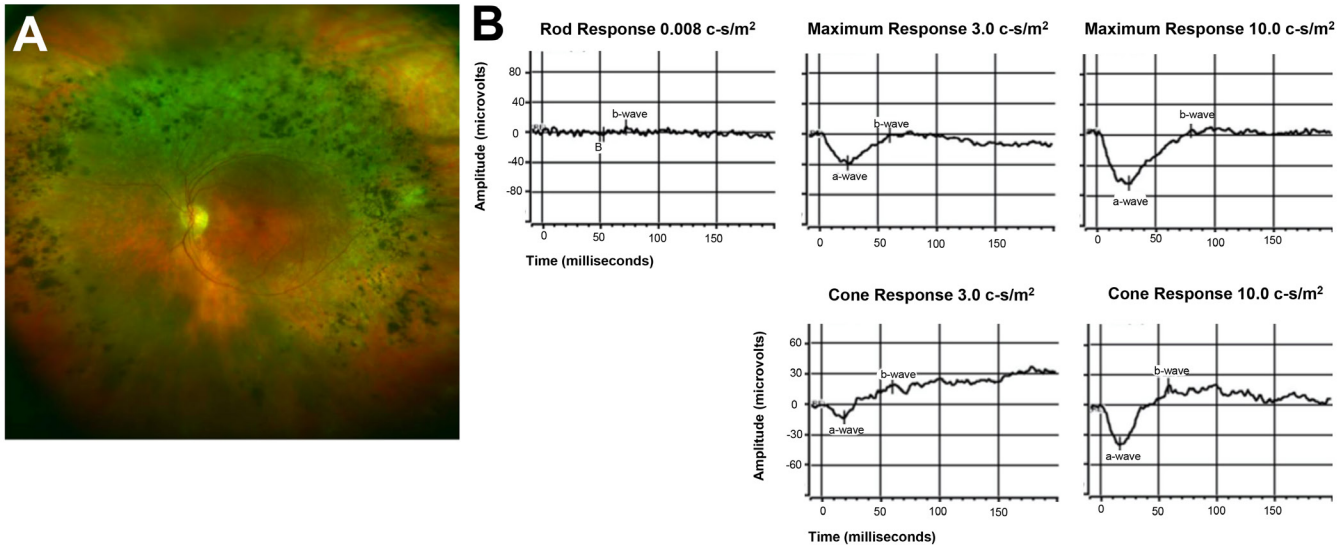

Figure 2. The patient with a clinical phenotype diagnostic of enhanced S-cone syndrome (ESCS) underwent targeted genetic testing, which confirmed homozygous (and previously reported) mutations in the *NR2E3* gene. **A**: The fundus photograph shows the typical nummular pigmentary changes throughout the mid-periphery typically associated with ESCS. **B**: The full-field flash electroretinography (ffERGs) results exhibited non-recordable rod responses with preserved mixed and cone responses showing electronegative waveforms characteristic of ESCS.

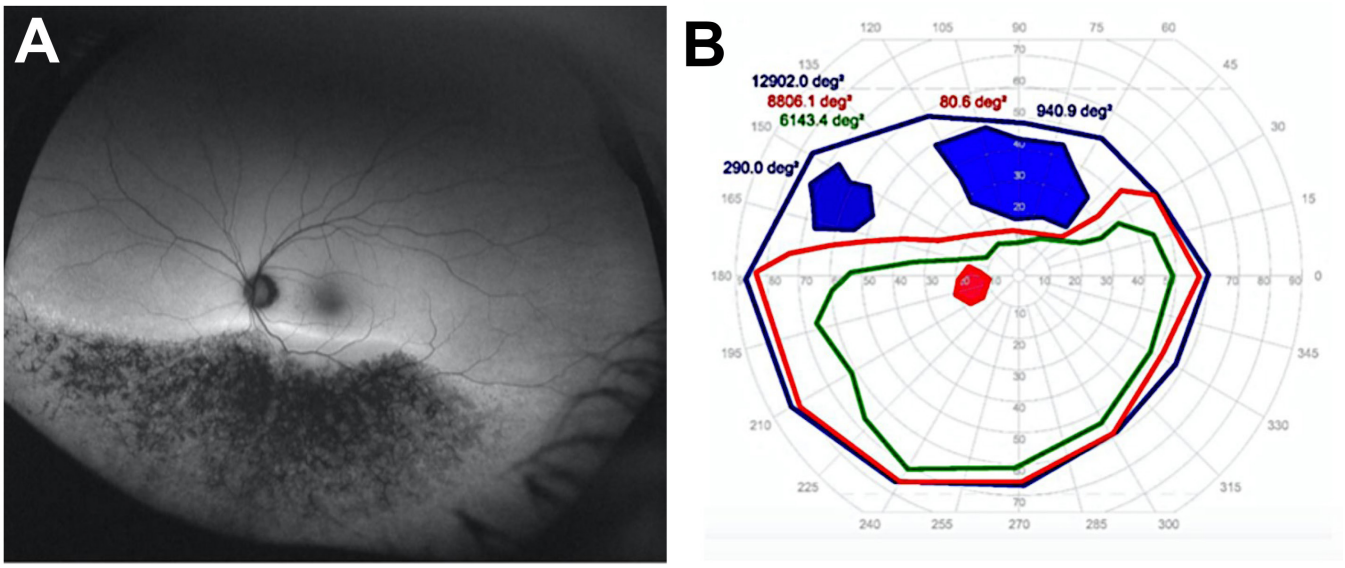

Figure 3. Targeted rhodopsin testing in a patient with a clinical phenotype highly suggestive of altitudinal autosomal dominant retinitis pigmentosa (ADRP) led to the identification of the well-known P23H *RHO* mutation. **A**: Fundus autofluorescence demonstrates altitudinal hypo-autofluorescent dense bone spicule deposits inferiorly, with a thin hyper-autofluorescent band, consistent with an altitudinal ADRP phenotype. **B**: Kinetic semi-automated perimetry exhibited superior visual field loss corresponding to the altitudinal inferior retinal degeneration observed clinically.

Center for Retinal Degenerations and Ophthalmic Genetic Diseases in 2014, she exhibited yellowish exudate-like lesions that did *not* show hyper-autofluorescence (AF) but rather were associated with patches of speckled hypo-AF. Cystoid macular edema (CME) was observed on ocular coherence tomography (OCT). In 2017, the exudate-like features disappeared, and the CME was minimal (not shown). Full-field flash ERG testing revealed a rod>cone (retinitis pigmentosa [RP]-like) pattern of retinal dysfunction of moderate severity.

An initial NGS-based broad panel screening revealed multiple changes. A novel *BEST1* c.75C>A, p.Tyr25Ter mutation stood out as very likely to be pathogenic. EOG, which typically exhibits an Arden ratio of <1.5 in Best's disease [14], was performed and supported this possibility in PT9. However, the clinically healthy mother and maternal uncle (i.e., her brother), who was affected with the vitelliform MD, did not exhibit this mutation, and their EOG results were normal. Del/dup microarray testing was therefore performed, revealing a second novel BEST1 exon 1–2 deletion (Table 1). This also shows that while of clear pathogenic potential, the *BEST1* exon 1–2 deletion harbored by the mother was phenotypically silent. The father of PT9 was also tested, and the Tyr25Ter *BEST1* presumed pathogenic mutation was confirmed to be paternally inherited. Thus, on the basis of these results, it could be convincingly concluded that the patient indeed had *BEST1*-related disease, as initially suggested by the NGS-based test results, albeit not a dominant one but a form of autosomal recessive bestrophinopathy (ARB) [15].

Consistent with this conclusion, the maternal uncle with the vitelliform MD phenotype was also found to be negative for the *BEST1* exon 1–2 deletion, which established that his condition was unrelated to the phenotype expressed by PT9 and was misleading with regard to the initially presumed autosomal dominant inheritance. This information was critically important not only diagnostically but also with regard to the reproductive risk counseling of the proband and other members of her nuclear family. The addition of a diagnostic method that permitted detection of the small deletion (below the typical CNV analysis detection threshold) also revealed a novel disease-causing change.

Pseudo-vitelliform occult macular dystrophy associated with an RP1L1 mutation: The pseudo-vitelliform occult macular dystrophy presentation associated with an *RP1L1* (gene ID: 94137; OMIM 608581) mutation in PT35 is perhaps the most challenging and least definitive of the cases in our series. The patient was a 26-year-old black man who presented with unexplained bilateral subretinal fluid and vitelliform changes on OCT with markedly depressed mfERG response amplitude (Figure 5) and normal EOG results (with an Arden ratio well above 1.5) [16]. The left eye had atypical hyporeflective subretinal pseudo-vitelliform lesions on OCT. Thus, this presentation was inconsistent with a *BEST1*-related disorder and, instead, suggested a possible variant of occult macular dystrophy (OMD), as this presentation was previously reported to occur in patients with OMD [17]. Consistent with this impression, a broad NGS-based panel failed to identify any *BEST1* gene changes but revealed a novel *RP1L1* change instead (Table 2 and Appendix 3). The clinical diagnosis of OMD could also be confirmed molecularly, at least in part, in this case [17]. The patient was found to also carry 3 VUS, all predicted to be benign using the PolyPhen-2 software and positive in the patient's asymptomatic mother. Two of the VUS were in the *ABCA4* (gene ID: 24; OMIM 601691) gene. The first was the c.618C>G, p.Ser206Arg change. Current evidence in the literature is conflicting regarding whether this variant is benign or pathogenic (ClinVar). At times, *ABCA4* variants have been reported to be associated with dominant inheritance. Thus, we examined the mother of our patient, who carried this variant, and found that she was asymptomatic and disease-free, providing no evidence that this variant may act dominantly. The other *ABCA4* VUS was the c.2546T>C, p.Val849Ala change, which was predicted to be completely benign using the Polyphen-2 software, with a near 0 pathogenicity score. It was also considered a benign polymorphism after evaluation using MutationTaster. The mother's proband did not carry this variant. Even if we postulate that the latter *ABCA4* variant could somehow have a pathogenic effect and that, coupled with the former variant, could be responsible for the patient's phenotype, the observed pseudo-vitelliform presentation is entirely inconsistent with an *ABCA4*-related phenotype. Thus, is this context, in silico and in vivo predictions were consistent with one another and supported a lack of association between the macular dystrophy and these *ABCA4* changes. Lastly, the patient also exhibited a *PROM1* (gene ID: 8842; OMIM 604365) c.1928C>G, p.Ala643Gly change. Our patient's asymptomatic and disease-free mother possessed this *PROM1* variant with no visual consequences. Both Polyphen-2 and MutationTaster predicted this VUS to be completely benign, with a near 0 pathogenicity score by the former and a benign polymorphism prediction by the latter. Thus, we feel fairly confident in predicting that only the *RP1L1* mutation was responsible for the phenotype found in this patient. To the patient's knowledge, his father did not have ocular symptoms, however his father was available for neither phase assessment nor eye examinations. Therefore we could not distinguish between the following scenarios: our patient exhibited a de novo mutation not carried by either parent, the father possessed the novel *RP1L1* variant but demonstrated no

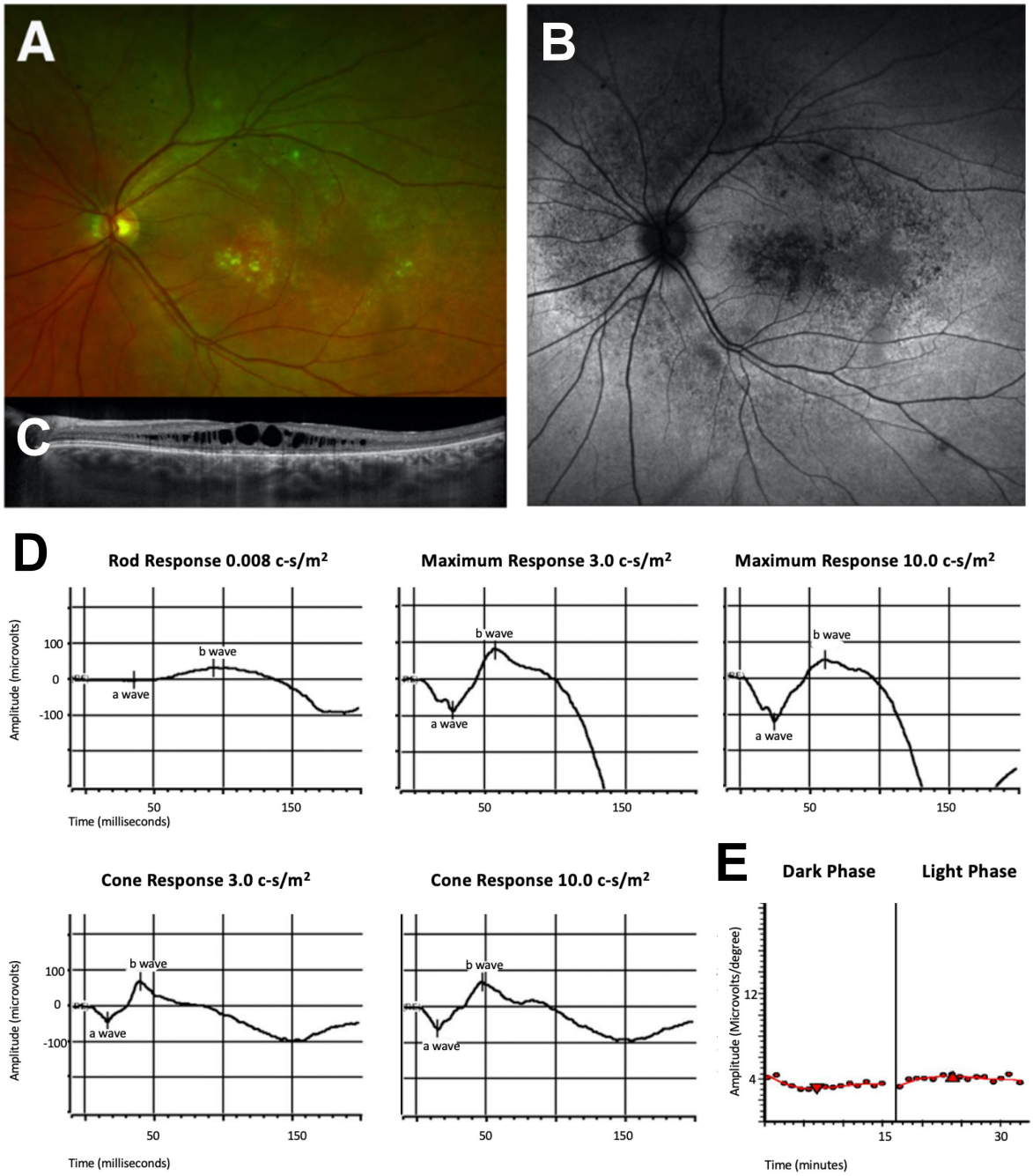

Figure 4. PT9 had an autosomal recessive bestrophinopathy (ARB). **A**: The fundus photograph depicts scattered subretinal yellowish lesions without classic vitelliform lesions. **B**: Fundus autofluorescence shows scattered hypo-autofluorescent punctate changes in the macular region and mid-periphery. **C**: The macular OCT image shows a schisis-like cystoid macular edema with fragmentation of the ellipsoid zone and RPE. **D**: The full field flash electroretinogram demonstrates a rod>cone pattern of retinal dysfunction. **E**: The electrooculogram shows an Arden ratio well below 1.5. All these features have been previously reported in patients with ARB. After a nonsense *BEST1* mutation was found initially via NGS sequencing, follow-up duplication/deletion testing revealed an exon 1–2 deletion in the *BEST1* gene, which was confirmed to be in trans using parental analysis, consistent with the diagnosis of ARB.

clinical features on ocular exam, or the father possessed the novel *RP1L1* variant with mild clinical phenotype detectable on ocular examination but was asymptomatic. Each of these scenarios has been previously reported in OMD families [18]. Thus, while this case remains especially challenging and with the aforementioned caveats, genetic testing revealed a plausible molecular explanation for the observed phenotype with potentially important implications due to the dominant nature of OMD.

Severe cone-rod dystrophy associated with ABCA4 mutations: The patient was a 30-year-old white man who presented with severe diffuse retinopathy, a clinical phenotype of profound loss of vision, minimal residual peripheral fields, and essentially nonrecordable ffERG responses in the presence of disseminated atrophic and pigmentary nummular lesions resembling a disseminated generalized multifocal chorioretinitis (Figure 6). However, he had a history of childhood-onset Stargardt disease. Consistent with this, a broad NGS panel revealed two *ABCA4* mutations, a deep intronic mutation (IVS38–10T>C) and a pathogenic synonymous change (p.Val2114Val), segregating correctly in the parents. Both mutations have been documented in the literature, with the synonymous change shown to introduce a premature splice leading to a large deletion in exon 46 [19]. After this discovery, wide-field fundus AF revealed a pattern of peripapillary AF sparing [20] (green arrow) and residual hyper-AF flecks in the far periphery (white arrow), entirely consistent with an *ABCA4*-linked phenotype but far more serious and advanced than the original diagnosis of Stargardt disease. Thus, despite the disease features that initially suggested more likely sequelae of a primary inflammatory disorder, genetic testing established an autosomal recessive condition instead. Anti-retinal autoantibody (AR-AAbs) testing and retinal immunohistochemistry were also performed for this patient. The results were positive for multiple AR-AAbs and retinal staining, strongly suggesting that a secondary autoimmune component (with supportive evidence of leakage on fluorescein angiography and improved vision by intravitreal steroids and a systemic immunomodulatory regimen, not shown) likely accounted, at least in part, for the disseminated chorioretinal lesions that, to our knowledge, are otherwise not outright typical for *ABCA4*-associated disease.

Usher syndrome type 2A associated with an exon 27 deletion in the second USH2A allele: PT43 (Figure 7, Table 2, and Appendix 3) was a 42 year-old white male patient with a history of congenital sloping high-frequency sensorineural hearing loss and RP that suggested Usher syndrome type 2. However, it could have been also a case of pseudo-Usher syndrome due to an *RPGR* (gene ID: 6103; OMIM 312610) mutation [1,21]. The clinical phenotype of typical RP exhibited by this patient was associated with disseminated peripheral nummular cobblestone-like atrophic spots, overall good central AF integrity with a perifoveal “bull's eye” pattern, and peripheral hypo-AF spots, but a good ellipsoid zone foveal residue on OCT (Figure 7). Despite the mild CME, the central acuity (20/25 in the left eye) was good. A close correspondence was observed between the area of best-preserved AF and the size of the III4e target (red) on Goldmann-style semi-automated kinetic Octopus perimetry (Figure 7). An initial NGS screening revealed multiple changes in various genes, including a known disease-causing, paternally inherited mutation in *USH2A* (gene ID: 7399; OMIM 608400; c.1256G>T, p.Cys419Phe), but no second mutation in this gene was detected. Subsequent del/dup microarray testing revealed that the other, hidden pathogenic allele was a novel large exon-27 *USH2A* deletion, which was maternally inherited, confirming the initially suspected diagnosis of Usher syndrome type 2A.

DISCUSSION

As gene therapy opportunities for IRDs continue to emerge and progress, and other gene-specific treatments are also being developed, far better therapeutic options for these previously incurable retinal disorders are becoming a reality [3,9]. Therefore, identifying the pathogenic genetic etiologies of IRDs is no longer medically necessary only for diagnostic, counseling, and reproductive risk assessment purposes but also for compelling therapeutic implications and is a top priority in the field of IRDs [4,7,22].

The ACMG guidelines emphasize the importance of genotype data with far less relevance placed on clinical phenotyping during the search for novel variants. This is an excellent general advice due to the lack of standardization of clinical data in many medical fields and the heterogeneity and often incompleteness of the clinical workups performed by many referring medical providers. Indeed, it has been our experience that the clinical diagnosis of suspected IRD in patients referred to our clinic is incorrect in approximately 50% of the cases.

Thus, from a strict laboratory perspective, the low level of relevance assigned by the ACMG guidelines to phenotypic information is completely understandable. When a novel genetic change that may be disease causing or is an outright VUS is found in a patient with IRD, it would be ideal to also perform a detailed in vitro or otherwise in vivo (e.g., in zebrafish, cultured inducible pluripotent stem cells, or retinal organoids) characterization of the various uncertain genetic changes. However, this is far beyond the scope of clinical

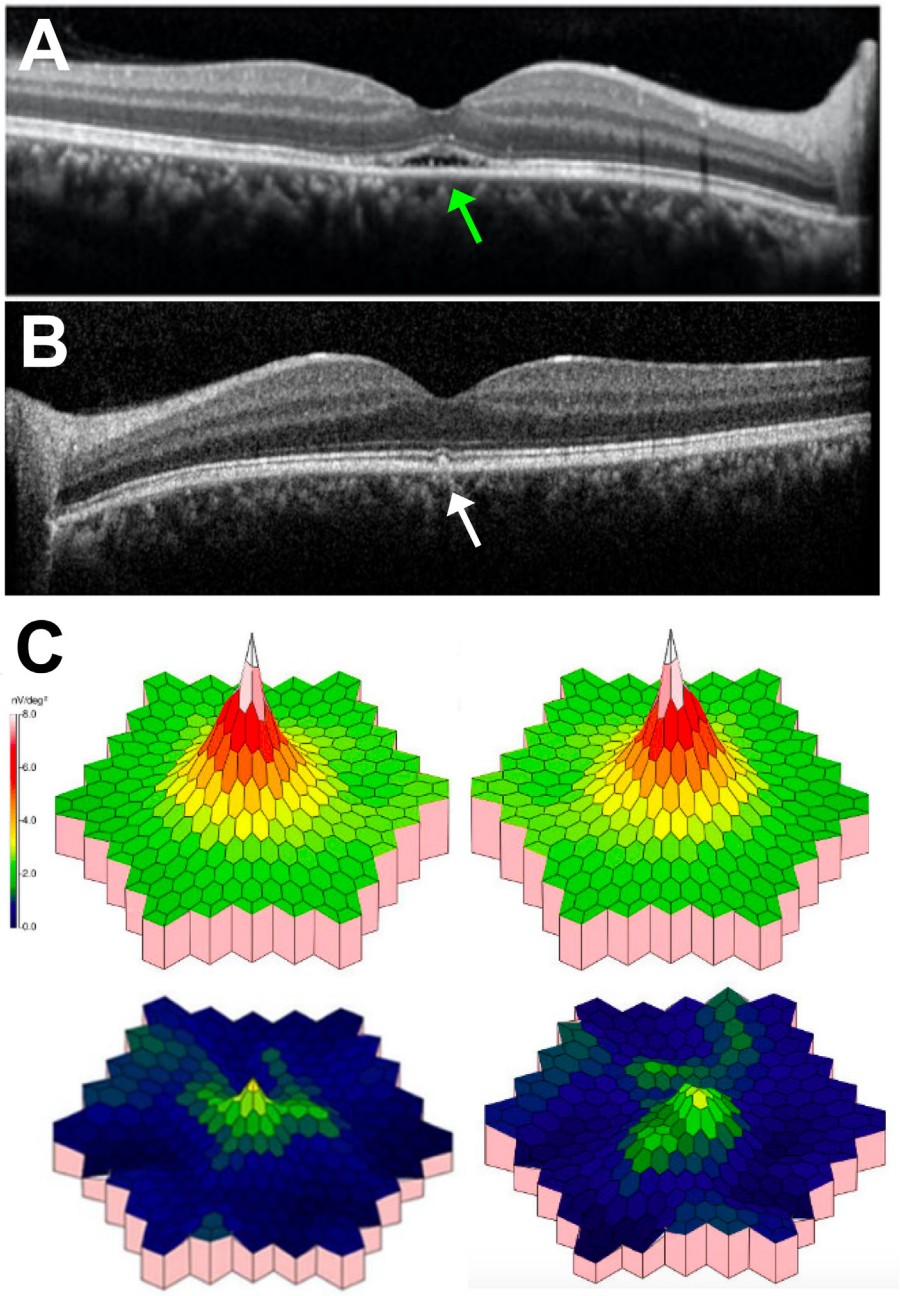

Figure 5. PT35 had a pseudo-vitelliform clinical phenotype compatible with occult macular dystrophy (OMD) and found to harbor a novel *RP1L1* change. **A**: The macular OCT image of the right eye, which was symptomatic of metamorphopsia, demonstrates a dome-shaped pocket of subfoveal hyporeflective fluid with a fragmented subretinal hyperreflective material (green arrow). **B**: The macular OCT image of the left eye, which was asymptomatic at presentation, shows a tiny pseudovitelliform subfoveal hyperreflective lesion (white arrow). **C**: Unlike the prediction from these focal findings, which were minimal in the left eye, the response densities of the multifocal electroretinogram were markedly depressed in both eyes, with partial foveal peak preservation, a common finding in OMD. The *BEST1* gene sequencing was normal, as was the EOG (not shown).

diagnostic testing and would be time-consuming and expensive to implement on a vast scale. IRDs are relatively unique compared with most other genetic disorders owing to the high accessibility of the human eye for detailed examination and testing. IRD specialists have already come together to define optimal approaches and protocols for IRD phenotyping [7,22] that are being broadly followed by IRD specialists worldwide. This affords IRD specialists an opportunity to maximize the throughput of high-quality, high-fidelity clinical and functional phenotype data. In turn, this information can then guide far more refined genotyping interpretations. Likewise, when genotyping information reveals paths to disease etiologies that had not been previously fully suspected, such as in the cases illustrated in Figure 4 and Figure 6, the current clinical and functional phenotyping capabilities afforded to IRD specialists permit the recognition, at times in retrospect, of disease features that had not been initially captured (e.g., the peripapillary sparing and peripheral flecks in the *ABCA4*-positive patient illustrated in Figure 6), which help confirm the molecular diagnosis. In PT9 (Figure 4), the initial and correct impression that the first *BEST1* mutation was predicted to be pathogenic in view of a vertical history of vitelliform disease was in fact misleading as to the true inheritance pattern and actual final diagnosis. It was not until the EOG results in the proband's parents were found to be normal that the pursuit of a possible second CNV in *BEST1* became evidently necessary. Once a del/dup microarray test confirmed that the atypical vitelliform phenotype of PT9 was recessive in nature (and thus an ARB) and not dominant, both the phenotype in the proband and the lack thereof in the mother, who carried the novel *BEST1* exon 1–2 deletion, which was phenotypically silent in the carrier state, were clarified. Thus, in the field of IRDs, a two-way interaction between IRD specialists and molecular genetic laboratories offers the unique opportunity to leverage the power of in-depth phenotyping. For both IRD specialists and laboratories, this allows for a greatly increased level of certainty in identifying novel disease-causing genetic variants without requiring complex, time-consuming, and expensive experiments on a systematic scale.

Taking advantage of this systematic approach, which is summarized schematically in the flowchart in Appendix 3, we identified 52 novel pathogenic variants in 27 IRD genes. The associated phenotypes are summarized in Appendix 4. The identified variants ranged from point mutations, splice site mutations, deletions, and duplications. We have illustrated cases in which our methodical approach to clinical phenotyping allowed for the identification of just a few genes (sometimes even just a single gene) that would be sufficient to

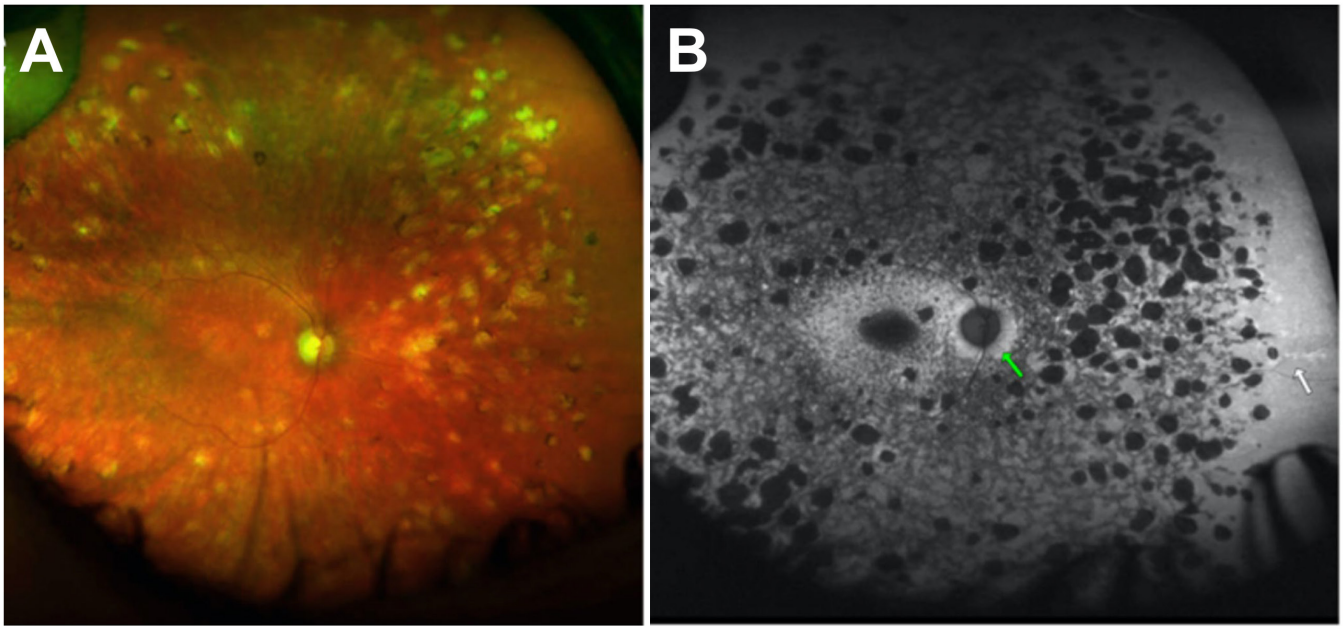

Figure 6. The patient had *ABCA4*-associated autosomal recessive cone-rod dystrophy (arCORD). **A**: This clinical phenotype was initially more suggestive of disseminated chorioretinitis, showing widespread peripheral punched-out chorioretinal lesions. However, the patient carried a childhood diagnosis of Stargardt disease, which had not been genetically confirmed. Broad NGS-based sequencing led to the identification of two previously reported *ABCA4* mutations, with phase determination confirming them to be in trans, consistent with *ABCA4*-associated arCORD. **B**: Upon further examination, the patient's clinical phenotype demonstrated characteristic peripapillary sparing (green arrow) and far peripheral hyper-autofluorescent flecks (white arrow), which are typical of an *ABCA4*-associated phenotype.

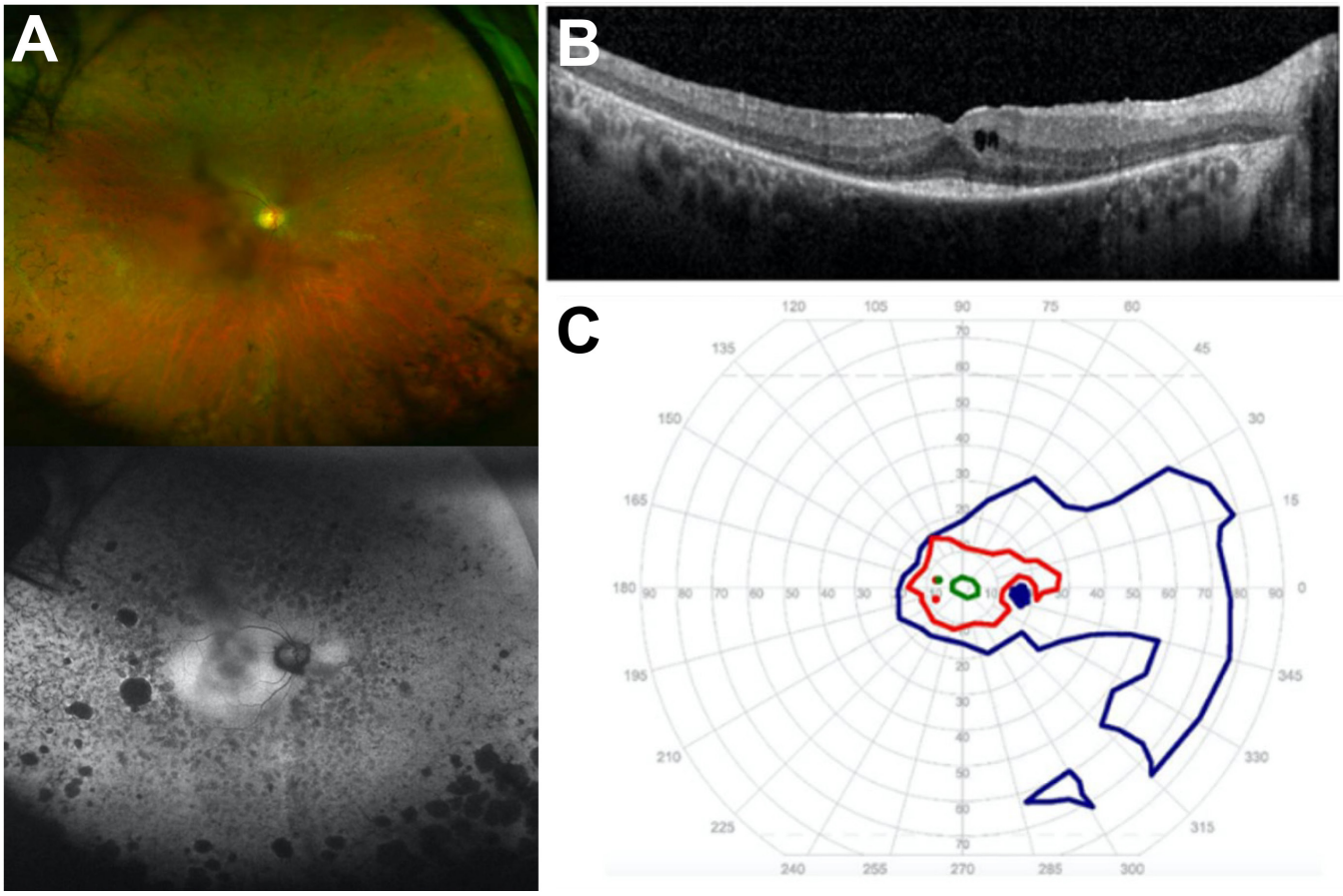

Figure 7. PT43 presented with Usher syndrome type 2 and two *USH2A* changes. The molecular diagnosis was confirmed only after follow-up del/dup testing, which revealed a large novel *USH2A* deletion encompassing exon 27, in trans to a heterozygous *USH2A* mutation initially detected using NGS-based panel testing. The patient had a clinical phenotype highly suggestive of Usher syndrome type 2 due to the association of retinitis pigmentosa (RP) with sensorineural hearing loss. **A**: The fundus photograph (top) demonstrates a waxy nerve pallor, attenuated retinal vessels, peripheral bone spicule deposits, and nummular RPE loss, typical of RP, presenting on autofluorescence (bottom) as disseminated hypo-autofluorescent fainter spots and darker, better-defined nummular areas, alongside central hyper-autofluorescence and a faint perifoveal ring of hypo-autofluorescence (incipient "bull's eye" maculopathy). **B**: The macular OCT image depicts a mild cystoid macular edema, an epiretinal membrane, thinning of the outer nuclear layer, and the ellipsoid zone sparing the fovea, also typical of RP. **C**: Kinetic semi-automated perimetry demonstrates peripheral vision loss with partial preservation of the temporal field in a pattern consistent with the observed clinical phenotype and the working diagnosis of *USH2A*-associated Usher syndrome type 2.

sequence and identify the causal variant in selected patients. The use of this approach led to significant savings in terms of both cost and time. We acknowledge that as sequencing techniques have become more streamlined and less expensive, many cases could have been reasonably identified by starting off with IRD-focused NGS-based panel testing, which is now widely available and far cheaper than even just a few years ago. These panels also often allow the identification of mutations and variants that may not be disease causing but may have relevance as phenotype modifiers and, at times, may be important for family-wide counseling. For these reasons, we presently advocate the utilization of CLIA-certified broad NGS-based panel testing as the first-line testing option.

For most other patients, phenotyping was followed by IRD-focused broad NGS-based panel testing. CNV determination became available as part of the initial panel sequencing partway through our study period. Whereas CNV analysis did not always lead to diagnosis in our cohort because it best detects genomic changes larger than 3 exons in size (i.e., larger than any novel variants found in our patients), it has been shown to be of conceptual utility in other studies and is emerging as an essential part of the genotyping approach. In many cases, careful reconciliation of clinical data with NGS panel-based sequencing was sufficient in diagnosing the genetic etiology. In certain cases, follow-up del/dup microarray testing was critical in establishing the diagnosis

and in finding novel variants, and can remain essential in identifying a second allele in autosomal recessive patients. The common thread in all of our cases was the initial step of in-depth clinical and functional phenotyping, the benefit of which cannot be sufficiently emphasized.

In summary, in the hands of IRD specialists, judicious use of meticulous, standardized, expert clinical-functional phenotyping combined with systematic, broad NGS panel-based genotyping was extremely effective for providing critical genetic diagnoses to patients with IRD and for the discovery of a large number of novel genetic IRD variants that could be characterized as causal with a high degree of confidence. Increasing these two-way interactions between IRD investigators and CLIA-certified molecular diagnostic laboratories warrants the continuous improvement in the yield of molecular genetic diagnostic testing in our field. This will ultimately facilitate the common goal of achieving genotypic characterization of all patients with IRD as we continue to remain steadily on the path to an increasing number of gene- and mutation-specific interventions, as well as treatments aimed at tackling gene- or mutation-specific driven mechanisms (e.g., translational readthrough-inducing drugs for stop-codon mutations or drugs interfering with vitamin A metabolism or recycling in *ABCA4*, *LRAT*, or *RPE65*-related diseases) [4,7,9,22].

APPENDIX 1. FLOWSHEET.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. SUPPLEMENTAL METHODS.

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

APPENDIX 3. NOVEL IRD VARIANTS.

To access the data, click or select the words "Appendix 3."

APPENDIX 4. PHENOTYPE NUMBER CASE SERIES.

To access the data, click or select the words "Appendix 4."

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