UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Biotechnology Doctoral Program

New Approaches in the Genetic Diagnosis of Inherited Retinal Dystrophies and Future Therapeutic Insights

Belén García Bohórquez

Doctoral Thesis

December 2024

SUPERVISORS:

José María Millán Salvador

Gema García García

PhD dissertation, 2024
Instituto de Investigación Sanitaria La Fe
Biotechnology Doctoral Program, Universidad Politénica de Valencia
Valencia, Spain

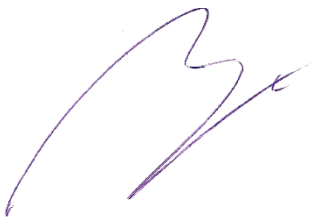
El Dr. José Mª Millán Salvador, Facultativo Especialista e Investigador Principal del grupo de Biomedicina Molecular, Celular y Genómica perteneciente al Instituto de Investigación Sanitaria La Fe.

La Dra. Gema García García, Investigadora Postdoctoral del Centro de Investigación Biomédica en Red de Enfermedades Raras y miembro del grupo de Biomedicina Molecular, Celular y Genómica perteneciente al Instituto de Investigación Sanitaria La Fe.

HACEN CONSTAR QUE:

El trabajo de tesis doctoral titulado *New approaches in the genetic diagnosis of inherited retinal dystrophies and future therapeutic insights* que presenta Belén García Bohórquez, Graduada en Biología con Máster en Investigación en Biomedicina Molecular, Celular y Genómica, para optar al título de Doctora por la Universitat Politècnica de València, ha sido realizado en el grupo de Biomedicina Molecular, Celular y Genómica del IIS La Fe bajo su dirección y que reúne las condiciones para ser defendida por su autora.

Para que conste, en cumplimiento de la legislación vigente, firman el presente certificado en Valencia, a 16 de noviembre de 2024.



Fdo. Dr. José Mª Millán Salvador

Fdo. Dra. Gema García García

A mis padres, siempre.

ACKNOWLEDGEMENTS (AGRADECIMIENTOS)

Desde que tengo uso de razón todos mis méritos los he compartido con las personas que quiero, porque siempre he creído que el apoyo de los que te rodean marca la diferencia y define, en cierta manera, quién eres.

Por eso, en primer lugar, como no podía ser de otra manera, quiero empezar dando las gracias a mis padres y a mi primo (mi choqui). Gracias por haberme demostrado un amor incondicional, por la dedicación absoluta que me habéis prestado, porque me lo habéis dado todo. Habéis sido el timón que me ha guiado siempre y las personas a las que les debo el orgullo que siento de haber llegado hasta aquí. Todo lo que consiga en mi vida es por y para vosotros, porque quiero ser la recompensa de todo vuestro esfuerzo.

Mamá, gracias por enseñarme que nunca es tarde para cumplir nuestros sueños, eres el mayor ejemplo de valentía y fuerza que cualquiera quisiera tener en su vida. Gracias por tener siempre una buena palabra cuando la necesito y que, en los momentos de tormenta, hayas sido mi calma. Gracias por mostrarme que el amor mueve montañas, por ayudarme a ser la mujer que soy hoy en día y por demostrarme que la vida contigo como madre, es más que bella. Cada paso que doy en mi vida es más firme si cuento contigo. Papá, gracias por compartir conmigo tu nobleza, tu forma de hacer las cosas y la pasión que le pones a todo lo que te gusta. He tenido la suerte de compartir contigo algunas de las que serán mis mayores aventuras y de que hayas dado todo por querer vivirlas conmigo. Gracias por escucharme y apoyarme siempre, por aconsejarme desde la prudencia y la bondad, y por permitirme cada día descubrir todo lo bonito que hay en ti. La vida contigo como padre es el mayor regalo que jamás pudiera imaginar. Sois unos padres ejemplares y así me lo demostráis cada día, con vuestra forma de quererme a mí y la forma de quereros entre vosotros. Os quiero muchísimo.

Sin embargo, nada de esto hubiera sido lo mismo si la vida no hubiera cruzado en mi camino a mi tercer pilar, a mi choqui. Gracias por formar parte de los recuerdos más bonitos de mi vida. Aquellos en los que yo interpretaba con ilusión los cuentos que tú me leías y en los que me llevabas al centro para ir a Nostromo y comer palodú. Mi choqui me ha hecho la friki con orgullo que soy hoy. Gracias por ser mi tercera e indispensable figura de apoyo, por

enseñarme a que se puede afrontar la vida con humor, sean cuales sean las circunstancias, y gracias, sobre todo, por convertirte en una parte esencial de mi vida. Esto también es para ti, choquito.

Me hubiera encantado poder agradeceros en persona lo que habéis influido en la persona que soy, mis abuelitos, y deciros lo mucho que aprendí de cada uno de vosotros. Por eso quiero intentarlo ahora. Gordita mía, cómo te digo que aún sigo sintiéndote conmigo, que miro tus fotos y tus videos y oigo tu risa a mi ladito. Solo tu historia es un ejemplo de vida del que todos deberíamos aprender. Me alegra haberte dado todo el amor que te merecías siempre que pude. Gracias por haber sido mi abuelita, tu bombón te adora. A mi abuela Elia, gracias por haber sido el mejor ejemplo de prudencia que conozco, por haber querido siempre a mi madre como una hija y por haber formado conmigo un vínculo único. Has sido la persona con la que más me gustaba ver los concursos de la tele y comer pipas de calabaza. Me enorgullece reconocerme en ti en muchas cosas. Y a mi abuelito, el que jugaba conmigo hasta más no poder cuando era una niña. Te ha tocado recorrer los últimos pasos en tu vida sin la compañía de tu bellotera, y has demostrado ser un gran ejemplo de valentía. Aún recuerdo cuando comprabas churritos el día de la lotería de Navidad y los días en los que me recogías del colegio. Siento que ninguno ha recibido durante su vida el cariño que hubiera merecido y no sé cómo agradeceros que yo haya tenido todo el amor del mundo gracias a los padres que me habéis dado. Por eso, mis logros son infinitamente vuestros.

Llegar hasta aquí ha sido un camino largo, el de toda mi vida. Por eso doy también las gracias a toda mi familia. Quiero dedicar este trabajo en especial a mis padrinos, a mi tito Ángel, a mi Lolilla y a mi tita Eli, que me han dado siempre todo el cariño del mundo. Gracias por quererme tanto, me habéis enseñado mucho y quiero seguir aprendiendo de vosotros cada día. A mis titos de Valencia, Gloria y Jose, gracias por haberme ayudado a sentirme siempre en casa, vuestro cariño ha sido esencial. Gracias también a mis primos, por haber formado parte de los recuerdos más bonitos de mi infancia. Además, como madrina, quiero que este trabajo sirva como un pequeño reflejo de lo bueno que te espera en la vida, mi Fran. Os adoro a todos.

Por supuesto, no quiero olvidarme de agradecer todo lo que he conseguido a mis amigos. Gracias mis “Templus Fugi”, a Pablo, a Natalia y a todos los que me han acompañado hasta

el día de hoy. Gracias por ser mi familia y por sentir que sois casa. A mi amiga Julia, gracias por haber sido la persona que el destino ha querido que me acompañase desde Sevilla hasta Valencia, con la que comparto vocación y la que siempre, siempre, está ahí. Y por si no fuera suficiente, Valencia me ha regalado grandes tesoros. Sin duda este ha sido el caso al encontrarme con vosotros en mis primeros pasos en Valencia y en la ciencia, Pepe y Yaiza; al sacarme bastante a flote en los momentos en los que la tesis puede hundirte un poco y al enseñarme que, a veces, la conexión entre las personas es inexplicable, Alba, Lidón y Amara. Me habéis sostenido en mis peores momentos y me habéis ayudado a continuar. Por eso este trabajo os lo agradezco y os lo dedico, porque vuestro cariño ha sido indispensable hasta llegar a conseguirlo. Gracias a todos aquellos amigos míos que no menciono aquí, porque no tendría espacio suficiente si quisiera nombrar a todas las personas tan bonitas que tengo en mi vida. Gracias a todos por haber dedicado tiempo de vuestras vidas a conocerme tan bien, casi mejor que yo misma, a quererme de una manera tan bonita y creer tanto en mí. Vuestro apoyo y amor son esenciales para mí. Os adoro y os quiero siempre conmigo. "*Es parentesco sin sangre una amistad verdadera*" – Pedro Calderón de la Barca.

Muchas gracias a Gema y a Chema, por confiar en mí desde el principio y darme la oportunidad de formar parte del grupo y dedicarme a la genética. Gracias también por vuestra escucha, cariño y por haber sido mis guías en este mundo de la ciencia. Llegué hace ocho años al laboratorio y me habéis educado profesionalmente hasta el día de hoy. Gracias siempre. Millones de gracias a todas mis compañeras de laboratorio, sin vuestras risas no hubiera sabido llevar tan bien los días. Con vosotras se ven las cosas de otra forma. Gracias también al resto de miembros del grupo, he tenido palabras de apoyo por parte de todos cuando lo he necesitado y os lo agradeceré siempre.

Y, por último, con especial ilusión, quiero darte las gracias a ti, Nico, mi chico. Gracias por aparecer en esta etapa de mi vida y acompañarme, comprenderme y apoyarme siempre. Sin duda has sido mi gran suerte, me has hecho el camino más fácil y los días más bonitos. Quiero compartir contigo todos mis logros y acompañarte y admirarte siempre en todos los tuyos. Te quiero mucho.

TABLE OF CONTENT

ABSTRACT

Inherited retinal dystrophies (IRD) encompass a wide range of genetic disorders characterized by the degeneration or dysfunction of photoreceptors or retinal pigment epithelium cells, leading to usually progressive vision loss. IRDs exhibit considerable genetic and clinical heterogeneity, making their genetic diagnosis a challenge. To date, over 280 genes have been associated to IRD, which hinders even more their diagnostic. However, the advent of high-throughput sequencing (HTS) has revolutionized the genetic diagnostic approach, enabling more efficient variant identification and offering patients more accurate genetic counselling and the possibility of accessing to clinical trials for gene-based therapies.

The first approach carried out in this thesis was based on the study of 92 IRD patients by sequencing two custom panels including IRD-causing genes. Both designs contained IRD-associated genes and intronic regions where pathogenic deep-intronic variants were identified to date. We successfully found the genetic cause in 57.6% of the cases, while 13% had only one mutation in genes known to follow an autosomal recessive inheritance pattern and 29.3% of patients remained undiagnosed. A total of 120 pathogenic or likely pathogenic variants were found, including 30 novel variants. Among patients with cone-rod dystrophy, *ABCA4* was the most frequently mutated gene, while mutations in *USH2A* were the most common among those with retinitis pigmentosa (RP). Additionally, 10 families carried mutations in multiple IRD-related genes, emphasizing the genetic complexity of these disorders. Due to the inclusion of several non-coding regions, two deep-intronic variants in *ABCA4* and *CEP290* previously known to be pathogenic were also identified. This accentuates the importance of studying non-coding regions in genetic diagnosis strategies. This approach has enabled the molecular diagnosis of a significant percentage of patients, thereby allowing them to make reproductive decisions and be included in clinical trials based on gene therapy from which they may probably benefit in the future.

Non-coding regions have gained a lot of significance becoming essential in genetic diagnosis. Particularly, deep-intronic variants in *USH2A* are being frequently described as pathogenic. This type of variants has been described to have an impact in splicing that alters the coding sequence. Thus, the second strategy designed for this thesis was focused on the

study of non-coding regions in USH genes in unsolved USH and non-syndromic autosomal recessive RP patients. For this reason and as a first standpoint, a screening of five *USH2A* deep-intronic variants previously described as pathogenic was performed in monoallelic patients. The genetic diagnosis was completed in the 30.95% of screened patients by identifying the second causing-variant among the five deep-intronic studied. Subsequently, in patients that remained unsolved and in those newly monoallelic cases, the sequencing of either the entire *USH2A* gene or all USH genes was performed. Sixteen variants were identified and predicted to have an impact in splicing, of which four turned out to actually affect splicing by minigenes assays. Two of the newly discovered variants were deep-intronic and a pseudoexon (PE) inclusion in the pre-mRNA was observed after the functional assay. The other two affected a regulatory cis-element. Afterwards, given their potential to modulate splicing, antisense oligonucleotides (ASOs) were designed to address the PE inclusion in three deep-intronic variants in *USH2A* (two identified in this study and one previously reported). The insertion of all PE was successfully corrected *in vitro* for the three variants. This highlights the critical role that deep-intronic variants play in the pathogenicity of *USH2A*-related diseases and demonstrates the therapeutic potential of ASOs for patients carrying these variants, offering a novel approach for a future treatment.

Inherited retinal dystrophies can also be considered as ciliopathies, a group of disorders caused by defects in cilia, which are essential structures for cell signalling and the function of various tissues. For example, mutations in genes like *USH2A* and *CEP290* disrupt ciliary function, leading to photoreceptor degeneration and vision loss. Particularly, the oro-facial-digital syndrome (OFD), is characterized by facial clefts, microphthalmia/anophthalmia, coloboma, and polydactyly, and is caused by mutations in genes involved in cilia formation. OFD type IX, was recently linked to the gene *TBC1D32*, which has also been associated with RP in certain families. Following this premise, the third line of study in this thesis was motivated by the study of a patient who displayed OFD type IX features, including RP and moderate bilateral sensorineural hearing loss (SNHL). This drove us to perform a clinical exome sequencing, which resulted in the identification of two heterozygous variants in *TBC1D32* confirmed to be in *trans* by segregation analysis. Since this is the first case diagnosed with SNHL with mutations in *TBC1D32,* we also performed an analysis of genes associated with SNHL, but no disease-causing variant was identified. Moreover, a wide

range of ciliopathies linked to abnormalities in inner ear cilia, often resulting in hearing impairment, have been reported. Altogether, it could be suggested that SNHL may be an additional symptom associated with OFD type IX caused by *TBC1D32* mutations. These findings imply a new possibility to broaden the clinical spectrum of *TBC1D32*-related disorders, highlighting the value of a combined genetic and clinical analysis in patients with a complex phenotype.

Collectively, these studies emphasize the importance of exploring both coding and non-coding regions to enhance the understanding of IRD. They also demonstrate how advanced genetic tools like HTS can significantly improve diagnosis and open new windows for personalized treatments, such as the use of ASOs, ultimately offering hope for patients with ultra rare variants. Furthermore, it is important to highlight the relevance of a proper clinical anamnesis and a careful review of previously reported cases that display similar clinical features to cases under study. This could eventually be decisive in unravelling the genetic diagnosis of a patient and ensuring proper follow-up of the case.

RESUMEN

Las distrofias hereditarias de retina (DHR) engloban un amplio espectro de trastornos genéticos caracterizados por la degeneración o disfunción de los fotorreceptores o las células del epitelio pigmentario, que conduce a una pérdida progresiva de la visión. Las DHR presentan una elevada heterogeneidad genética y clínica, lo que hace que su diagnóstico genético sea un reto. Hasta la fecha, más de 280 genes se han asociado a las DHR, lo que dificulta aún más su diagnóstico. Sin embargo, la llegada de la secuenciación de nueva generación o *High-throughput Sequencing* (HTS) ha supuesto una revolución para el diagnóstico genético, permitiendo una identificación de variantes más eficiente, ofreciendo a los pacientes un asesoramiento genético más preciso y la posibilidad de acceder a ensayos clínicos basados en terapia génica.

La primera parte de esta tesis se basó en el estudio 92 pacientes con DHR mediante la secuenciación de dos paneles personalizados que incluían genes asociadas a estas patologías. Ambos diseños contenían genes asociados a DHR y regiones intrónicas donde se habían identificado variantes intrónicas profundas patogénicas hasta la fecha. Identificamos la causa genética en el 57,6% de los casos, mientras que el 13% tenía solo una mutación en genes que siguen un patrón de herencia autosómico recesivo y el 29,3% de los pacientes permanecieron sin un diagnóstico molecular. Se encontraron un total de 120 variantes patogénicas o probablemente patogénicas, incluidas 30 variantes noveles. Entre los pacientes con distrofia de conos y bastones, *ABCA4* fue el gen mutado con mayor frecuencia, mientras que las mutaciones en *USH2A* fueron las más comunes entre aquellos pacientes con Retinosis Pigmentaria (RP). Además, 10 familias portaban mutaciones en múltiples genes relacionados con la distrofia de conos y bastones, lo que resalta la complejidad de estas patologías a nivel genético. Gracias a haber incluido ciertas regiones no codificantes en el diseño del panel, también se identificaron dos variantes intrónicas profundas en *ABCA4* y *CEP290* descritas previamente como patogénicas. Esto subraya la importancia de estudiar las regiones no codificantes en las estrategias de diagnóstico genético. Este enfoque ha permitido el diagnóstico molecular de un alto porcentaje de pacientes, permitiéndoles así tomar decisiones reproductivas y ser incluidos en ensayos clínicos basados en terapia génica de los que probablemente podrán beneficiarse en el futuro.

Las regiones no codificantes han adquirido una gran importancia y su estudio ha pasado a ser esencial para el diagnóstico genético. En particular, se ha identificado un número importante de variantes intrónicas profundas en el gen *USH2A* que se han clasificado como patogénicas. Se ha descrito que este tipo de variantes tienen un impacto en el splicing que altera la secuencia codificante. Por lo tanto, la segunda parte de esta tesis se centró en el estudio de las regiones no codificantes de los genes USH en pacientes no resueltos con USH y con RP autosómica recesiva no sindrómica.En un primer paso, se realizó un cribado de cinco variantes intrónicas profundas en *USH2A,* previamente descritas como patogénicas, en pacientes monoalélicos para este gen. El diagnóstico genético se completó en el 30,95% de los pacientes seleccionados identificando la segunda variante causante de enfermedad entre las cinco intrónicas profundas estudiadas. Posteriormente, en los pacientes que permanecieron sin resolver y en aquellos nuevos casos monoalélicos, se realizó la secuenciación completa de *USH2A* o de todos los genes USH. Se identificaron 16 variantes en las que se predijo una alteración en el splicing, de las cuales cuatro mostraron un efecto al splicing mediante ensayos funcionales con minigenes. Dos de las variantes identificadas en este estudio eran intrónicas profundas y el ensayo funcional mostró la inclusión de un pseudoexón (PE) en el pre-ARNm. Las otras dos alteraban un elemento regulador en *cis*. Posteriormente, dado su potencial para modular el splicing, se diseñaron oligonucleótidos antisentido (ASO) para revertir la inclusión del PE en tres variantes intrónicas profundas del gen *USH2A* (dos identificadas en este estudio y una descrita previamente). La inserción de todos los PE se corrigió con éxito *in vitro* en las tres variantes. Estos resultados resaltan el papel fundamental que tienen las variantes intrónicas profundas en la patogenicidad de estas enfermedades y demuestra el potencial terapéutico de los ASO para los pacientes portadores de estas variantes.

Las DHR también pueden ser consideradas como ciliopatías, un grupo de trastornos causados por defectos en los cilios, que son estructuras esenciales para la señalización celular y la función de varios tejidos. Por ejemplo, las mutaciones en genes como *USH2A* y *CEP290* alteran la función ciliar, lo que conlleva a la degeneración de los fotorreceptores y la pérdida de visión. En particular, el síndrome oro-facial-digital (OFD) se caracteriza por hendiduras faciales, microftalmia/anoftalmia, coloboma y polidactilia, y está causado por mutaciones en genes involucrados en la formación de los cilios. El OFD tipo IX se vinculó

recientemente con el gen *TBC1D32*, que también se ha asociado a RP en ciertas familias. Siguiendo esta línea, el tercer artículo de esta tesis ha sido resultado del estudio de un paciente que presentaba una clínica compatible con OFD tipo IX, incluyendo RP e hipoacusia neurosensorial bilateral moderada (*Sensorineural Hearing Loss*, SNHL). Esto nos llevó a realizar una secuenciación del exoma clínico en el que se identificaron dos variantes en heterocigosis en el gen *TBC1D32.* Posteriormente, el análisis de segregación familiar demostró que se encontraban en *trans*. Dado que este es el primer caso diagnosticado con SNHL con mutaciones en *TBC1D32*, también realizamos un análisis de genes asociados con SNHL, pero no identificamos ninguna variante relevante que pudiese explicar la hipoacusia. Además, se ha descrito un amplio número de ciliopatías vinculadas a anomalías en los cilios del oído interno que a menudo resultan en pérdida auditiva. En base a los hallazgos encontrados en el paciente, se podría sugerir que la SNHL podría ser un síntoma adicional asociado con OFD tipo IX causado por mutaciones en *TBC1D32*. Estos resultados implican la posibilidad de ampliar el espectro clínico de los trastornos relacionados con *TBC1D32*, destacando la importancia de realizar un análisis genético y clínico conjunto en pacientes con un fenotipo complejo.

En resumen, los resultados obtenidos en la tesis subrayan la importancia de analizar tanto las regiones codificantes como las no codificantes para mejorar la comprensión de DHR. También demuestran cómo las herramientas como la HTS pueden mejorar significativamente el diagnóstico y abrir nuevas vías para tratamientos personalizados, tales como el uso de ASO, abriendo una posible vía terapéutica para los pacientes con variantes ultra raras. Además, cabe destacar la importancia de una anamnesis clínica adecuada y una buena revisión de los casos previamente publicados que muestran características clínicas similares a nuestros casos de estudio ya que podría ser decisivo para conseguir el diagnóstico genético de un paciente y continuar con un buen seguimiento del caso.

RESUM

Les distròfies hereditàries de retina (DHR) engloben un ampli espectre de trastorns genètics caracteritzats per la degeneració o disfunció dels fotoreceptors o les cèl·lules de l'epiteli pigmentari, que conduïx a una pèrdua progressiva de la visió. Les DHR presenten una elevada heterogeneïtat genètica i clínica, la qual cosa fa que el seu diagnòstic genètic siga un repte. Fins a la data, més de 280 gens s'han associat a les DHR, la qual cosa dificulta encara més el seu diagnòstic. No obstant això, l'arribada de la seqüenciació de nova generació o *High-throughput Sequencing* (HTS) ha suposat una revolució per al diagnòstic genètic, permetent una identificació de variants més eficiente, oferint als pacients un assessorament genètic més precís i la posibilitat d'accedir a assajos clínics de teràpies basades en gens.

La primera la primera part d'aquesta tesi es va basar en l'estudi 92 pacients amb DHR mitjançant la seqüenciació de dos panells personalitzats que incloïen gens associats a aquestes patologies. Tots dos dissenys contenien gens associats a DHR i regions intròniques on s'havien identificat variants intròniques profundes patogèniques fins a la data. Identifiquem la causa genética en el 57,6% dels casos, mentres que el 13% tenia només una mutació en gens que seguixen un patró d'herència autosòmic recessiu i el 29,3% dels pacients van romandre sense un diagnòstic molecular. Es van trobar un total de 120 variants patogèniques o probablement patogèniques, incloent 30 variants novelles. Entre els pacients amb distròfia de cons i bastons, *ABCA4* va ser el gen mutat amb major freqüència, mentres que les mutacions en *USH2A* van ser les més comuns entre aquells pacients amb Retinosis Pigmentària (RP). A més, 10 famílies portaven mutacions en múltiples gens relacionats amb la distròfia de cons i bastons, la qual cosa ressalta la complexitat genètica d'aquestas patologies. Gràcies al fet d'haver inclòs certes regions no codificants en el disseny del panell, també es van identificar dos variants intròniques profundes en *ABCA4* i *CEP290* descrites prèviament com a patogèniques. Això subratlla la importància d'estudiar les regions no codificants en les estratègies de diagnòstic genètic. Aqueste enfocament ha permés el diagnòstic molecular d'un alt percentatge de pacients, permetent-los així prendre decisions reproductives i ser inclosos en assajos clínics basats en teràpia gènica dels quals probablement podran beneficiar-se en el futur.

Les regions no codificants han adquirit una gran importància i el seu estudi ha passat a ser essencial per al diagnòstic genètic. En particular, s'ha identificat un nombre important de variants intròniques profundes en el gen *USH2A* que s'han classificat com a patogèniques. S'ha descrit que aqueste tipus de variants tenen un impacte en el *splicing* que altera la seqüència codificant. Per tant, la segona part d'aquesta tesi es va centrar en l'estudi de les regions no codificants dels gens USH en pacients no resolts amb USH i amb RP autosòmica recessiva no sindròmica. En un primer pas, es va realitzar un cribratge de cinc variants intròniques profundes en *USH2A*, prèviament descrites com a patogèniques, en pacients monoalèlics per a aqueste gen. El diagnòstic genètic es va completar en el 30,95% dels pacients seleccionats identificant la segona variant causant de malaltia entre les cinc intròniques profundes estudiades. Posteriorment, en els pacients que van romandre sense resoldre i en aquells nous casos monoalèlics, es va realitzar la seqüenciació completa d'*USH2A* o de tots els gens USH. Es van identificar 16 variants en les quals es va predir una alteració en el *splicing*, de les quals quatre van mostrar un efecte al *splicing* mitjançant assajos funcionals amb minigens. Dos de les variants identifiques en aqueste estudi eren intròniques profundes i l'assaig funcional va mostrar la inclusió d'un pseudoexó (PE) en el pre-ARNm. Les altres dos alteraven un element regulador en *cis*. Posteriorment, donat el seu potencial per a modular el *splicing*, es van dissenyar oligonucleòtids antisentit (ASO) per a revertir la inclusió del PE en tres variants intròniques profundes del gen *USH2A* (dos identificades en aqueste estudi i una descrita prèviament). La inserció de tots els PE es va corregir amb èxit *in vitro* en les tres variants. Aquestes resultats ressalten el paper fonamental que tenen les variants intròniques profundes en la patogenicitat d'aquestes malalties i demostra el potencial terapèutic dels ASO per als pacients portadors d'aquestes variants.

Les DHR també poden ser considerades com ciliopatíes, un grup de trastorns causats per defectes en els cilis, que són estructures essencials per a la senyalització cel·lular i la funció de diversos teixits. Per exemple, les mutacions en gens com *USH2A* i *CEP290* alteren la funció ciliar, la qual cosa porta a la degeneració dels fotoreceptors i la pèrdua de visió. En particular, la síndrome oro-facial-digital (OFD), es caracteritza per clivelles facials, microftalmia/anoftalmia, coloboma i polidactília, i és causada per mutacions en gens involucrats en la formació dels cilis. El OFD tipus IX es va vincular recentment amb el gen

TBC1D32, que també s'ha associat amb RP en unes certes famílies. Seguint aquesta línia, el tercer article d'aquesta tesi ha resultat de l'estudi d'un pacient que presentava una clínica compatible amb OFD tipus IX, incloent RP i hipoacúsia neurosensorial bilateral moderada (*Sensorineural Hearing Loss*, SNHL). Això ens va portar a realitzar una seqüenciació del exoma clínic, en el qual es van identificar dos variants en heterocigosi en *TBC1D32*. Posteriorment, l'anàlisi de segregació familiar va demostrar que es trobaven en trans. Atés que este és el primer cas diagnosticat amb SNHL amb mutacions en *TBC1D32*, també realitzem una anàlisi de gens associats amb SNHL, però no identifiquem cap variant rellevant que poguera explicar la hipoacúsia. A més, s'ha descrit un ampli número de ciliopatíes vinculades a anomalies en els cilis de l'orella interna que sovint resulten en pèrdua auditiva. Sobre la base de les troballes identificades en el pacient, es podria suggerir que la SNHL podria ser un símptoma addicional associat amb OFD tipus IX causat per mutacions en *TBC1D32*. Aquestes resultats impliquen la possibilitat d'ampliar l'espectre clínic dels trastorns relacionats amb *TBC1D32*, destacan la importància de realitzar una anàlisi genètica i clínica en conjunt en pacients amb un quadre clínic complex.

En resum, els resultats obtinguts en la tesi subratllen la importància d'analitzar tant les regions codificants com les no codificants per a millorar la comprensió de DHR. També demostren com les ferramentes com la HTS poden millorar significativament el diagnòstic i obrir noves vies per a tractaments personalitzats, com ara l'ús de ASO, obrint una possible via terapèutica per als pacients amb variants ultres rares. A més, cal destacar la importància d'una anamnesi clínica adequada i una bona revisió dels casos prèviament publicats que mostren característiques clíniques similars als nostres casos d'estudi, ja que podria ser decisiu per a aconseguir el diagnòstic genètic d'un pacient i continuar amb un bon seguiment del cas.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2'-O-MOE	2'-O-methoxyethyl
3'ss	3' splice site
5'ss	5' splice site
AAV	adeno-associated virus
ABCA4	ATP-binding cassette transporter retina specific protein
ACMG	American college of medical genetics and genomics
AD	Autosomal dominant
ADHD	Attention-deficit/hyperactivity disorder
ALMS	Alstrom syndrome
AR	Autosomal recessive
ARRP	Autosomal recessive retinitis pigmentosa
ASO/AON	Antisense oligonucleotide
aUSH	Atypic Usher syndrome
BBS	Bardet-bield syndrome
BCVA	Best corrected visual acuity
BPD	Butterfly-shaped Pigment Dystrophy
BPS	Branch point site
BWA	Burrows-Wheeler Aligner
Cas	CRISPR associated protein
CC	Connecting cilium
CD	Cone dystrophy
cDNA	Complementary DNA
CES	Clinical exome sequencing
cGMP	Cyclic guanosine monophosphate
ChIP-seq	Chromatin immunoprecipitation sequencing
ChR2	Channelrhodopsin 2
CNV	Copy number variation
CRD	Cone-rod dystrophy
CRISPR	Clustered regularly interspaced short palindromic repeats
DFNA	Dominant deafness
DFNB	Recessive deafness
DMD	Duchenne muscular dystrophy
DNA	Deoxyribonucleic acid
EOSRD	Early onset severe retinal dystrophy

ERG	Electroretinography
ESC	Embryonic stem cell
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
ExAC	Exome aggregation consortium
GC-rich	Guanine cytosine rich
gnomAD	Genome aggregation database
HDR	Homology directed repair
HGMD	Human gene mutation database
HITI	Homology-independent targeted integration
hnRNP	Heterogeneous nuclear ribonucleoproteins
HPO	Human phenotype ontology
HSF	Human splicing finder
HTS	High throughput sequencing
IFT	Intraflagellar transport
iPSC	Induced pluripotent stem cell
IRBP	Interstitial retinol binding protein
IRD	Inherited retinal dystrophy
IS	Inner segment
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
JBTS	Joubert syndrome
LCA	Leber congenital amaurosis
LOVD	Leiden open variation database
LRAT	Lecithin-retinol acyltransferase
LV	Lentivirus
MAF	Minor allele frequency
MD	Macular dystrophy
MG	Müller glia
MLPA	Multiplex ligation-dependent probe amplification
mmASO	Mismatched antisense oligonucleotide
MMEJ	Microhomology-mediated end joining
MOE	Methoxyethyl
mORF	Main open reading frame
MRI	Magnetic resonance image
mRNA	Messenger RNA

MSC	Mesenchymal stem cell
MUT	Mutant
NCBI	National center for biotechnology information
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NMD	Nonsense mediated decay
NpHR	*Natromonas pharaonic* halorhodopsin
nsRP	Non-syndrome retinitis pigmentosa
OCT	Ocular coherence tomography
OFD	Oro-facial-digital syndrome
OMIM	Online mendelian Inheritance in man
ORF	Open reading frame
OS	Outer segment
P1	First custom USH panel
P2	Second custom USH panel
PacBio	Pacific biosciencies
PAS	Polyadenylation signal
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Pseudoexon
PHARC	Polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract
PMO	Phosphorodiamidate morpholino oligomer
PPT	Polypyrimidine tract
pre-mRNA	Premature messenger RNA
PS	Phosphoriate
PTC	Premature termination codon
PV1	Panel version 1
PV2	Panel version 2
qPCR	Quantitative PCR
RBP	RNA-binding protein
RCD	Rod-cone dystrophy
RD	Rod dystrophy
RDH12	Retinol dehydrogenase 12
RDH5	Retinal dehydrogenase 5
RetNet	Retinal information network
RNA	Ribonucleic acid

RNA-SEQ	RNA SEQUENCING
RP	RETINITIS PIGMENTOSA
RPC	RETINAL PROGENITOR CELL
RPE	RETINAL PIGMENT EPITHELIUM
RT	REAL TIME
SECORD	SEVERE EARLY CHILDHOOD-ONSET RETINAL DYSTROPHY
SEL-TCP-SEQ	SELECTIVE TRANSLATION COMPLEX PROFILING SEQUENCING
SMA	SPINAL MUSCULAR ATROPHY
smMIPs	SINGLE-MOLECULE INVERSION PROBES
SMRT	SINGLE MOLECULE REAL-TIME
SNHL	SENSORINEURAL HEARING LOSS
SNV	SINGLE NUCLEOTIDE VARIANT
SR	SERINE-RICH
SSL	SENIOR-LØKEN SYNDROME
STGD	STARGARDT DISEASE
STR	SHORT TANDEM REPEAT
SV	STRUCTURAL VARIANT
TALEN	TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASE
TE	TRANSPOSABLE ELEMENT
U7SNRNA	U7 SMALL NUCLEAR RNA
uORF	UPSTREAM OPEN READING FRAME
USH	USHER SYNDROME
USH1	USHER SYNDROME TYPE 1
USH2	USHER SYNDROME TYPE 2
USH3	USHER SYNDROME TYPE 3
UTR	UNTRANSLATED REGION
VUS	VARIANT OF UNKNOWN SIGNIFICANCE
WES	WHOLE EXOME SEQUENCING
WGS	WHOLE GENOME SEQUENCING
WT	WILD-TYPE
XL	X-LINKED
ZFN	ZINC FINGER NUCLEASE

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. THE VISUAL SYSTEM

The visual system let us perceive a visual stimulus around us, which will be processed as nerve signals eventually generating interpretable images in the brain. The eyeball (principal visual organ) is composed by two different liquids: the aqueous humour, which is responsible for feeding and getting oxygenated the retinal layers that lack a blood supply; and the vitreous humour, which is quite dense and works as a structural support (Figure 1). Additionally, the eye is also formed by the crystalline lens and three concentric overlapping layers as follows (Figure 1):

- Fibrous layer. This is the most outer layer of the eye and it is responsible for protecting and holding the eyeball. It is formed by two continuous structures called sclera and cornea (Figure 1). The sclera is a whitish anterior membrane made up by collagen fibers, while the cornea is a transparent anterior membrane that permits the light path and protects the iris and lens.
- Vascular layer. This layer is also called uvea and it is formed by the choroid in the posterior pole (Figure 1), which is a membrane with blood vessels and connective tissue that irrigates the rest of layers. In the anterior pole, the ciliary body is implicated in the production of aqueous humour and holds the lens (Figure 1). This layers also contains the iris (Figure 1), which is a coloured and circular membrane that controls the amount of light entering the eye.
- Nervous layer. This is the inner layer and it is known as retina (Figure 1). It is constituted by photosensitive cells covered by epithelial cells that contain melanin. Its principal function is to transform the light stimulus into a nerve impulse.

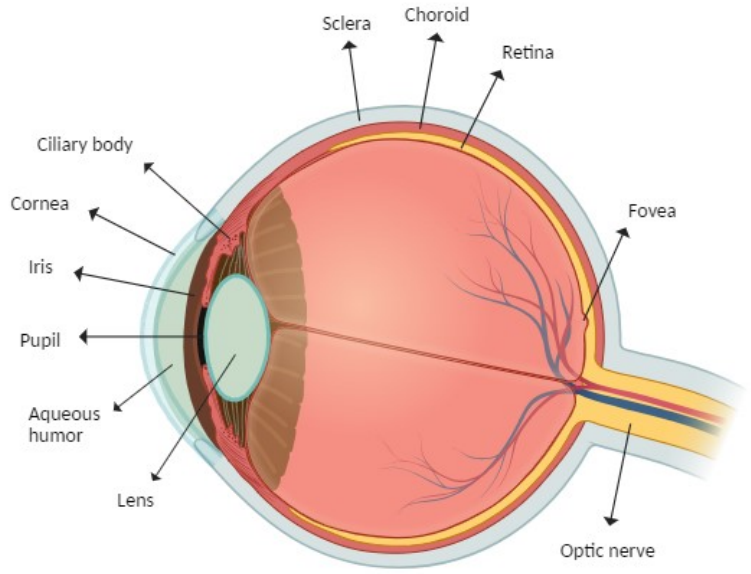


Figure 1. Human eye structure. Schematic representation of the different layers of the eye.

1.1. The retina

The retina is the innermost layer of the eyeball (Figure 1). As it is the photosensitive segment of the eye, its function relies on transforming light into neural signals that will be send to the brain in order to be interpreted. It is localised in the posterior segment of the eye and two regions can be differentiated:

- The anterior or blind section. This region covers the anterior part of the ciliary body and iris (Figure 1).
- The posterior or sensitive section. This region harbours the optic spot, where the optic nerve converges and which is lack of sensitive cells; the macula, which is yellowish oval area and occupies the centre of the retina; and the fovea, an avascular hollow in the centre of the macula that is the region with the greatest visual acuity (Dowling, 1987) (Figure 1).

The human retina shows a complex layered structure, which can be thought of as a succession of neuronal relays. The first neuron in the visual pathway is the photoreceptor (cone-rod layer/outer nuclear layer). The photoreceptors capture the light signal and establish synapses in the outer plexiform layer with the following neuron: the bipolar cell (inner nuclear layer) that receives the information, sent by the first neuron, and emits its axon to the inner plexiform layer, establishing contact with the third neuron: the retinal

ganglion cell (ganglion cell layer). Finally, ganglion cell axons are directed towards the optic nerve papilla forming the optic nerve fiber layer. In the outer pole, the photoreceptors are embedded in the retinal pigment epithelium (RPE) which is in charge of transporting nutrients to the photoreceptors, protecting the retina from the photo-oxidation and the renewal of the photoreceptors. Two other types of neurons in the retina, the horizontal cells and the amacrine cells, have their cell bodies in the inner nuclear layer and are primarily responsible for lateral interactions within the retina. Besides, there are glial cells (Müller cells, microglia, astrocytes and oligodendrocytes) that play a crucial role in maintaining the structural and functional stability of the retina (Figure 2A).

1.2. Photoreceptors

The photoreceptors are the neuron cells responsible for turning the luminous energy into an electric signal through the phototransduction. There are two different types of photoreceptors, cones and rods (Figure 2B). They can be differentiated by its morphology, the localization along the retina and their functions. On one hand, rods are the most abundant and they are localized in the whole surface, excepting the fovea. These cells are implicated in the peripheric vision, low-luminosity vision (scotopic vision) and black and white vision. They contain the rhodopsin as visual pigment. On the other hand, cones are condensed to the macula, being the only photoreceptor found in the fovea. Cones are responsible for colour perception, visual acuity and high-luminosity vision (photopic vision) and they contain three types of opsins (Stockman and Sharpe, 2000). The photoreceptor function is modulated by horizontal cells (Figure 2A). Both cones and rods have a similar structure organised as shown in Figure 2B:

- The Outer segment (OS) is where the photosensitive pigment is localized and where the phototransduction occurs. This segment is a specialized primary cilium with membranous discs that contain photopigment. These discs are continuously renewed through a process in which old discs are phagocytosed by RPE cells, while new discs are generated.
- Connecting cilium (CC) is a narrow region between OS and IS that facilitates the intracellular transport of vesicles and other metabolites. This segment is necessary for the renewal of the discs in the OS.

- The inner segment (IS) is differentiated in the ellipsoid and myoid zones. The first accumulates mitochondria and in the second resides the protein synthesis cell machinery. This segment also harbours the cellular body, which comprises the cellular nucleus.
- Synaptic region, terminal area where neurotransmitters are released towards bipolar and horizontal cells.

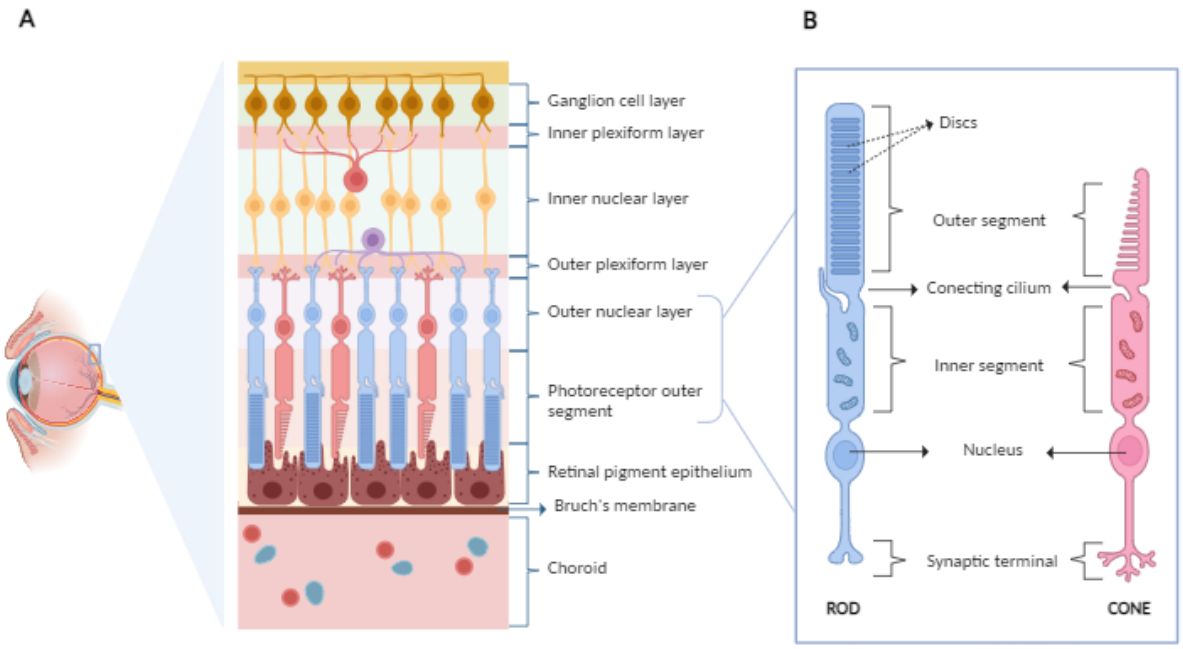


Figure 2. Human retina structure. (A) Transversal section of the retina representing the specific stratification of the layers. The different cell layers are depicted from ganglion cell layer, the inner layer of the retina, to the choroid. (B) Representation of the photoreceptor and their different segments.

1.3 Phototransduction and visual cycle

The phototransduction is the process by which the luminous stimulus is absorbed by the pigments in the photoreceptors, amplified and transformed into a nerve signal that will be interpreted by the brain. This process relies on the constant production of photosensitive pigments, which are located in the membranous discs in the OS, place where the signalling cascade begins. The light absorption produces a chemical modification in the photopigments that activates a signal cascades which consequence is the closure of cyclic guanosine monophosphate (cGMP)-dependent cation channels hyperpolarization of the

membrane, and thus, the release of neurotransmitters in the synaptic regions of photoreceptors.

The process that regenerates the retinal chromophore is known as retinoid/visual cycle (Figure 3). The visual cycle in rods is carried out by a canonical pathway, while the cones can also follow a non-canonical pathway (Kefalov, 2012). It has been speculated that this distinction could be due to the hight demand of chromophores by cones, so that, they compete with rods (Mata et al., 2002; Wang et al., 2009; Wang and Kefalov, 2011; Kiser et al., 2018).

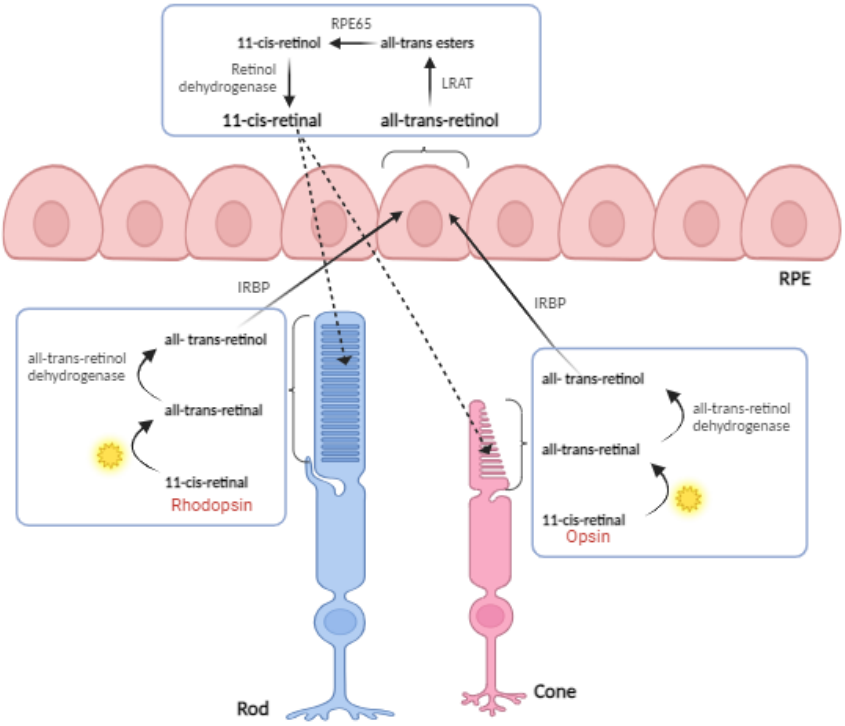


Figure 3. Canonical visual cycle in rods and cones. The image represents the recycling of the all-cis-retinal after being activated by light and separated from the opsin. LRAT: Lecithin-retinol acyltransferase; IRBP: Interstitial retinol binding protein; RPE: Retinal pigment epithelium.

Since visual cycle has been wider studied in rods comparing with cones, canonical visual cycle pathway is detailed bellow. After the photoreceptor activation by light, the all-trans retinal is released from the opsin, conjugated with the lecithin-retinol acyltransferase (LRAT) and is transported from the interior of the disc to the cytoplasm of the photoreceptor by the ATP-binding cassette transporter retina specific protein (ABCA4; ABCR proteins family).

Following, all-trans retinal is reduced to all-trans retinol by the enzyme all-trans retinol dehydrogenase 12 (RDH12) and is transported to the RPE by the interstitial retinol binding protein (IRBP) where it acquires its initial conformation (11-cis retinal) by the all-cis retinal dehydrogenase 5 (RDH5) (Den Hollander et al., 2010; Wright et al., 2010).

2. INHERITED RETINAL DYSTROPHIES

Inherited retinal dystrophies (IRDs) are a group of rare disorders characterized by either dysfunction or death, mainly progressive, of photoreceptor and RPE cells. Subsequently, this results in loss of vision and, in some cases, legal blindness. IRDs affect around 1.5 million people worldwide (Heath Jeffery et al., 2021), and they represent the first cause of legal blindness in the population aged from 16 to 64 (Liew et al., 2014; Hanany et al., 2020; Heath Jeffery et al., 2021). However, they are individually considered rare diseases since the incidence is 1 in 3,000-5,000 (Simunovic et al., 2019; Malvasi et al., 2023). IRDs follow many different modes of inheritance, from autosomal recessive (AR), autosomal dominant (AD), X-linked (XL) and even mitochondrial inheritance, uniparental isodisomy and digenic inheritance (Kajiwara et al., 1994; Dryja et al., 1997; Rivolta et al., 2002; Ayuso and Millan, 2010; Parmeggiani et al., 2011; Neveling et al., 2012).

2.1 Heterogeneity and classification of IRDs

IRDs are characterized by a high genetic heterogeneity with 316 genes and 345 *loci* implicated in the pathogenicity of these diseases (RetNet in December 2024; https://retnet.org/). The genes implicated in IRDs and the corresponding proteins are responsible for many functions such as phototransduction, cilia formation and trafficking, morphogenesis of photoreceptors outer segment discs, visual cycle, phagocytosis, among others (Karali and Banfi, 2015; Farrar et al., 2017; Manley et al., 2023). Furthermore, many genes have been described as causative for the same phenotype and one single gene may be responsible for different clinical entities (Méjécase et al., 2019). It has also been described that IRDs can show a high inter- and intra-familiar variability with variable symptoms expression and even incomplete penetrance (Farrar et al., 2017).

IRDs can be classified depending on several criteria, such as the region of the retina that is initially affected (peripheral/central), the presence of an associated extraocular pathology (syndromic/non-syndromic), whether photoreceptor degeneration is progressive or not, among others. Depending on the first photoreceptor type affected at the age of onset, we can distinguish among cone dystrophies (CD), cone-rod dystrophies (CRD), rod dystrophies (RCD), macular dystrophies (MD), diseases that primarily affect the choroid and vitreoretinopathies (Tatour and Ben-Yosef, 2020; Georgiou et al., 2021; Fenner et al., 2022; Moseley et al., 2024). Likewise, IRDs can be associated with other symptoms as part of the 80 syndromes associated with retinal dystrophy (Tatour and Ben-Yosef, 2020), or manifest in isolation as occurs in 70-80% of cases.

2.1.1 Cone dystrophies and Cone-Rod dystrophies

The CD are characterized by primary degeneration of cones, which are located to the central area of the retina and it has an incidence of 1/30,000 individuals (Michaelides, 2004; Hamel, 2007). Nonetheless, in advanced stages of the disease, the death of cones also causes the damage of rods and eventually its death. When the rod death occurs in early stages we can define it as CRD, which affects 1 person per 40,000 (Michaelides, 2004; Hamel, 2007). The severity of the symptoms and the average age when patients end up legally blind varies between CD to CRD (48 years old for CD and 24 for CRD) (Thiadens et al., 2012). The first symptoms of cones degeneration are a decrease of visual acuity, followed by photophobia, central scotoma and the alteration in colours perception. This usually appears within the first and second decade of life eventually leading to legal blindness (Thiadens et al., 2012).

The cone dystrophies could be either progressive, as it has been described for CD and CRD, or stationary, such as congenital achromatopsia (Aboshiha et al., 2016; Gill et al., 2019). The main symptoms of achromatopsia entail reduced visual acuity, photophobia, colour blindness and infantile nystagmus (Baxter and Borchert, 2024).

Thirty-two genes have been described as causative for CD/CRD, most of them with an AR pattern of inheritance. Six genes have been mostly associated to CD and 22 generally involved in CRD, existing an overlap among most of them (Gill et al., 2019).

2.1.2 Macular Dystrophies

Macular dystrophies (MD) are a heterogeneous group of IRDs that generally cause bilateral central vision loss in different levels and they are not as much related to nyctalopia as the rest of IRDs (Chiang and Yu, 2023). In every MD there is a peripheral vision preservation and a progressive degeneration of the retina and the macular RPE. In some cases, electrophysiological examination does not show any alteration until later stages of the disease (Rahman et al., 2020).

Macular diseases can be grouped relying on various pigment deposition patterns within the macula. The primary affected layer of the retina is the RPE. In several pattern dystrophies there is an accumulation in the form of lipofuscin.

Based on the pigment pattern distribution there are five main categories (Gass, 1997):

- Adult-onset Foveomacular Vitelliform Dystrophy
- Butterfly-shaped Pigment Dystrophy (BPD)
- Reticular Dystrophy
- Multifocal Pattern Dystrophy Simulating Stargardt's Disease
- Fundus Pulverulentus

One pattern may turn progressively into another in the same patient. Some patients may have a different a pattern in each eye and some families may demonstrate different phenotypes despite sharing the same pathogenic mutation.

Stargardt disease (STGD) is the most prevalent MD with an incidence of 1/8000-10000 cases (Tsang and Sharma, 2018). The STGD displays a high variability in the age of onset and progression of the symptoms, but it leads to legal blindness or severe damage in all cases around the fourth and seventh decade of life (Cremers et al., 2020). The course of the disease starts with visual acuity and central vision decrease in combination with difficulties in dark adaptation. The STGD is also known under the name of *Fundus Flavimaculatus* as a reference to the drusen and flecks as yellowish stains observed in the RPE (Khan and Cremers, 2020). The macular atrophy occurs in later stages, despite the fact that many

patients still preserve the fovea and relatively good visual acuity in advanced stages (Khan and Cremers, 2020).

STGD follows an AR pattern of inheritance and it is caused by biallelic pathogenic variants in *ABCA4*. The protein encoded by *ABCA4* is localized to the OS of photoreceptors and it is implicated in the visual cycle. It should be mentioned that there is a certain genotype-phenotype correlation for variants identified in this gene relying on the resulting amount of functional protein (Runhart et al., 2018). Despite the fact that the age of onset of the most severe phenotypes is prior to 10 years of age, there is a wide intervention window for therapies due to the slow progression of the disease.

2.1.3 Rod Dystrophies

Rod dystrophies (RD) or rod-cone dystrophies (RCD) are characterized by the initial degeneration of rods, which being mostly in the retinal periphery lead to nyctalopia (night blindness), followed by the reduction of the peripheric vision (tunnel vision at advanced stages) (Hamel, 2006). However, as the disease progresses, the cones are subsequently affected and ultimately cause the loss of central vision. RCD could either be stationary or progressive. Night blindness is the stationary form of RCD and there are two different types depending on whether the photoreceptors or the bipolar cells are affected.

Retinitis pigmentosa (RP) is the most frequent progressive RCD with an incidence of 1/4000 worldwide (Bruninx and Lepièce, 2020; Hanany et al., 2020). It is also described as the most prevalent IRD causing the 85-90% of all cases (Ayuso and Millan, 2010). The age of onset is usually within the second decade of life and the progression of the symptoms varies among patients, even among those whose causative gene is the same (Jespersgaard et al., 2020). Even so, RP patients used to display nyctalopia and visual field reduction. With the progression of the disease, pigment accumulations (bone spicules) can be observed in the eye fundus derived from the cell damage. Arteriolar attenuation and waxy disc pallor are also present in the clinical phenotype. It is necessary to mention that there is a directly proportional relationship between the age of onset and the severity and progression of the symptoms (Hamel, 2006; Hartong et al., 2006), being the symptoms more severe and with rapid progression in cases with an earlier onset.

RP follows different modes of inheritance such as AR (70% of all RP cases), AD (25%) and XL (5%) according to an epidemiologic study carried out in Spain (Perea-Romero et al., 2021). Around 30% of cases are sporadic (Parmeggiani et al., 2011). In XL cases, an earlier onset of the disease can also be observed, reaching legal blindness within the third-fourth decade (Hamel, 2006; Bhardwaj et al., 2022).

To date, 93 genes have been described as causative, which are implicated in the phototransduction cascade, the visual cycle and the maintenance of the photoreceptors structure. In Spain, *RHO* (31.4%) and *PRPF31* (16.4%) are the most frequently mutated genes for AD cases, while *USH2A* (19.1%) is the most frequently mutated gene in AR RP cases and *RPGR* (41.2%) in XL cases (Bernal, 2003; Perea-Romero et al., 2021). Moreover, more than 80 genes have been described in association with AR RP (including candidate and causative genes), around 30 for AD RP and 3 for XL cases (RetNet in November 2024; https://sph.uth.edu/retnet/home.htm) (Bhardwaj et al., 2022). Particularly, 6 genes have been associated with both AR and AD RP (*BEST1, NRL, NR2E3, RHO, RP1* and *RPE65*) (Diakatou et al., 2019). Due to the higher number of AR RP cases, *USH2A* happens to be the most frequently mutated gene. Interestingly, variant c.2276G>T in *USH2A* has also been described as the most common pathogenic variant in the European population (Dreyer et al., 2000; DuPont et al., 2018; Han et al., 2024).

Leber Congenital Amaurosis (LCA) is the most severe form of progressive RCD as the degeneration progresses so rapidly that cones death is observed in early stages of the disease. Within the first six weeks of life nystagmus and reduced vision are displayed and oscillate until the first and second decade, where the vision starts decreasing (Koenekoop, 2004). Nevertheless, in three quarters of cases the vision is stationary (Koenekoop, 2004). Furthermore, LCA is also characterised by photophobia and nyctalopia with further complications such as keratoconus and cataracts (Hanein et al., 2004; Huang et al., 2021). In the most severe cases of LCA, this leads to blindness in the first decade of life as every function is compromised from the onset. It has an incidence of 1/30,000 – 1/81,000 and it comprises a 5% of all the IRDs and a 20% of school age children cases (Koenekoop, 2004; Koenekoop et al., 2007; Stone et al., 2017; Chen et al., 2021; Huang et al., 2021). The LCA follows an AR and AD modes of inheritance and at least 28 genes have been described as

causative for this disease (Kumaran et al., 2017) (RetNet in December 2024; https://sph.uth.edu/retnet/home.htm). These genes are involved in phototransduction, visual cycle, ciliary transport, photoreceptors differentiation, etc. The frequency of genes in which mutations are identified in LCA cases varies relying on the population, being *CEP290*, *GUCY2D*, and *RPE65* the most frequently mutated in Caucasian population (Sweeney et al., 2007; Coppieters et al., 2010; Astuti et al., 2016).

Among early-onset cases, "Early Onset Severe Retinal Dystrophy" (EOSRD) and "Severe Early Childhood-Onset Retinal Dystrophy" (SECORD) were defined as different groups of diseases (Weleber et al., 2011). In contrast to LCA onset that occurs within the first months of life, EOSRD and SECORD are described as severe IRDs that usually display after infancy and before the age of 5 years (Kumaran et al., 2017). However, there is an overlap of the causative genes between EOSRD and LCA, although some of them are mostly associated with LCA (*GUCY2D*, *NMNAT1*, *CEP290* and *AIPL1*), while others with EOSRD (*RPE65*, *LRAT* and *RDH12*) (Kumaran et al., 2017). Particularly, *RPE65* is responsible for 4-16% of EOSRD (Aoun et al., 2021) and a gene therapy has been developed and approved by both FDA and EMA, which has already been administrated to pediatric patients with great results in accordance with clinical trials (Deng et al., 2022).

2.1.4 Other retinopathies

There are ocular diseases that involve other structures besides the retina, which can be classified into those who first affect the choroids and the vitreoretinopathies.

2.1.4.1 Ocular diseases that primarily affect the choroid

Choroideremia is an IRD with an incidence of 1/50,000-100,000 in Europe characterized by the degeneration of the retina (RPE and photoreceptors) and choroid (Zhang et al., 2015a). Choroideremia follows a XL recessive mode of inheritance, and it is caused by pathogenic variants in the *CHM* gene which encodes the protein REP-1. The lack of RPE-1 in RPE, photoreceptors or choroid, triggers their degeneration (MacDonald et al., 2009; Zhang et al., 2015a; Jo et al., 2023). Due to its XL inheritance pattern, differences in the clinic can be observed between male and female. While male patients usually display reduction of visual field and nyctalopia with reduced visual acuity in advanced stages, female do not usually

present severe symptoms except for changes in retinal pigmentation (Jauregui et al., 2019). Nonetheless, more severe cases have been described in female patients associated with the skewed inactivation of X chromosome (Bonilha et al., 2008). The age of onset is around the second decade of life and the disease progresses slowly until the fifth decade when patients usually go blind.

Gyrate atrophy is also an IRD that affect the choroid and its incidence has been estimated to be around 1/50,000 (Mäntyjärvi and Tuppurainen, 1998). Chorioretinal atrophic patches can be observed in patients with gyrate atrophy during the first decade with further signs of visual field constriction, nyctalopia and, sometimes in later stages, a slightly reduction of visual acuity (Takki and Milton, 1981; Hayasaka et al., 1986; Elnahry et al., 2018). It is an AR IRD caused by alterations in the *OAT* gene, whose defects lead to the accumulation of several substances that result in a toxic environment particularly for the eye (Khan et al., 1994). Gyrate atrophy is frequently associated with the development of cataracts and cystic macular edema (Takki and Milton, 1981; Elnahry et al., 2018).

2.1.4.2 Vitreoretinopathies

This group of IRDs is characterized by vitreous anomalies with poor vascularization and retinal detachment (Nixon et al., 2024). Vitreoretinopathies can be divided into exudatives or non-exudatives (Nixon et al., 2024). In patients with non-exudative vitreoretinopathies, the vitreous also present a fibrillar degeneration associated to early onset cataracts and different entities have been included in this group (Goldmann-Favre; Snowflake vitreoretinopathy degeneration; AD vitreoretinochoroidopathy; Wagner syndrome; Erosive vitreoretinopathy) (Ankala et al., 2018; Komro et al., 2022; Ashkenazy et al., 2023; Dass et al., 2023; Borella et al., 2024).

On the other hand, the familiar exudative vitreoretinopathy is an entity in which non-vascular areas on the peripheral retina lead to vascularization issues such as fibrosis, retinal detachment, peripheric exudation, preretinal neovascularization, etc. Nonetheless, the clinical spectrum can vary from vascularization problems without visual impairment to legal blindness as a consequence of retinal detachment (Tian et al., 2019; Chen et al., 2020; Tao

et al., 2021). This group of disorders can follow different modes of inheritances such as AR, AD or XL, and around 16 genes have been associated with this disease (Wang et al., 2023).

2.2 Syndromic IRDs

Even though IRDs are displayed solely in most of the cases, around 20-30% can be associated with other clinical symptoms as a part of one of the 80 syndromic forms that have been described (Ayuso and Millan, 2010; Tatour and Ben-Yosef, 2020), for which at least 200 genes have been described as causative (Tatour and Ben-Yosef, 2020). The majority of syndromic IRDs are rare, follow an AR mode of inheritance and can be classified into two groups: inborn errors of metabolism and ciliopathies (Tatour and Ben-Yosef, 2020). The most prevalent ciliopathies are the Usher Syndrome (USH), the Bardet-Bield Syndrome (BBS), the Joubert Syndrome (JBTS), the Senior-Løken Syndrome (SSL) and the Alstrom Syndrome (ALMS). Among all, USH, BBS and JBTS can be pointed out as they are characterised by the highest genetic heterogeneity. In these cases, the proteins encoded by the responsible genes build protein networks in the retina that explain the similarity in the phenotype caused by pathogenic variants in different genes (Tatour and Ben-Yosef, 2020).

2.2.1 Usher Syndrome

The Usher Syndrome is a rare AR disorder that associates vision loss due to RP, sensorineural hearing loss (SNHL) and, in some cases, vestibular dysfunction. It is considered a syndromic IRD and it has an incidence of 4-17 per 100,000 (Delmaghani and El-Amraoui, 2022), being the most prevalent genetic cause of deaf-blindness cases. USH is responsible for more than 50% of inherited deaf-blindness and for 3-6% of cases during childhood (Hope et al., 1997; Kimberling et al., 2010). It is also characterised by its high clinical and genetic heterogeneity, which will be explained in detail in sections below.

2.2.1.1 Clinical and genetic classification of USH

The USH can be classified into three clinical subtypes depending on the age of onset and the severity and progression of the symptoms (Smith et al., 1994):

- USH type 1 (USH1): It is the most severe type accounting for of 25-44% of USH cases (Bonnet et al., 2016; Jouret et al., 2019; Toms et al., 2020b; Fuster-García et al.,

2021). Patients present with stable and prelocutive SNHL ranging from severe to profound, vestibular dysfunction and RP of prepuberal onset. Six genes have been described as causative of USH1 (*CDH23*, *MYO7A, PCDH15, USH1C, USH1G* and *CIB2*), and each of them, except for *USH1G*, also cause non-syndromic AR deafness (DFNB) and, in the case of *MYO7A* non-syndromic AD deafness (DFNA) (Weil et al., 1997; Bork et al., 2001; Ahmed et al., 2002, 2003; Ouyang et al., 2002; Mburu et al., 2003; Riazuddin et al., 2012; Maria Oonk et al., 2015; Giese et al., 2017; Jacoszek et al., 2017; Wang et al., 2017). Particularly, *CIB2* is still controversial as USH1 causative as it has been proposed that mutations in this gene can cause phenocopies of USH1 (Booth et al., 2018).

- USH type 2 (USH2): This subtype is the most prevalent among all USH cases, being responsible for two-thirds of the USH cases (Bonnet et al., 2016; Jouret et al., 2019; Toms et al., 2020a; Fuster-García et al., 2021). The SNHL is congenital and stable and varies from moderate to severe in low frequencies and severe to profound in high frequencies (Abadie et al., 2012). It had always been considered that USH2 patients did not exhibit vestibular alterations; however, this assumption has recently been called into question (Wafa et al., 2021; Amorim et al., 2024). In the USH2 patients the RP onset occurs during the second decade of life (Aarem et al., 1995; Millán et al., 2011; Pierrache et al., 2016; Toms et al., 2020a). *ADGRV1, USH2A* and *WHRN* are responsible for USH2. Moreover, *USH2A* causes non-syndromic RP (nsRP) and *WHRN* is causative for DFNB (Mustapha et al., 2002; Tlili et al., 2005; Lenassi et al., 2015).
- USH type 3 (USH3): Being the less frequent subtype, USH3 is responsible for 2-4% of all cases although a higher incidence has been observed in Finnish and Ashkenazi Jews populations due to a founder effect (Adato et al., 2002; Ben-Yosef and Friedman, 2003; Ness, 2003; Malm et al., 2011; Millán et al., 2011; Marouf et al., 2022). It is also the most variable subtype as vestibular function can be either preserved or altered, the SNHL is progressive with variable onset and the RP onset also varies around second and third decade of life (Adato et al., 2002; Ness, 2003; Herrera et al., 2008; Malm et al., 2011; Khan et al., 2017). Only *CLRN1* gene has been clearly associated to USH3 and pathogenic variants in this gene also cause nsRP (Khan et al., 2011).

- Atypic USH (aUSH): This last subtype is constituted by USH patients whose clinical symptoms do not agree the standards of any of the three previous subtypes. Even though the definition of aUSH is not consistent in the bibliography, it can be characterised by moderate expression levels of SNHL and CRD. Different genes have been slightly associated with aUSH (*HARS*, *CEP250, CEP78, ARSG, ESPN*) (Khateb et al., 2014b, 2018; Tiwari et al., 2016; Fu et al., 2017; Ahmed et al., 2018; Fuster-García et al., 2018).

Interestingly, monoallelic pathogenic variants in *PDZD7* have been identified in a USH family and it has been proposed as an *USH2A* phenotype modifier and even responsible for digenic inheritance with monoallelic *ADGRV1* variants (Ebermann et al., 2010). Nevertheless, these concepts are quite controversial and there is insufficient evidence that these mechanisms have a clear role in the pathogenicity of USH.

2.2.1.2 Usher interactome

Usher proteins are organised as a protein network called USH interactome (Figure 4), which is localised in the inner ear and the retina. This is why altering any of these proteins results in similar clinical features. It has been proved that the USH interactome is essential for the auditive function development and for the photoreceptor maintenance (Figure 4) (El-Amraoui and Petit, 2005, 2014; Kremer et al., 2006; Maerker et al., 2008; Bonnet and El-Amraoui, 2012; Mathur and Yang, 2015; Géléoc and El-Amraoui, 2020). Specifically, several studies have described that this interactome is mainly localised to the stereocilia of ciliated cells and the periciliary area of photoreceptors, but it can also be found in the synaptic region of photoreceptors.

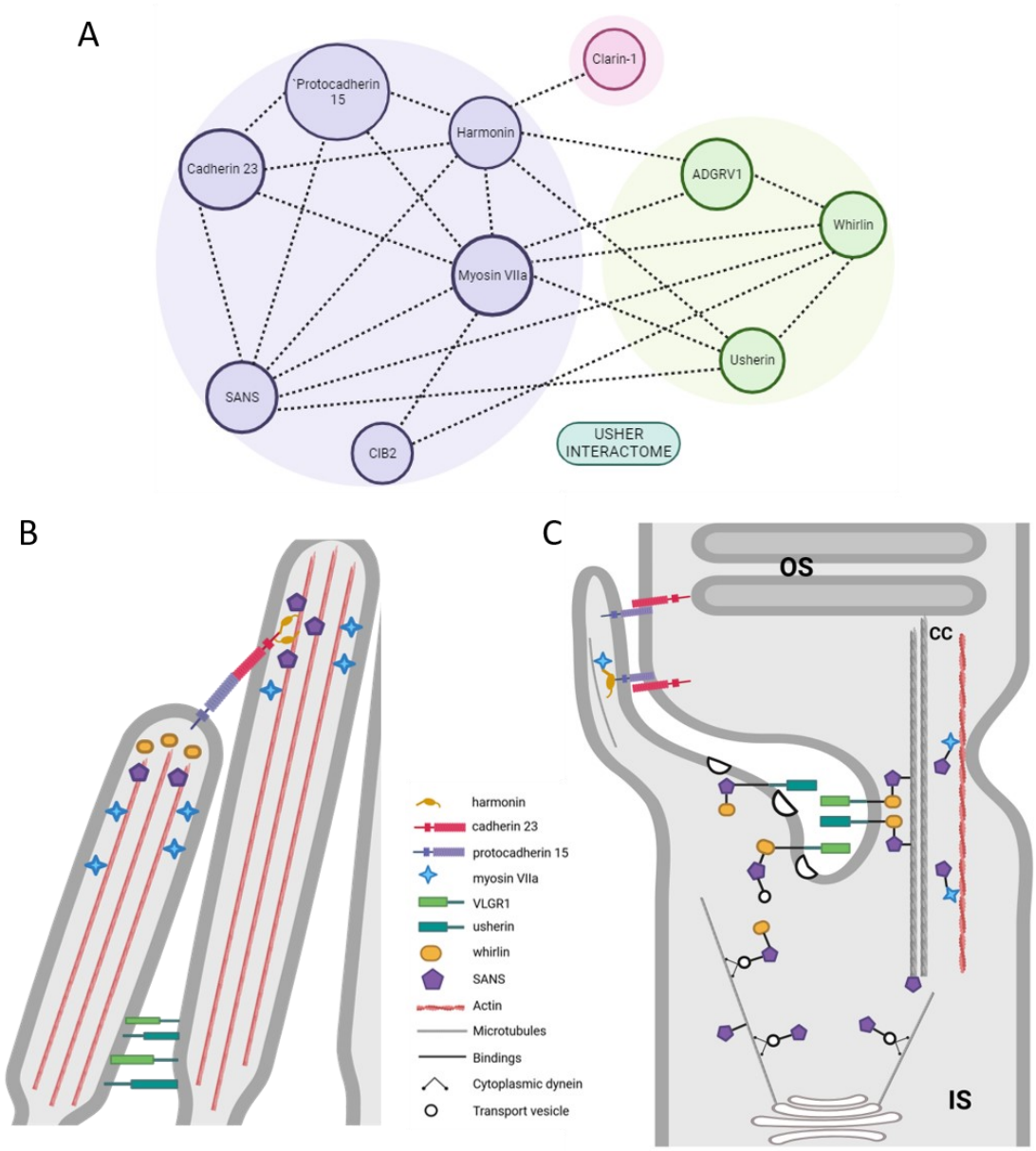


Figure 4. Usher interactome and tissues where it is expressed. (A) Usher proteins are colour-organized depending of the USH subtype. USH1 proteins are depicted in purple, USH2 in green and USH3 represented by *CLRN1* in pink. (B) USH proteins in inner ear hair cells. (C) USH proteins in photoreceptor outer segment (OS), connecting cilium (CC) and inner segment (IS). The Golgi complex (at the bottom of the photoreceptor in IS) generates vesicles that are directly directed to the apical IS membrane. The different USH proteins are detailed in the legend, which is common for (B) and (C) sections Clarin 1 is not represented in the figure as it expresses in the synapse areas in both cochlea and photoreceptors.

3. GENETIC DIAGNOSIS APPROACH

The genetic diagnosis of IRDs means a challenge especially due to their high genetic heterogeneity. Moreover, the clinical overlap of IRDs and their association with multiple syndromic forms, complicate an accurate clinical diagnosis and a patient-tailored follow-up.

The lack of genetic diagnosis hinders the possibility of anticipating to the first symptoms (in syndromic cases) and preventing some issues that could be detrimental for certain diseases (for instance STGD and vitamin A supplementation) (Radu et al., 2008). Giving a proper genetic diagnosis offers beneficial assistance such as cochlear implants in patients with SNHL (Rahman et al., 2022) and, even more, future genetic therapies that will be explained in detail in next sections. Unravelling the genetic cause can confirm or, in some cases, re-classify the clinical diagnosis, which also guides the familiar study in order to provide genetic counselling. Eventually, it should be noted that despite significant advancements in sequencing techniques, 30-40% of IRD patients still lack a genetic diagnosis (García Bohórquez et al., 2021; Ganapathi et al., 2022; Nash et al., 2022).

3.1 High throughput sequencing

The development of High-throughput Sequencing (HTS) has improved the genetic diagnosis of high heterogeneous diseases such as IRDs with efficient results (Britten-Jones et al., 2023b; Kocaaga et al., 2023). The HTS analysis can be carried out by different strategies based on sequencing of target regions or genes, the whole exome sequencing (WES) or the whole genome sequencing (WGS).

3.1.1 Targeted sequencing

Targeting HTS is based on the sequencing of a panel including specific regions of the genome such as certain genes, coding and non-coding regions, regulatory regions, etc. This sequencing approach provides the possibility of a previous enrichment step in order to select and increase the coverage of the regions of interest to perform the targeted analysis. The regions enrichment can be assessed by hybridization method, by multiplex PCR or even by single-molecule molecular inversion probes (smMIPs) method. The hybridization method is based on the use of probes which enable the capture of regions of interest. This methodology has offered a great advantage overtime, which has been observed in the increase of the diagnostic ratio in the genetic diagnosis of hearing loss and IRDs (Choi et al., 2013; Dockery et al., 2017). The smMIPs method is based on single-stranded DNA that hybridizes to the target region and can be used with DNA polymerase in order to fill the gaps (Lizardi et al., 2011). The effectiveness of smMIPs approach has also been shown in cases of a clear clinical diagnosis as it is very useful detecting low frequencies variants (Khan

et al., 2020b). During the first years of HTS development, targeted sequencing was the most used technique as it allows the specific analysis of regions of interest, facilitating the posterior analysis and provides less unexpected findings. It also offers a good solution in terms of low storage capacity, being a cost and time effective approach. Nevertheless, the diagnosis percentage obtained by targeted sequencing does not exceed 50-70% (García Bohórquez et al., 2021; Ganapathi et al., 2022; Nash et al., 2022). Additionally, targeted sequencing needs periodic updates of the genes included. Thus, this strategy is being progressively substituted by techniques such as WES or WGS.

3.1.2 Whole Exome Sequencing

The WES permits the analysis of the coding regions of all genes. Since 85% of pathogenic variants are located in coding regions, this approach has become the best option to analyse these regions as well as for the identification of new candidate genes. Nevertheless, the diagnostic yield for IRD by WES is over 50-80% (Tiwari et al., 2016; Haer-Wigman et al., 2017; Hussain et al., 2023) depending on the clinical entity studied. This outcome is comparable with the success obtained with targeted sequencing. Moreover, the identification of some types of structural variants (SVs) remains a challenge with this approach and intronic regions or regulatory elements cannot be analysed, except for some versions of WES which may include specific non-coding regions. On the other side, an interesting option for the clinical practice is the clinical exome, focusing on sequencing coding regions of genes associated exclusively with known inherited pathologies. (LaDuca et al., 2017). It has proven to be a very robust technique and a very cost-effective approach for genetic diagnosis (Stark et al., 2016; Stellacci et al., 2024).

3.1.3 Whole Genome Sequencing

The WGS approach offers a solution for those cases in which targeted sequencing and WES have not unravelled the genetic cause of the disease. In this line, WGS preparation does not require the capture of target regions, resulting in a better and more homogeneous coverage. Moreover, this approach enables the study of non-coding regions that can have an impact in the pathogenicity of the disease, combining the benefits of WES with the ability to identify new genes associated with the disease (Reurink et al., 2023). This is why an increase in the diagnostic yield of cases previously analysed by WES has been described

when applied WGS, in which SVs and deep-intronic variants have been identified (Karam et al., 2023; Liu et al., 2024b; Stellacci et al., 2024; Zeuli et al., 2024). Although the cost of WGS has traditionally been a significant barrier, it has now become one of the most used techniques for genetic diagnosis. As its cost continues to decrease, WGS is becoming competitive enough to be considered the first-line strategy in certain cases.

3.2 Third-Generation Sequencing: new insights for genetic diagnosis

Despite the advance that WES and WGS have meant to genetic diagnosis, short reads-based HTS strategies face some difficulties. These include the identification of large rearrangements, sequencing of repetitive regions and short tandem repeats (STRs) and also the establishment of haplotypes and pseudogenes are dismissed (Mantere et al., 2019). A great solution that overcomes these limitations is the third-generation sequencing, which offers the possibility of generating long reads (over 10Kb) (van Dijk et al., 2018; Mantere et al., 2019). The first technique to be developed was the single molecule real-time (SMRT) by PacBio (Pacific Biosciences), followed by Nanopore Sequencing by Oxford Nanopore (Eid et al., 2009; Magi et al., 2017). Both coincide in a PCR-free process that require a minimum amount of DNA and provide results in a few hours (Eid et al., 2009). In spite of its benefits, third-generation sequencing has faced some problems such as concentration steps to sequence a single molecule of DNA by real-time and the complications of the long-read sequencing itself (Schadt et al., 2010). Initially, although LR was a very useful tool for SVs identification, it used to generate many errors when detecting SNVs. Nonetheless, LR approaches has improved significantly, even becoming comparable with SR in the detection of SNVs (Kono and Arakawa, 2019).

3.3 Variants in non-coding regions

3.3.1. Splicing alterations

Splicing is the process by which introns are removed from newly transcribed RNA or pre-mRNA and exons are joined together to form mature mRNA. Eventually, the mRNA sequence will be translated into protein. Splicing takes place in the nucleus and it is catalysed by a protein complex called the spliceosome, which is formed by five ribonucleoproteins (U1, U2, U4–U6) and many other associated proteins (Anna and Monika, 2018).

Splicing elements can be classified as *trans-acting* or *cis-acting* elements (Figure 5). The spliceosome ribonucleoproteins are *trans-acting* elements that interact with *cis-acting* elements present in the RNA sequence. Within *cis* elements, which define the exonic and intronic sequence, we find donor (5'ss) and acceptor (3'ss) splice sites, the branch point site (BPS), polypyrimidine tract (PPT) and some auxiliary elements such as exonic or intronic splicing silencers (ESSs/ISSs) and enhancers (ESEs/ISEs) (Glisovic et al., 2008) (Figure 5). In this line, some of the associated proteins of the spliceosome recognize the ESE/ESS and ISE/ISS, which boost the correct intron cleavage. Generally, the serine and arginine-rich proteins (SR) are in charge of recognizing the ESE/ESS promoting the junction with the BPS (Cáceres and Kornblihtt, 2002; Lee and Rio, 2015), while the ISE and ISS recognition happens by heterogenous nuclear ribonucleoproteins (hnRNP) (Wagner and Garcia-Blanco, 2001) (Figure 5). The recognition of the different *cis-acting* elements within the same pre-mRNA sequence enables splicing to occurs alternatively, a process knowns as the alternative splicing, which generates different mRNA transcripts (WANG et al., 2015). Several alternative splicing events have been reported, with exon skipping and intron retention being the most frequent (Black, 2003; Chen and Manley, 2009).

Splicing is essential for the production of correct mRNA, which directly affects protein synthesis. Indeed, it is estimated that 15-50% of identified variants have a pathogenic effect in splicing (Wang and Cooper, 2007) (Figure 6). Splicing variants can be classified into canonical or non-canonical, depending on their position. Canonical variants are those located at nucleotides ±1 and ±2 of the splice sites. Variants located outside canonical positions are referred as non-canonical and can affect the branch point as well other of *cis* regulatory elements, such as ESE/ESS and ISE/ISS.

Variants located deep within the intron can cause part of intron to be recognised as coding sequence, leading to its inclusion in the mRNA as a pseudoexon (PE) or a cryptic exon (Figure 6). These variants typically activate cryptic splice site, create new splice sites or disrupt existing splicing regulatory elements such as ISE/ISS motifs and branch points. In addition, over 25% of variants that alter the splicing process, have been described to have an impact in splicing enhancers and silencers, promoting the recognition of cryptic sites or even causing exon-skipping when the variant is flanking an exon (Cartegni et al., 2002; Davis et

al., 2009; Sterne-Weiler et al., 2011). It is estimated that non-canonical events cause 3.5% of all IRD cases (Soens et al., 2017). In most cases, missplicing results in the creation of a premature stop/termination codon (PTC) which may lead to the mutant transcript being targeted for nonsense-mediated decay to (NMD) degradation or, alternatively, the synthesis of a non-functional protein (Dhir and Buratti, 2010; Popp and Maquat, 2013; Romano et al., 2013; Fraile-Bethencourt et al., 2019).

Deep-intronic variants causing the inclusion of a PE have been identified and described in several IRD-associated genes, with *USH2A* and *ABCA4* being the genes in which they have been most frequently described (Den Hollander et al., 2006; Littink et al., 2010; Vaché et al., 2012a; Webb et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015; Liquori et al., 2016; Baux et al., 2017; Khan et al., 2017, 2019b; Fadaie et al., 2019; Sangermano et al., 2019). Particularly, more than 30 deep-intronic variants have been identified in *ABCA4* (HGMD, accessed on 20th of December), being the gene that accumulates the highest number of deep-intronic variants identified so far. Thus, the study of non-coding regions and the analysis of these type of variants has happened to be essential for the proper genetic approach of IRD cases.

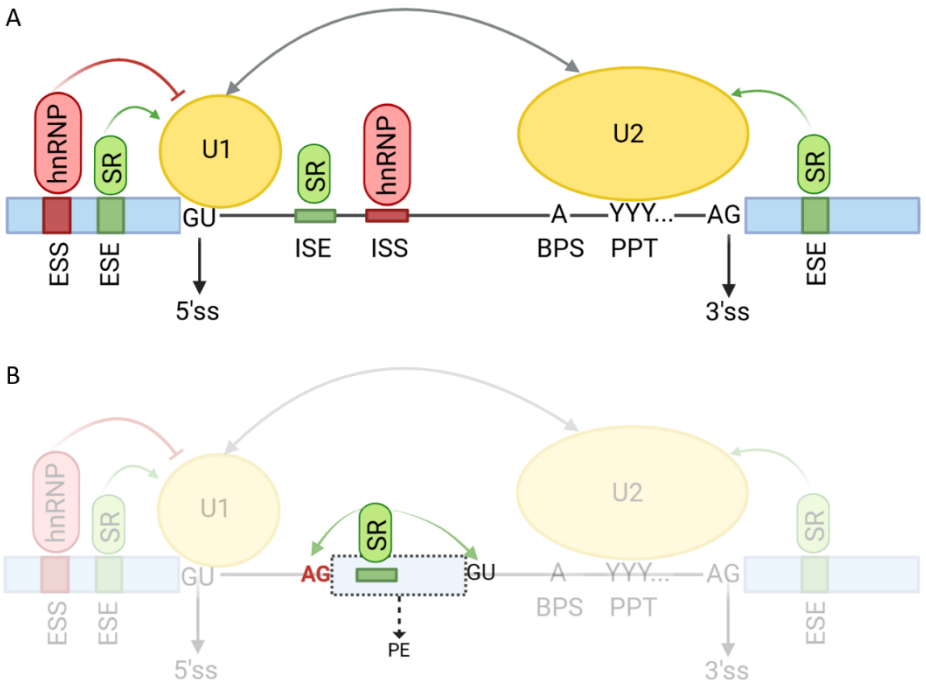


Figure 5. Scheme of the cis- and trans-acting splicing elements. (A) Cis-regulatory elements sequences encompass donor (5'ss) and acceptor (3'ss) splice sites, the branch point site (BPS), the polypyrimidine tract (PPT), as well as splicing silencers and enhancers (ESS; ESE; ISE; ISS). The donor and acceptor sites are represented by "GU" and "AG", respectively, as they are highly conserved across evolution. In contrast, the BPS and PPT exhibit greater sequence variability. Together, these *cis-acting* sequences are recognized by *trans-acting* elements, some of which are part of the spliceosome (here only U1 and U2 are depicted) and some are splicing activators and repressors (SR and hnRNP). Blue boxes in the extremes represent the exons. (B) This section depicts the recognition of part of the intron. When a deep-intronic mutation activates a cryptic splice site (in red the activation of a 3'ss) and there is a close ISE (green horizontal box) a SR protein can bind to this enhancer and allow this region to be finally recognized as a PE.

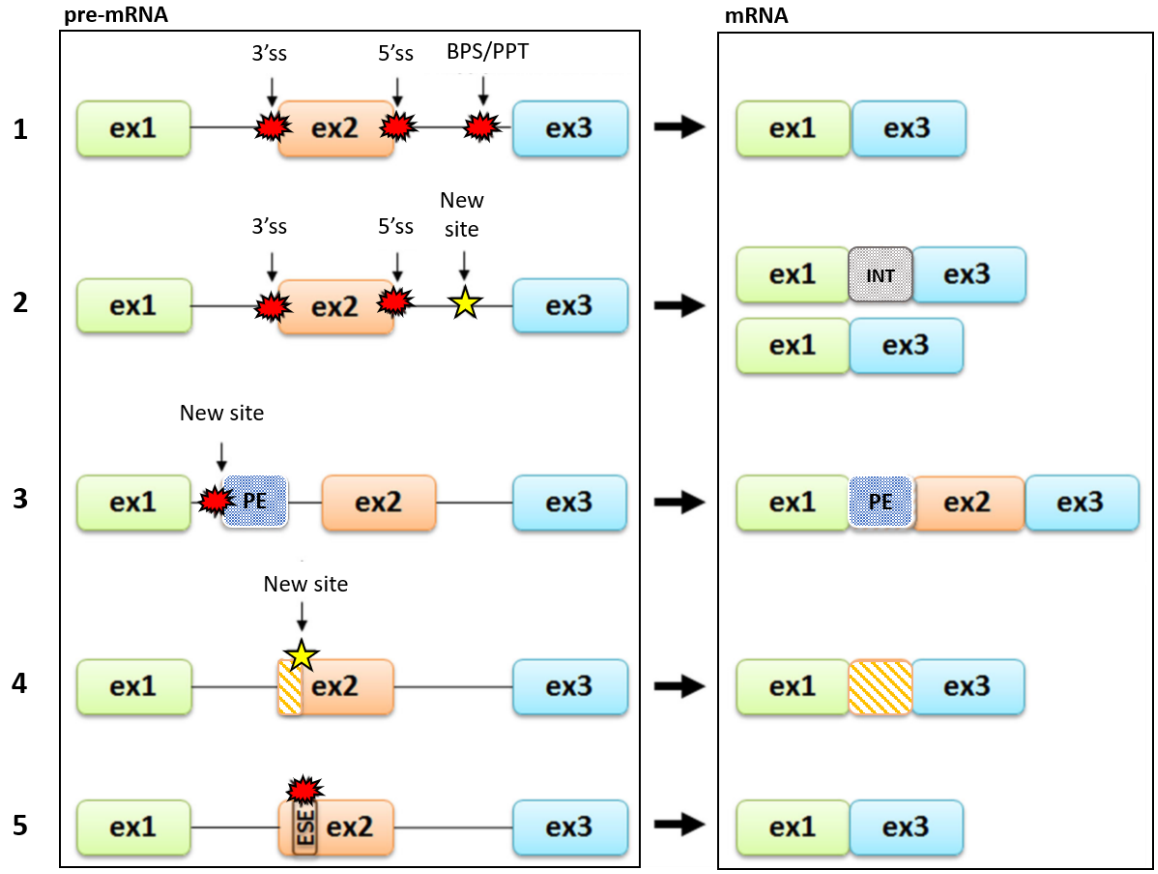


Figure 6. Missplicing caused by variants in canonical and non-canonical splicing positions. (1) Canonical splicing variants altering a splice site (3'ss or 5'ss) and non-canonical splicing variants affecting either a branch point site (BPS) or polypyrimidine tract (PPT) result in an exon-skipping. (2) Exon skipping and intron retention caused by canonical splicing variants altering a splice site in combination with the creation of a new splicing site within the intron. (3) A deep-intronic variant creates a new splice site or activates a cryptic splice site and causes a pseudoexon (PE) inclusion. (4) Variants within the coding region creates a new splice site causing partial exon-skipping. (5) Non-canonical splicing variant within the coding region alters a *cis* element, particularly an exonic splicing enhancer (ESE), producing an exon skipping effect. This figure has been obtained and modified from (Anna and Monika, 2018).

3.3.2. Splicing variants detection and analysis

Given the impact of splicing variants in the pathogenicity of the IRD, predicting their effect is the first challenge. Different *in silico* predictors have been developed for this purpose, such as Human Splicing Finder (HSF, https://www.genomnis.com/access-hsf), SpliceAI and MaxEnt (https://mobidetails.iurc.montp.inserm.fr/MD/), NNSplice (https://www.fruitfly.org/seq_tools/splice.html), VarSeak (https://varseak.bio/), regSNP-intron (https://regsnps-intron.ccbb.iupui.edu/), NetGene2 (NetGene2 - 2.42 - Services -

DTU Health Tech), ESEfinder (ESEfinder 3.0 -- (cshl.edu), , SVM-BPfinder (http://regulatorygenomics.upf.edu/Software/SVM_BP/) and LaBranchoR (available in https://genome.ucsc.edu/).

Despite the benefits of the study of these variants *in silico*, the splicing regulation is a complex process, and predictors do not have enough sensitivity and specificity to provide unequivocal results. For this reason, the most accurate way of unravel the effect is through the study of patient RNA. Nevertheless, the expression of many genes involved in IRDs is tissue-specific and the access to those tissues is complicated. In order to solve this issue, the use of minigenes to perform *in vitro* functional assays provides a good alternative, which use is already widespread (Baralle, 2003; Singh and Cooper, 2006; Rodriguez-Muñoz et al., 2022).

3.3.3 Variants in UTR 5' and 3'

Untranslated regions (UTRs) are segments found flanking a gene's coding sequence. While they do not code for amino acids, they are still transcribed into pre-mRNA (Hinnebusch et al., 2016). The 5' UTR, also known as the leader sequence, is located on the 5' end of the gene. It plays a crucial role in ribosome binding and recognizing the start codon in addition to translation efficiency. The 3' UTR, also referred to as the trailer sequence, is a significant regulatory element that influences the speed of protein translation and is located to the 3' extreme of the gene (Schwerk and Savan, 2015).

Beyond those variants that have a direct impact on the function of a protein, an alteration in the regulation of the mRNA also has a huge impact in terms of gene expression and function. Regions 5' and 3' UTR are in charge of the proper RNA regulation, stability, translation and many more processes. Variants in UTR regions have been previously associated with IRD (Coppieters et al., 2015; de Sousa Dias et al., 2015; Ghanbari et al., 2017).

For instance, 5' UTR regions contain upstream open reading frames (uORF), ribosome-specific motifs and stability motifs, and they are involved in many processes such as the initiation of the translation as well as the post-transcriptional regulation of the mRNA through uORFs and other regulatory structures (Calvo et al., 2009; Araujo et al., 2012).

Additionally, they have the ability to form secondary structures that intervene in translation and, hence, in gene expression (Leppek et al., 2018; Schuster and Hsieh, 2019). The uORFs are responsible of producing uPeptides and also control the main ORF translation (mORF) (Renz et al., 2020). uORFs can be initiated from either canonical or non-canonical start codons (McGillivray et al., 2018), the use of which relies on stress conditions and are related to several regulatory systems (Andreev et al., 2022).

On the other hand, 3' UTR regions are also implicated in processing, stabilize, translate and localization of the mRNA. Moreover, these regions harbour motifs for RNA-binding proteins (RBP) that, in complexes with other proteins, control all those processes (Mayya and Duchaine, 2019; Gebauer et al., 2021). Polyadenylation signals (PAS) are also localized to 3' UTR and have a role in the cleavage and polyadenylation of the mRNA through micro-RNA and RBPs. Within the 3' UTR, alternative PAS can be found, whose coordination with alternative splicing have been recently described (Zhang et al., 2023).

Despite that these regions have not been commonly studied by HTS and the *in silico* analysis do not provide fully trustable information, an increase in variants identified in 5' UTR has been observed. This comprises those variants altering ORFs, most of them affecting their start codon and so the protein production, and also those that have an impact in gene regulation (Whiffin et al., 2020; Ruberto et al., 2021; Trizuljak et al., 2024). So far, only some studies related to IRD have taken these regions into account and only some variants have been reported as IRD-causing in these regions (Coppieters et al., 2015; Ruberto et al., 2021; Daich Varela et al., 2023; Dueñas Rey et al., 2024). Nonetheless, it is still a challenge to predict the effect of variants located within these regions by the existing bioinformatic tools (Zhang et al., 2021).

Yet, the UTR sequences of some genes are not well characterised in order to predict the impact in the gene expression and their implication in the disease. One of the most traditionally used techniques to characterize promoter regions or validate the effect of variants within these regions has been the luciferase assay (Kim et al., 2022; Malka et al., 2024). It allows cloning the whole promotor region of a transcript of interest, and so the promotor activity can be valued *in vitro* by measuring its luminescence. Furthermore, different approaches have been proposed lately with the aim of finding ORFs and

determining their function (Kute et al., 2022). These include ribosome footprinting or ribo-seq (Ingolia et al., 2009), which allow the study of small mRNA fragments that are protected by ribosomes. They are further converted to DNA in order to perform deep sequencing for elucidating not only those regions occupied by ribosomes but the translation efficiency (Kute et al., 2022). Nonetheless, discovering novel ORFs is often challenging, so that, approaches based on translation initiation blockers together with ribosome profilins are used (Kute et al., 2022). Other complementary approaches to ribosome profiling consist of mass spectrometry strategies in order to detect functional peptides originated from certain small ORFs, or even a selective ribosome profiling based on selective translation complex profiling sequencing (sel-TCP-seq) (Bohlen et al., 2020; Wagner et al., 2020; Kute et al., 2022). Particularly, small ORFs can be shorter than 300 nucleotides and can differ from ORFs, for instance, in their start codon sequence (Ingolia et al., 2011; Lee et al., 2012). Although their identification has always been challenging, some developments in computational analysis through diverse pipelines are also been assessed (Kute et al., 2022).

4. THERAPEUTIC APPROACHES AND CURRENT THERAPIES

The search for a treatment has always been a subject of interest and the great development of diagnosis strategies for IRD has offered new possibilities to explore even further. However, due to the high heterogeneity of IRDs, as well as the multiple modes of inheritance associated with these pathologies, multiple therapeutic strategies are needed to address the various underlying pathogenic mechanisms. Furthermore, not all of them are applicable at all stages of the disease, which highlights the importance of developing personalized approaches. These include pharmacological therapy, gene therapy, cell-based treatments, and optogenetics approaches, that can provide effective and tailored solutions for each case (Figure 7).

4.1 Pharmacological therapy

The photoreceptor death triggers some effects such as inflammation, apoptotic signals and oxidative stress that contribute to this cell death. With the aim of overcoming this situation, some drug-based treatments are being assessed to act against all these effects and prevent the progression of the disease (Pinilla et al., 2022). Pharmacological therapies aim to slow the progression of IRDs and preserve vision by employing neuroprotective, anti-apoptotic,

antioxidant, and anti-inflammatory methods (Jayakody et al., 2015; Olivares-González et al., 2021). The anti-inflammatory therapies are focused on reducing the cell death and maintaining the integrity of the retina by inhibiting TNFα and microglia and by the administration of hormones (Roche et al., 2018; Benlloch-Navarro et al., 2019; Olivares-González et al., 2021). Other strategies based on inhibiting cell apoptosis and other cell death mechanism are the focus of "Anti-Cell Death" therapies (Olivares-González et al., 2021). Another approach based on vitamins supplementation, such as nutraceutical molecules that promote photoreceptor maintenance, and compounds with mixed mechanisms of action have also shown effectiveness in patients with RP (Olivares-González et al., 2021).

Several clinical trials have been initiated to treat IRD by pharmacological therapy. Antioxidant, antiapoptotic and anti-inflammatory compounds are being developed for RP, LCA, USH and STGD. These molecules include N-acetylcysteine, saffron, minocycline, melatonin, lutein, lutein plus vitamine A, vitamine A and/or E, etc (Pinilla et al., 2022).

Interestingly, some drugs have been studied in order to block PTCs promoting the translation of the whole protein. This is a genotype-dependent strategy, where the molecule interacts with the ribosome for ignoring the PTC, which enables the translation of the complete protein (Goldmann et al., 2011, 2012). As a consequence, a random amino acid is introduced relying on the stop codon, which means that the amino acid of the consensus sequence does not have to rigorously be introduced (Dabrowski et al., 2018). This pharmacogenomics strategy could serve as an effective treatment, though it may sometimes only alleviate the condition by producing alternative, functionally similar proteins instead of the wild-type version. This has been tried for variant p.Arg31Ter of *USH1C* gene with PTC124 molecule, although all stop codons could be the target for this treatment (Goldmann et al., 2011, 2012).

4.2 Gene therapy

The genetic therapy is focused on treating genetic disorders either providing a functional gene copy by gene augmentation strategies, correcting the DNA using different editing tools or even modulating gene expression. As we will detail later, some of these strategies are

already yielding very good results, and others under development are highly promising. However, gene therapy is usually focused on patients with a genetic diagnosis of the disease and sufficient cell viability. In addition, the eye gathers great immunological conditions and it is an idoneal environment for the application of genetic tools due to the isolation from the rest of organs. In this section, gene augmentation, gene editing and the use of ASOs for splicing modulation will be detailed.

4.1.1 Gene augmentation

Gene augmentation or replacement is based on the introduction of a functional copy of the gene and it is mostly focused on diseases caused by loss-of-function mutations. This can be carried out by the use of vectors (viral and non-viral), many of which have been already evaluated (Sundaram et al., 2012; Ziccardi et al., 2019). Among viral vectors, adeno-associated viruses (AAVs) have gained prominence in gene therapy due to their safety profile and capacity for long-term gene expression. Compared to other viral vectors, AAVs have low immune response, long-term expression, low frequency of host genome integration, easy manipulation and cell-type specificity (Buch et al., 2008; Hauswirth, 2014; Battu et al., 2022). Additionally, AAVs can effectively transduce non-dividing cells, such as retinal and neuronal cells, making them suitable for treating a variety of genetic disorders (Pham et al., 2024). Likewise, the ability of targeting multiple retina layers is also a benefit (Vingolo et al., 2024). Despite these advantages, AAVs face several challenges. A key limitation is their restricted packaging capacity, which is limited to 4.5 kb and obstacles the supplementation of large genes such as *MYO7A, ABCA4* or *USH2A*, for which dual-vector therapy is now being assessed (Dyka et al., 2019; Nuzbrokh et al., 2021; Battu et al., 2022; Shchaslyvyi et al., 2023). Although its low immunogenicity, it should be mentioned that there is a preexisting human immune response to AAV that could reduce therapeutic effectiveness and generate concerns about safety (Falese et al., 2017; Ghosh et al., 2020; Mendell et al., 2022). While the low integration capacity of AAVs is generally an advantage, it occurs randomly and can either disrupt or limit other gene expression (Ghosh et al., 2020).

In spite of the challenges that AAV present, a treatment for patients with biallelic mutation in *RPE65* has been developed and approved by the FDA and EMA under the name of LUXTURNA. A recombinant AAV2 vector delivers a functional *RPE65* cDNA copy to RPE cells

that compensates the biallelic mutation. Eventually, this enables the production of the RPE65 protein and its administration is performed by subretinal injection (Russell et al., 2017; Shahryari et al., 2019; Shchaslyvyi et al., 2023).

An alternative for AAV could be lentivirus (LV). LV possess a high transgene capacity, enabling the integration of sequences up to 8 kb, thereby facilitating sustained long-term expression. However, LVs present certain limitations, including the risk of insertional mutagenesis, the complexity of their production process, and their relatively large diameter (Cavalieri et al., 2018; Battu et al., 2022). Furthermore, in spite of the fact that gene delivery through LV in RPE showed good effectiveness, differentiated photoreceptors may not be transduced with good results (Bennett, 2017).

In recent years, non-viral vectors are gaining importance as a very good alternative to viral vectors (Jain and Daigavane, 2024). The use of nanoparticles, liposomes or even naked-DNA showed good results in animal models (Sundaram et al., 2012; Ziccardi et al., 2019; Sun et al., 2020). Non-viral vectors offer the advantage of minimizing immune responses in comparison with AAV, while broadening the spectrum of diseases that can be addressed through gene therapy. Nonetheless, there are several limitations that need to be overcome, such as achieving efficient and prolonged gene delivery, as well as adjusting the targeting and reception by retinal cells. (Battu et al., 2022; Jain and Daigavane, 2024).

The final success of gene therapy in IRD depends on the efficacy of delivery methods. Subretinal injection turns out to be the principal option for delivering therapeutic vectors (Irigoyen et al., 2022; Jain and Daigavane, 2024). It offers direct vector delivery to photoreceptor as the injection occurs in the space between retina and RPE, being very efficient (Battu et al., 2022). Despite this, this is an invasive approach and some procedural difficulties can occur, such as a temporary retinal detachment and several vitrectomy complications (Battu et al., 2022). Moreover, reaching the subretinal space can be addressed by a suprachoroidal delivery system, which is a more precise approach but requires a specialized system and can cause suprachoroidal or subretinal haemorrhage (Battu et al., 2022). Another delivery method is the intravitreal injection, which is an easier and less invasive technique but also less efficient in reaching the target cells, since several physical barriers need to be traversed (Battu et al., 2022; Irigoyen et al., 2022). This is of

main importance as accessing photoreceptors or RPE cells requires a vector size that is able to cross different retinal layers (Teo et al., 2018; Tavakoli et al., 2020).

4.1.2 Gene editing

Gene editing strategies directly correct mutations in the host genome. This seeks to overcome gene augmentation therapies limitations, which are focused on the treatment without DNA modifications of loss-of-function mutations and haploinsufficiency cases.

Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the first generation of nucleases employed in gene editing (Gaj et al., 2013; Hirakawa et al., 2020). These tools rely on protein–DNA interactions to achieve sequence-specific binding. Nevertheless, their intricate design poses significant challenges for their development, thereby limiting their broader application in both research and therapeutic contexts (Pulman et al., 2022). Ultimately, the clustered regularly interspaced short palindromic repeats (CRISPR) system has become the principal gene editing tool as it is more straightforward to use (Farrar et al., 2012; Athanasiou et al., 2018). This technology is based on the use of an RNA guide that leads the machinery to a double-strand break in the target sequence through an endonuclease. Over time, different nucleases have been used for this purpose. Some of them are CRISPR-associated (Cas) proteins, with Cas9 as the key enzyme in this group (Fernández et al., 2017).

After the DNA break, different cell repair mechanisms such as non-homologous end joining (NHEJ) or homology directed repair (HDR) are activated (Doudna and Charpentier, 2014). The NHEJ system causes indels because of the non-precise DNA repair and it is mostly used for gene-knockouts. On the other hand, HDR is a more precise repair mechanism but requires the presence of a DNA template by which the break can be repaired (Doudna and Charpentier, 2014). Despite this use, HDR system is restricted to cells in division, which limits its use in retina and has led the researchers to evaluate other different repair systems like homology-independent targeted integration (HITI) or microhomology-mediated end joining (MMEJ) (Sfeir and Symington, 2015; Suzuki et al., 2016; Nishiguchi et al., 2020). These alternatives have been tested in murine models for correcting mutations in *Mertk* and *Gnat1* genes (Suzuki et al., 2016; Nishiguchi et al., 2020).

The CRISPR system has been used for editing some IRD genes such as *USH2A, RHO* and *PDE6B* among others, the last two performed in a murine model (Fuster-García et al., 2017; Hu et al., 2023). These achievements have entailed the development of clinical trials (Hernández-Juárez et al., 2021). This is case of the clinical trial called EDIT-101 has been developed to correct the recurrent mutation c.2991+1655A>G in *CEP290* responsible for Leber congenital amaurosis 10 (LCA10) through CRISPR (Pierce et al., 2024). The system includes the RNA guide and a SaCas9 endonuclease (from *Staphylococcus aureus*) and is directly delivered via AAV5 vector by subretinal injection (Fenner et al., 2022). The trial is in Phase 1/2, focusing on evaluating the safety, tolerability and preliminary efficacy of the treatment in patients with this IRD (Pierce et al., 2024). Initial results seem to be promising with some participants experiencing improvement in visual function, though the study is ongoing to further assess long-term outcomes (Pierce et al., 2024).

4.1.3 Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are short, synthetic DNA/RNA strands designed to bind specifically to complementary sequences. By hybridizing with target pre-mRNA, ASOs can modulate gene expression through various mechanisms, including splicing modulation (Crooke et al., 2017; Bennett, 2019), which makes them a powerful tool in therapeutic applications for genetic disorders such as IRD.

ASOs have the capacity to modulate splicing through various mechanisms. They can block the inclusion of a PE caused by a deep-intronic mutation, which has been already described for the variant c.2991+1655A>G in *CEP290* (Cideciyan et al., 2023). This is possible due to the inhibition of certain splice sites or regulatory elements, so they prevent the recruitment of splicing factors (Collin and Garanto, 2017; Xue and MacLaren, 2020). Furthermore, they are able to induce the inclusion of exons, the lack of which causes a disease, just like occurs with spinal muscular atrophy (SMA) (Haque and Yokota, 2024). On the other hand, ASOs can eliminate exons with pathogenic variants, generating mRNA with open reading frame that codify relatively functional proteins. For instance, exon 13 of *USH2A* turned out to be the most frequently mutated region of this gene. Eventually, it has been proven that the lack of this exon do not completely alter the protein function and a clinical trial is currently studying this mechanism (Dulla et al., 2021; Girach et al., 2022).

Nonetheless, ASOs can also treat AD diseases. This is case of AD RP caused by *RHO* mutations. Since the variant c.68C>A is one of the most frequent in USA in this gene (Daiger et al., 2015), an ASO was designed to repress the formation of the mutant allele in order to retain the expression of the wild-type allele (Biasutto et al., 2019; Woods and Pfeffer, 2020).

This is a safe and cost-effective strategy, together with the advantage of the intravitreal delivery and the capacity of penetrating the outer layers of the retina (Chi et al., 2017; Xue and MacLaren, 2020). With the purpose of increasing the cell penetrance capacity of these molecules and improving their *in vitro* stability, some developments in nuclease resistance were assessed. This led to a nonpolyanion backbone modification known as phosphorodiamidate morpholino oligomers (PMO) (Smith and Zain, 2019).

4.3 Cell therapy

Cell therapy is an approach that involves the use of living cells to repair or replace damaged tissues and/or cells. Cell replacement therapy focuses on substituting damaged host cells with healthy donor cells through transplantation of autologous or allogeneic cells (El-Kadiry et al., 2021). Cell therapy is a mutation-independent strategy and remains effective even when almost all photoreceptors have died so there is not target for gene therapy (Battu et al., 2022). Following this, it was demonstrated that RPE cells transplantation has a great potential to improve visual acuity (Binder et al., 2002; Battu et al., 2022). The development of general therapies genotype-independent such as cellular therapy is becoming essential, since 30-40% of patients remain without genetic diagnosis and a single strategy can be applied in many different cases.

Retinal transplantation can be achieved through two primary methods: by grafting retinal sheets onto the residual neural tissue or by injecting dissociated cells directly into the target region. These transplanted cells may originate from various sources, including stem cells, induced pluripotent stem cell (iPSC)-derived retinal progenitors, photoreceptor precursor cells, or more advanced structures such as retinal organoids (Gasparini et al., 2019). In addition to this, patient-derived human iPSC can be modified with CRISPR tools followed by an autologous transplant that provides higher histocompatibility (El-Kadiry et al., 2021;

Battu et al., 2022). It has been also described the activation of endogenous stem cells like Müller Glia (MG) cells as an alternative option (Liu et al., 2024a).

Different delivery methods encompassing subretinal, intravitreal, or suprachoroidal vias are being contemplated (Battu et al., 2022). There are several approaches under evaluation for IRD treatment, although some aspects are still understudy. These include the ability of the transplanted cells to establish new synaptic connections with RPE neurons, for instance, in retinas deserted of photoreceptors. Additionally, it is still unclear which are the best differentiation cells stages for transplanting, but clinical trials in phase 1/2 for Age-related MD and STGD are using either human embryonic stem cells (ESC)/iPSC-derived RPE cells or human ESC-derived RPE cells, respectively (Schwartz et al., 2012, 2015; Surendran et al., 2021; Battu et al., 2022).

4.4 Optogenetics approaches

A different area of study focused on vision restoration is optogenetics. This approach merges genetics with optical technology to control neural cells using light stimulation. As a result of the photoreceptors death, the cells suffer several morphology changes. Thus, the remaining cones that have already loss their outer segment tend to stablish ectopic synaptic connections with rods and bipolar cells forming new clusters and changing their activity (Peng et al., 2000; Haq et al., 2014). Despite this, the bipolar and ganglion cells in the retina still survive until late stages of the disease. This is used as an advantage for vision restoration approach by delivering transgenes encoding a light-sensitive protein (McClements et al., 2020; Lindner et al., 2022; Parnami and Bhattacharyya, 2023). This optogenetic strategy has been applied in animal models for RP showing an improvement in electrophysiology response (Gilhooley et al., 2022; Prosseda et al., 2022).

In the context of patients with RP, several studies have been assessed to convert still-functioning cells in damaged retinas, such as surviving cone bodies or bipolar cells, into artificial photoreceptors. This is achieved by introducing opsins, like channelrhodopsin 2 (ChR2), *Natromonas pharaonic* halorhodopsin (NpHR), melanopsin, or human rhodopsin, into these cells. There is a current clinical trial in Phase I/II with RP patients in late stages of the disease (NCT02556736), which faces some challenges regarding high intensity of light

that results toxic for the retina. Concerning this, different perspectives have been takin into consideration. First, the use of opsins can intrinsically increase the sensitivity to light (Parnami and Bhattacharyya, 2023), although the light response does not suit movement objects. Another recent focus aimed to target bipolar cells rather than ganglion cells and some differences has been observed in visual restoration (Gilhooley et al., 2021).

Another option for patients with almost no light perception, consists in stimulating the inner layers of the retina through artificial photosensors. Two implants have already been accepted by the FDA (Argus II by Second Sight Medical Products, Sylmar, California; Alpha AMS by Retina Implant AG, Alemania) (da Cruz et al., 2016; Stingl et al., 2017).

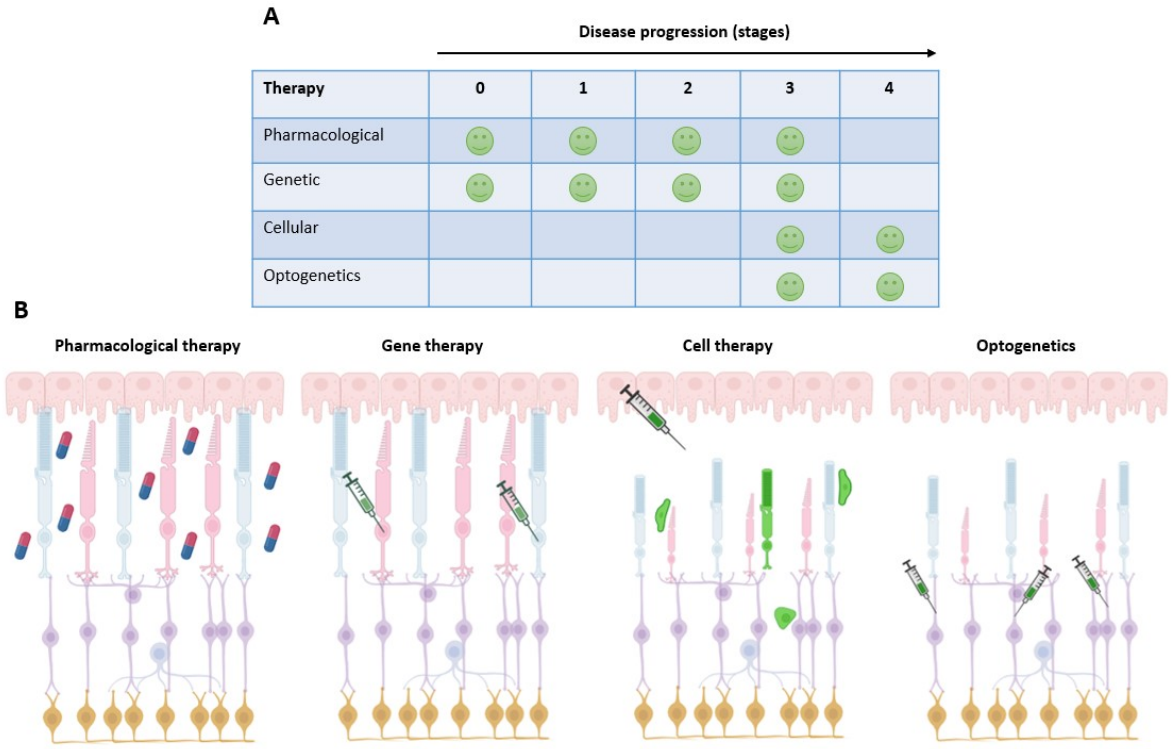


Figure 7. Scheme of the different therapy approaches currently being studied for treating IRDs. A) Different stages of the retinal degeneration are represented. Stage 1: thickness of the retina as the first symptom with normal layer organization and function; stages 2 and 3: loss of photoreceptors nucleus, shortened IS and OS and depigmented RPE; stage 4: slimming of retina with severe cell death and loss of laminar architecture of retina layers. This table has been adapted from (Jacobson and Cideciyan, 2010). B) Different therapies are depicted within the retina layers. While cell therapy provides new cells, the gene therapy corrects or supplies the photoreceptors with functional gene copies and optogenetics converts other cells into photosensitive cells.

OBJECTIVES

OBJECTIVES

I. To improve the genetic diagnosis of IRD patients by identifying new molecular defects and candidate genes that may be causative for these diseases using different HTS-based genes panels.

II. To explore the non-coding regions of USH genes in non-syndromic RP and USH patients who already carry a pathogenic variant in heterozygous state and to perform *in vitro* functional assays to validate the aberrant effect.

III. To correct the aberrant splicing caused by deep-intronic variants identified and validated in the previous objective, using specific ASOs as a potential genetic therapeutic approach.

CHAPTER I

CHAPTER I

Updating the Genetic Landscape of Inherited Retinal Dystrophies

Belén García Bohórquez[1,2], Elena Aller[1,2,3], Ana Rodríguez Muñoz[1,2], Teresa Jaijo[1,2,3], Gema García García[1,2], José M. Millán[1,2].

[1]Molecular, Cellular and Genomics Biomedicine. Health Research Institute La Fe, Valencia Spain. [2]CIBER of Rare Diseases. Madrid Spain. [3]Unit of Genetics, University Hospital La Fe, Valencia Spain.

Frontiers in cell and developmental biology – Volume 9, 645600 (2021)

10.3389/fcell.2021.645600

(Adapted to meet the format of this document)

Abstract

Inherited retinal dystrophies (IRD) are a group of diseases characterized by the loss or dysfunction of photoreceptors and a high genetic and clinical heterogeneity. Currently, over 270 genes have been associated with IRD which makes genetic diagnosis very difficult. The recent advent of next generation sequencing has greatly facilitated the diagnosis process, enabling to provide the patients with accurate genetic counselling in some cases.

We studied 92 patients clinically diagnosed with IRD with two different custom panels. In total, we resolved 53 patients (57.6%), in 12 patients (13%) we found only one mutation in a gene with known autosomal recessive pattern of inheritance and 27 patients (29.3%) remained unsolved. We identified 120 pathogenic or likely pathogenic variants; 30 of them were novel. Among the cone-rod dystrophy patients, ABCA4 was the most mutated gene, meanwhile, USH2A was the most prevalent among retinitis pigmentosa patients.

Interestingly, 10 families carried pathogenic variants in more than one IRD gene, and we identified two deep-intronic variants previously described as pathogenic in *ABCA4* and *CEP290*.

In conclusion, the IRD study through custom panel sequencing demonstrates its efficacy for genetic diagnosis, as well as the importance of including deep-intronic regions in their design. This genetic diagnosis will allow patients to make accurate reproductive decisions, enrol in gene-based clinical trials and benefit from future gene-based treatments.

Introduction

Inherited retinal dystrophies (IRD) are a group of diseases characterized by the progressive death or dysfunction of photoreceptors that leads to vision loss and, in some cases, legal blindness. IRDs have a prevalence of one case in 3000 individuals (Sahel et al., 2015). Depending on the photoreceptor initially affected, IRD can be classified into cone, rod-cone or cone-rod dystrophies in those cases in which both are affected at one time. Moreover, they can manifest as either isolated (70-80% of the total) or part of one of the 80 syndromes that have been estimated to be associated with IRD (Ayuso and Millan, 2010; Tatour and Ben-Yosef, 2020).

This group of diseases has a wide clinical spectrum and number of involved genes, currently reaching 271 for syndromic and non-syndromic forms (Retnet, https://sph.uth.edu/retnet/sum-dis.htm#A-genes, December 2020); explaining why IRDs have such high clinical and genetic heterogeneity. Furthermore, there is high inter and intrafamily variability, variable expression and incomplete penetrance (Farrar et al., 2017). IRDs can follow different patterns of inheritance, including autosomal recessive, autosomal dominant, X-linked, mitochondrial mode and some other less common, such as, uniparental isodisomy or digenic inheritance (Kajiwara et al., 1994; Dryja et al., 1997; Rivolta et al., 2002; Ayuso and Millan, 2010; Parmeggiani et al., 2011; Neveling et al., 2012). These issues together with the fact that 50% are sporadic cases, make it even more complicated to determine the mode of inheritance, genetic diagnosis and genetic counselling (Perea-Romero et al., 2021).

With the advent of next generation sequencing (NGS), the ratio of diagnosis has risen to 50-70%, which was difficult to achieve a few years ago (Carss et al., 2017; Sanchis-Juan et al., 2018; Rodríguez-Muñoz et al., 2020). Some different approaches, such as, custom panel designs, whole exome sequencing (WES) and whole genome sequencing (WGS), have been implemented to study the molecular mechanisms of IRD. Currently, these new sequencing techniques are essential to obtain an early and accurate genetic diagnosis, which is necessary to offer a correct genetic counselling to patients and their families (Salmaninejad et al., 2019).

In this study, we analyzed 92 patients, previously diagnosed clinically with IRD, with two custom panels for the main aim of achieving genetic diagnoses.

Materials and Methods

Cohort Selection

We selected 92 patients, belonging to 90 different Spanish families, with a clinical diagnosis of non-syndromic IRD, except for one who had clinical suspicion of a syndromic IRD. Patient DNA was isolated from peripheral blood with the automatic extractor QIAsymphony (QIAGEN).

Patients underwent complete ophthalmologic examinations, including OCT (Heidelberg Spectralis OCT Bluepeak, Heidelberg, Germany; Topcon 3D OCT 2000, Tokyo, Japan; CIRRUS OCT Zeiss, Oberkochen, Germany), ERG (Roland RETI-port/scan21, Brandenburg, Germany), eye fundus (Visucam NM/FA Zeiss, Oberkochen, Germany), visual acuity measure (BCVA) and evoked potentials and visual fields (Carl Zeiss Humphrey Field Analyzer, Oberkochen, Germany). A clinical questionnaire, which collected the main IRD characteristics, and an informed consent, were completed by each patient. This study was approved by the Hospital La Fe Ethics Committee, in agreement with the Declaration of Helsinki.

Panel Design and Sequencing

Sixty-three patients were sequenced with a gene panel version (PV1) that included 117 genes involved in non-syndromic IRD, and their flanking intronic regions (+/- 25 base pairs) (Rodríguez-Muñoz et al., 2020). Moreover, the panel of genes contained five intronic regions of *ABCA4*, *OFD1*, *USH2A*, *CEP290*, *PRPF31*, in which pathogenic variants had been previously identified (Den Hollander et al., 2006; Littink et al., 2010; Vaché et al., 2012; Webb et al., 2012; Braun et al., 2013) (Table S1).

The remaining 29 cases were analyzed with an updated version of the custom panel (PV2) that had 114 genes and all the deep-intronic variants described in the last few years in *ABCA4* and *USH2A* (Vaché et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bauwens et

al., 2015, 2019; Liquori et al., 2016; Baux et al., 2017; Fadaie et al., 2019; Khan et al., 2019; Sangermano et al., 2019) (Table S2).

The patients' libraries were prepared in accordance with the SureSelect QXT protocol (Agilent Technologies) and sequenced on a MiSeq platform (Illumina, San Diego, CA) in 300 cycles with 2x150 base pairs reads.

Data Analysis

The reads alignment against the reference hg19 genome, variant calling and annotation of all the identified variants were carried out with the *Alissa* resource (Agilent Technologies). The obtained variants were filtered based on a MAF (minor allele frequency) ≤ 0.01 according to the ExAC (http://exac.broadinstitute.org/) and gnomAD (https://gnomad.broadinstitute.org/) databases. In order to evaluate the pathogenicity of the detected variants, we also evaluated specific databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Locus Specific Data Base (https://grenada.lumc.nl/LSDB_list/lsdbs) and HGMD professional (https://portal.biobase-international.com/cgi-bin/portal/login.cgi). To evaluate the potential effect of novel missense variants we used the *in silico* predictors included in Varsome (https://varsome.com/). The putative effect on the splicing process was performed with HSF (Human SplicingFinder; http://www.umd.be/HSF), NNSplice (https://www.fruitfly.org/seq_tools/splice.html), and SpliceAI (https://mobidetails.iurc.montp.inserm.fr/MD/). Finally, IGV view finder (http://software.broadinstitute.org/software/igv/) allowed the examination of all detected variants in every read.

Novel variants identified in this study were classified according to standards of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015).

Copy Number Variation Analysis

A copy number variations (CNV) analysis was performed using the DECoN bioinformatic tool version 1.0.2 (Fowler et al., 2016).

We also study large rearrangements by Multiplex Ligation-dependent Probe Amplification (MLPA; MRC Holland) in patients harboring one pathogenic variant in *USH2A* (probemixes P361 and P362), *EYS* (probemix P328-A3) and *ABCA4* (probemixes P151 and P152). The multiplex ligation-dependent probe amplification results were analyzed by Coffalyser.Net software version 140721.1958 (MRC-Holland).

Sanger Sequencing and Segregation Analysis

The candidate variants identified in each patient were validated by Sanger sequencing (Big Dye Terminator v1.1 or v3.1, Applied Biosystems by Life Technologies). Moreover, the deep-intronic variants already described as pathogenic in *USH2A* and *ABCA4* (Table S3) (Vaché et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015, 2019; Liquori et al., 2016; Baux et al., 2017; Fadaie et al., 2019; Khan et al., 2019; Sangermano et al., 2019), were studied by Sanger sequencing in patients analyzed with PV1 who carried only one pathogenic variant in either or both genes.

Segregation analysis was performed when relatives' DNA was available (Figure S1).

Results

We obtained a mean depth of 190x per patient. In 98.6% of patients, at least 88% of the bases were covered with a sequencing depth of coverage ≥ 50x, and 95% of bases were covered with at least 20x. Moreover, we obtained a 70% of on-target reads.

We solved 53 patients belonging to 52 families (53/92, ratio of 57,6%): 17 (of 17 families), which had disease-causing variants in autosomal dominant genes, 33 (of 32 families) with autosomal recessive genes, two patients (of two families) who carried a pathogenic variant in an X-linked gene and one patient who carried two different pathogenic variants in two different autosomal dominant genes and two additional variants in an autosomal recessive

gene (RPN-670). We reported five cases with more than two pathogenic variants in the same autosomal recessive gene, and we also described six patients who carry pathogenic variants in more than one IRD gene (Table 1). Among the unresolved patients, 13% carried one pathogenic variant in an autosomal recessive IRD gene, with a higher prevalence of cases with a heterozygous variant in *ABCA4,* followed by *USH2A* and *RPGRIP1* (Table 2). In the 29,3% remaining cases, no pathogenic variant was identified.

We identified a total of 120 pathogenic or likely pathogenic variants (of which 85 were unique), including: 65 missense, 15 nonsense, 17 frameshift, 16 splice-site variants, two pathogenic deep-intronic variants, one in-frame deletion, one synonymous and three CNV (Figure 1). Twenty-nine variants were first described in this study (Tables 1-3). In this line, we identified a high number of mutated-genes, 9 autosomal dominant genes (highest prevalence of *PRPH2*), 19 autosomal recessive genes among which *ABCA4* and *USH2A* stand out, and finally, one X-linked gene, *RPGR* (Figure 2). All identified novel variants were classified as pathogenic or likely pathogenic according to ACMG criteria except for variant c.2470_2478del (p.Lys824_Glu826del) in *PDE6B*, which remained as variant of uncertain significance. This patient harbored another heterozygous pathogenic variant in the same gene, however, we could not carry out the segregation analysis to confirm it as likely pathogenic.

Table 1. Pathogenic variants identified in solved patients.

Family	Patient	Clinical diagnosis	Gene	Nucleotidechange	Protein change	Zygosity	Reference
$FRPN-51[1]	RPN-129	STG	*ABCA4* (NM_000350.3)	c.1804C>T	p.(Arg602Trp)	heterozygous	(Lewis et al., 1999)
				c.982G>T	p.(Glu328*)	heterozygous	(Fishman, 2003)
	RPN-544	STG	*ABCA4* (NM_000350.3)	c.5882G>A	p.(Gly1961Glu)	heterozygous	(Allikmets et al., 1997)
				c.982G>T	p.(Glu328*)	heterozygous	(Fishman, 2003)
$FRPN-244[1]	RPN-646	RP	*RHO* (NM_000539.3)	c.328T>C	p.(Cys110Arg)	heterozygous	(To et al., 2004)
FRPN-246[1]	RPN-649	MD/STG	*ABCA4* (NM_000350.3)	c.5917del	p.(Val1973*)	homozygous	(Rivolta et al., 2000)
$FRPN-252[1]	RPN-657	STG	*ABCA4* (NM_000350.3)	**c.5714+1G>A**	**p.?**	**heterozygous**	**This study**
				c.3386G>T	p.(Arg1129Leu)	heterozygous	(Allikmets et al., 1997)
FRPN-254[1]	RPN-659	RP	*EYS* (NM_001142800.2)	**c.7736_7742del**	**p.(Thr2579Lysfs*36)**	**homozygous**	**This study**
FRPN-255[1]	RPN-660	RP	*USH2A* (NM_206933.4)	c.4732C>T	p.(Arg1578Cys)	heterozygous	(Le Quesne Stabej et al., 2012)
				c.1214del	p.(Asn405Ilefs*3)	heterozygous	(Schwartz et al., 2005)
$FRPN-256[1]	RPN-661	RP/LCA	*CRB1* (NM_201253.3)	c.2416G>T	p.(Glu806*)	homozygous	(Corton et al., 2013)
FRPN-258[1]	RPN-663	STG	*ABCA4* (NM_000350.3)	c.3386G>T	p.(Arg1129Leu)	homozygous	(Allikmets et al., 1997)
				c.6718A>G	p.(Thr2240Ala)	heterozygous	(Zernant et al., 2011)
FRPN-261[1]	RPN-666	Reverse BCAMD/STG/RP	*ABCA4* (NM_000350.3)	c.5929G>A	p.(Gly1977Ser)	heterozygous	(Rozet et al., 1998)
				c.5882G>A	p.(Gly1961Glu)	heterozygous	(Allikmets et al., 1997)
FRPN-263[1]	RPN-668	CD	*CRB1* (NM_201253.3)	c.1604T>C	p.(Leu535Pro)	heterozygous	(Corton et al., 2013)
				c.2843G>A	p.(Cys948Tyr)	heterozygous	(den Hollander et al., 1999)
FRPN-265[1]	RPN-670	NA	*RP1* (NM_006269.2)	c.3157del	p.(Tyr1053Thrfs*4)	heterozygous	(Jacobson et al., 2000)
			PRPH2 (NM_000322.5)	c.623G>A	p.(Gly208Asp)	heterozygous	(Kohl et al., 1997)
			USH2A (NM_206933.4)	**c.6957+1G>C**	**p.?**	**heterozygous**	**This study**
				c.4955C>T	**p.(Pro1652Leu)**	**heterozygous**	**This study**
FRPN-266[1]	RPN-671	RP	*EYS* (NM_001142800.2)	c.5928-2A>G	p.?	homozygous	(González-del Pozo et al., 2011)
FRPN-267[1]	RPN-672	RP	*CNGB1* (NM_001297.5)	**c.2492+2T>G**	**p.?**	**homozygous**	**This study**
FRPN-268[1]	RPN-673	RP	*USH2A* (NM_206933.4)	c.2276G>T	p.(Cys759Phe)	heterozygous	(Rivolta et al., 2000)
				c.13894C>T	**p.(Pro4632Ser)**	**heterozygous**	**This study**
FRPN-269[1]	RPN-675	RP	*USH2A* (NM_206933.4)	c.4732C>T	p.(Arg1578Cys)	heterozygous	(Le Quesne Stabej et al., 2012)

				c.12575G>A	p.(Arg4192His)	heterozygous	(Ávila-Fernández et al., 2010)
FRPN-273[1]	RPN-679	STG	*ABCA4* (NM_000350.3)	**c.4880del**	**p.(Leu1627Argfs*35)**	**heterozygous**	**This study**
				c.5714+5G>A	p.?	heterozygous	(Cremers, 1998)
				c.2953G>A	**p.(Gly985Arg)**	**heterozygous**	**This study**
FRPN-276[1]	RPN-682	MD	*BEST1* (NM_004183.4)	c.247G>T	p.(Val83Phe)	heterozygous	(Kinnick et al., 2011)
FRPN-277[1]	RPN-683	RP	*RPGR* (NM_001034853.2)	**c.1366del**	**p.(Gln456Lysfs*20)**	**hemizygous**	**This study**
FRPN-278[1]	RPN-684	RP	*EYS* (NM_001142800.2)	c.9468T>A	p.(Tyr3156*)	homozygous	(Collin et al., 2008)
FRPN-279[1]	RPN-685	NA	*PRPH2* (NM_000322.5)	c.658C>T	p.(Arg220Trp)	heterozygous	(Payne et al., 1998)
FRPN-280[1]	RPN-686	RP	*USH2A* (NM_206933.4)	c.12575G>A	p.(Arg4192His)	homozygous	(Ávila-Fernández et al., 2010)
FRPN-281[1]	RPN-687	CRD/STG	*CRB1* (NM_201253.3)	**c.481G>A**	**p.(Ala161Thr)**	**homozygous**	**This study**
FRPN-284[1]	RPN-692	RP Punctata albensces	*ABCA4* (NM_000350.3)	c.3386G>T	p.(Arg1129Leu)	heterozygous	(Allikmets et al., 1997)
				c.6148G>C	p.(Val2050Leu)	heterozygous	(Lewis et al., 1999)
$FRPN-286[1]	RPN-694	NA	*PRPH2* (NM_000322.5)	**c.440dup**	**p.(Gly148Trpfs*29)**	**heterozygous**	**This study**
$FRPN-289[1]	RPN-697	STG	*ABCA4* (NM_000350.3)	c.3386G>T	p.(Arg1129Leu)	heterozygous	(Allikmets et al., 1997)
				c.634C>T	p.(Arg212Cys)	heterozygous	(Gerber et al., 1998)
FRPN-293[1]	RPN-701	RP	*PRPH2* (NM_000322.5)	**c.647C>A**	**p.(Pro216His)**	**heterozygous**	**This study**
FRPN-294[1]	RPN-702	MD	*ELOVL4* (NM_022726.4)	c.59A>G	p.(Asn20Ser)	heterozygous	(Hu et al., 2020)
FRPN-296[1]	RPN-704	CD	*GUCA1A* (NM_000409.5)	**c.66C>A**	**p.(Tyr22*)**	**heterozygous**	**This study**
FRPN-298[1]	RPN-706	CRD	*CRB1* (NM_201253.3)	c.613_619del	p.(Ile205Aspfs*13)	homozygous	(Lotery, 2001)
				c.2291G>A	p.(Arg764His)	heterozygous	(Corton et al., 2013)
FRPN-299[1]	RPN-707	RP	*USH2A* (NM_206933.4)	c.13811+2T>G	p.?	heterozygous	(Besnard et al., 2014)
				c.2276G>T	p.(Cys759Phe)	heterozygous	(Rivolta et al., 2000)
FRPN-300[1]	RPN-708	NA	*OTX2* (NM_001270525.2)	**c.638T>A**	**p.(Leu213*)**	**heterozygous**	**This study**
FRPN-301[1]	RPN-709	RP	*PROM1* (NM_006017.2)	**deletion exon 11**	**p.?**	**homozygous**	**This study**
FRPN-302[1]	RPN-710	MD/BVMD	*PRPH2* (NM_000322.5)	c.641G>A	p.(Cys214Tyr)	heterozygous	(Trujillo et al., 2001)
FRPN-303[1]	RPN-711	RP	*USH2A* (NM_206933.4)	c.14803C>T	p.(Arg4935*)	heterozygous	(Baux et al., 2007)
				c.2332G>T	p.(Asp778Tyr)	heterozygous	(Lenassi et al., 2015)
FRPN-307[1]	RPN-715	RP	*RHO* (NM_000539.3)	c.512C>A	p.(Pro171Gln)	heterozygous	(Antiñolo et al., 1994)
$FRPN-308[2]	RPN-717	RP	*ABCA4* (NM_000350.3)	c.6148G>C	p.(Val2050Leu)	heterozygous	(Allikmets et al., 1997)
			PRPF31 (NM_015629.3)	**Gene deletion**	**p.?**	**heterozygous**	**This study**
FRPN-309[2]	RPN-718	RP	*USH2A* (NM_206933.4)	c.2276G>T	p.(Cys759Phe)	homozygous	(Rivolta et al., 2000)
FRPN-312[2]	RPN-721	RP	*RHO* (NM_000539.3)	c.512C>A	p.(Pro171Leu)	heterozygous	(Stone et al., 2017)

FRPN-315[2]	RPN-725	MD/STG	*ABCA4* (NM_000350.3)	**c.6310C>T**	**p.(Gln2104*)**	**heterozygous**	**This study**
				c.3386G>T	p.(Arg1129Leu)	heterozygous	(Allikmets et al., 1997)
FRPN-316[2]	RPN-726	RP	*NRL* (NM_001354768.3)	c.149C>T	p.(Ser50Leu)	heterozygous	(Koyanagi et al., 2019)
			ABCA4 (NM_000350.3)	c.5908C>T	p.(Leu1970Phe)	heterozygous	(Rozet et al., 1998)
FRPN-318[2]	RPN-728	Joubert syndrome	*CEP290* (NM_025114.4)	c.4966_4967del	p.(Glu1656Asnfs*3)	heterozygous	(Sheck et al., 2018)
				c.2817G>T	p.(Lys939Asn)	heterozygous	(Srivastava et al., 2017)
FRPN-320[2]	RPN-730	MD/STG	*PRPH2* (NM_000322.5)	c.537G>A	p.(Trp179*)	heterozygous	(Diñeiro et al., 2020)
FRPN-322[2]	RPN-732	RP	*PDE6B* (NM_000283.4)	**c.1920+1G>A**	**p.?**	**heterozygous**	**This study**
				c.2470_2478del	**p.(Lys824_Glu826del)**	**heterozygous**	**This study**
FRPN-323[2]	RPN-733	MD	*PRPH2* (NM_000322.5)	c.421T>C	p.(Tyr141His)	heterozygous	(Diñeiro et al., 2020)
			ABCA4 (NM_000350.3)	c.5908C>T	p.(Leu1970Phe)	heterozygous	(Rozet et al., 1998)
FRPN-324[2]	RPN-734	MD/CD	*ABCA4* (NM_000350.3)	c.3113C>T	p.(Ala1038Val)	heterozygous	(Rozet et al., 1998)
				c.4539+2064C>T	[p.?;p.(=, Arg1514Leufs*36)]	heterozygous	(Zernant et al., 2014; Bauwens et al., 2019)
				c.1364T>A	p.(Leu455Gln)	heterozygous	(Salles et al., 2018)
FRPN-325[2]	RPN-735	NA	*EYS* (NM_001142800.2)	**c.8854del**	**p.(Thr2973Leufs*23)**	**heterozygous**	**This study**
				c.1194del	**p.(Gly399Aspfs*22)**	**heterozygous**	**This study**
			RDH5 (NM_002905.3)	c.712G>T	p.(Gly238Trp)	heterozygous	(Gonzalez-Fernandez et al., 1999)
FRPN-328[2]	RPN-737	MD/STG	*PRPH2* (NM_000322.5)	c.641G>A	p.(Cys214Tyr)	heterozygous	(Trujillo et al., 2001)
FPRN-327[2]	RPN-738	MD/STG	*BBS1* (NM_024649.4)	c.1169T>G	p.(Met390Arg)	homozygous	(Nishimura et al., 2010)
FRPN-335[2]	RPN-745	RP	*RPGR* (NM_001034853.2)	c.935-2A>G	p.?	hemizygous	(Koyanagi et al., 2019)
			ABCA4 (NM_000350.3)	c.5908C>T	p.(Leu1970Phe)	heterozygous	(Rozet et al., 1998)
			PDE6A (NM_000440.3)	**c.2144T>C**	**p.(Met715Thr)**	**heterozygous**	**This study**
FRPN-337[2]	RPN-747	RP	*RHO* (NM_000539.3)	**c.670G>A**	**p.(Gly224Arg)**	**heterozygous**	**This study**
FRPN-339[2]	RPN-749	STG	*ABCA4* (NM_000350.3)	c.3386G>T	p.(Arg1129Leu)	heterozygous	(Allikmets et al., 1997)
				c.3210_3211dup	p.(Ser1071Cysfs*14)	heterozygous	(Nasonkin et al., 1998)
				c.560G>A	p.(Arg187His)	heterozygous	(Cornelis et al., 2017)
[$]FRPN-340[2]	RPN-750	LCA	*CEP290* (NM_025114.4)	c.2991+1655A>G	p.?	homozygous	(Den Hollander et al., 2006)

Novel pathogenic variants are highlighted in bold font. FRPN: Family number; FRPN-"1": Families studied with PV1; FRPN-"2": Families studied with PV2; [$]FRPN: Families in which segregation analysis was performed; RPN: Patient number; RP: Retinitis pigmentosa; MD: Macular dystrophy; p.?: Unknown protein effect; STG: Stargardt; LCA: Leber congenital amaurosis; BCAMD: Benign concentric annular macular dystrophy; CD: Cone dystrophy; CRD: Cone-rod dystrophy. BVMD: Best vitelliform macular dystrophy; NA: Not Available.

Table 2. Patients in which only one pathogenic variant in a recessive gene has been identified.

Family	Patient	Clinic diagnosis	Gene	Nucleotide change	Protein change	Zygosity	Reference
FRPN-243[1]	RPN-645	RP	*USH2A* (NM_206933.4)	c.2276G>T	p.(Cys759Phe)	heterozygous	(Rivolta et al., 2000)
			CEP290 (NM_025114.4)	**c.7394_7395del**	**p.(Glu2465Valfs*2)**	**heterozygous**	**This study**
FRPN-272[1]	RPN-678	STG	*ABCA4* (NM_000350.3)	c.288C>A	p.(Asn96Lys)	heterozygous	(Stenirri et al., 2004)
			USH2A (NM_206933.4)	c.754G>T	p.(Gly252Cys)	heterozygous	(Bravo-Gil et al., 2016)
FRPN-283[1]	RPN-691	RP Punctata albensces	*CDHR1* (NM_033100.3)	c.783G>A	(p.Pro261=)	heterozygous	(Glockle et al., 2014)
			POC1B (NM_172240.3)	**c.1079_1080del**	**p.(Pro360Argfs*8)**	**heterozygous**	**This study**
FRPN-287[1]	RPN-695	NA	*ABCA4* (NM_000350.3)	c.6089G>A	p.(Arg2030Gln)	heterozygous	(Lewis et al., 1999)
FRPN-288[1]	RPN-696	NA	*ABCA4* (NM_000350.3)	c.6148G>C	p.(Val2050Leu)	heterozygous	(Allikmets et al., 1997)
FRPN-304[1]	RPN-712	RP	*RPGRIP1* (NM_020366.3)	**c.3339+5G>C**	**p.?**	**heterozygous**	**This study**
FRPN-305[1]	RPN-713	RP	*SAG* (NM_000541.5)	c.577C>T	p.(Arg193*)	heterozygous	(Maw et al., 1998)
			GUCY2D (NM_000180.4)	**c.1991A>G**	**p.(His664Arg)**	**heterozygous**	**This study**
FRPN-308[2]	RPN-717	RP	*ABCA4* (NM_000350.3)	c.6148G>C	p.(Val2050Leu)	heterozygous	(Allikmets et al., 1997)
FRPN-311[2]	RPN-720	RP (early onset)	*RPGRIP1* (NM_020366.3)	c.767C>G	p.(Ser256*)	heterozygous	(Jamshidi et al., 2019)
FRPN-313[2]	RPN-722	RP	*RBP3* (NM_002900.3)	c.3238G>A	p.(Asp1080Asn)	heterozygous	(den Hollander et al., 2009)
FRPN-314[2]	RPN-724	RP	*EYS* (NM_001142800.2)	**c.6882_6883del**	**p.(Gln2294Hisfs*3)**	**heterozygous**	**This study**
FRPN-319[2]	RPN-729	RP	*CNGA3* (NM_001298.3)	**c.673+5G>T**	**p.?**	**heterozygous**	**This study**
FRPN-336[2]	RPN-746	MD	*PCARE* (NM_001271441.2)	**c.656_665dup**	**p.(Ala223Argfs*38)**	**heterozygous**	**This study**

Novel pathogenic variants identified are highlighted in bold font. FRPN: Family number; FRPN-"1": Families studied with PV1; FRPN-"2": Families studied with PV2; RPN: Patient number; RP: Retinitis pigmentosa; STG: Stargardt; NA: Not Available; p.?: Unknown protein effect; SNHL: Sensorineural hearing loss; XL: X-linked.

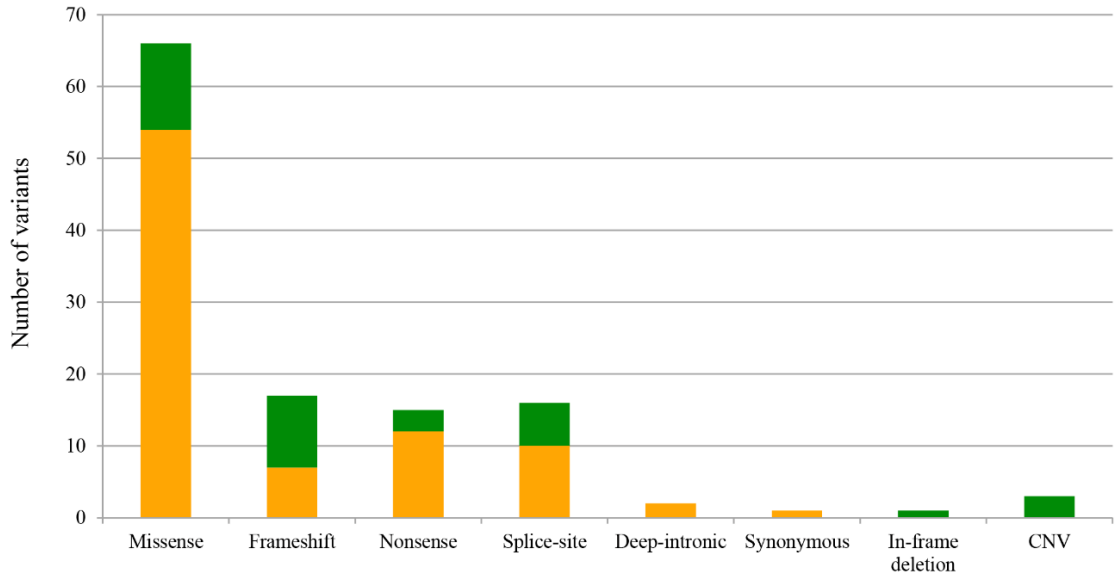


Figure 1. Representation of the total alleles identified in this study classified according to the alteration type. The X-axis refers to the different types of variants identified in this study, and the Y-axis concerns the total allele count for each type of variant. Pathogenic variants identified in this study are represented in green, while those previously described are in orange.

Copy number variants analysis with the bioinformatic tool allowed us to detect two putative deletions: a deletion in *PRPF31,* which was properly validated with MLPA (patient RPN-717) and a homozygous deletion of exon 11 of *PROM1* in patient RPN-709. In this last patient, the exon 11 did not have reads in the alignment and did not amplify by PCR, however, there was PCR amplification for the flanking exons, thus reinforcing the hypothesis of a homozygous deletion of the exon.

The individual RPN-728 presented nephropathy and alterations in the brain MRI (mild hypoplasia of the cerebellar vermis and slightly elongated superior cerebellar peduncles) in addition to retinal degeneration, suggesting a Joubert syndrome. The panel sequencing allowed us to identify two pathogenic variants in *CEP290*, c.4966_4967delGA (p.Glu1656Asnfs*3) and c.2817G>T(p.Lys939Asn). On the other hand, the panel analysis in the RPN-708, revealed a pathogenic variant in the *OTX2* gene (Table 1). Although this gene has been implicated in other diseases such as microphthalmia and retinal degeneration with pituitary dysfunction (OMIM: 610125; 610125), our patient only referred a macular dystrophy (MD) phenotype.

Table 3. Criteria considered for the pathogenicity classification of the novel identified variants.

Gene	Mutation		Classification	Frequency (gnomAD Ex)	Pathogenicity scores[a]	Conservation score (GERP)[b]	Reputable source	
	Nucleotide change	Protein change					ClinVar	HGMD[c]
ABCA4 (NM_000350.3)	c.2953G>A	p.(Gly985Arg)	Likely pathogenic	NF	12 of 13	5,7199	NA	NA
	c.4880del	p.(Leu1627Argfs*35)	Pathogenic	0,0000159	NA	5,6399	Pathogenic	NA
	c.5714+1G>A	p.?	Pathogenic	0,00000398	NA	4,7399	Likely pathogenic	NA
	c.6310C>T	p.(Gln2104*)	Pathogenic	NF	NA	6,0799	NA	NA
CEP290 (NM_025114.4)	c.7394_7395del	p.(Glu2465Valfs*2)	Pathogenic	0,000598	NA	5,42	NA	NA
CNGA3 (NM_001298.3)	c.673+5G>T	p.?	Likely pathogenic	0,0000199	NA	5,09	NA	NA
RPGRIP1 (NM_020366.3)	c.3339+5G>C	p.?	Likely pathogenic	NF	2 of 2	NA	Likely pathogenic	NA
CNGB1 (NM_001297.5)	c.2492+2T>G	p.?	Pathogenic	NF	NA	5,4499	NA	NA
CRB1 (NM_201253.3)	c.481G>A	p.(Ala161Thr)	Likely pathogenic	NF	12 of 13	5,5199	Uncertain significance	NA
PCARE(NM_001271441.2)	c.656_665dup	p.(Ala223Argfs*38)	Likely pathogenic	0,0000579	NA	1,8514	NA	NA
EYS (NM_001142800.2)	c.1194del	p.(Gly399Aspfs*22)	Pathogenic	NF	NA	6,07	NA	NA
	c.6882_6883del	p.(Gln2294Hisfs*3)	Pathogenic	NF	NA	5,38	NA	NA
	c.7736_7742del	p.(Thr2579Lysfs*36)	Pathogenic	0,0000127	NA	4,01	Likely pathogenic	NA
	c.8854del	p.(Thr2952Leufs*23)	Pathogenic	NF	NA	4.57	NA	NA
GUCY2D (NM_000180.4)	c.1991A>G	p.(His664Arg)	Likely pathogenic	NF	11 of 12	5.35	NA	NA
GUCA1A (NM_000409.5)	c.66C>A	p.(Tyr22*)	Likely pathogenic	0,00000795	NA	5,75	Uncertain significance	NA
OTX2 (NM_001270525.2)	c.638T>A	p.(Leu213*)	Pathogenic	NF	NA	5,53	NA	NA
PDE6A (NM_000440.3)	c.2144T>C	p.(Met715Thr)	Likely pathogenic	0,000231	12 of 13	5,32	Uncertain significance	NA
PDE6B (NM_000283.4)	c.1920+1G>A	p.?	Pathogenic	NF	NA	4,19	NA	NA
	c.2470_2478del	p.(Lys824_Glu826del)	Uncertain significance	0,000128	NA	4,1599	NA	NA

POC1B (NM_172240.3)	c.1079_1080del	p.(Pro360Argfs*8)	Pathogenic	0,0000145	NA	5,69	NA	NA
PROM1 (NM_006017.2)	exon 11 del	p.?	Pathogenic	NF	NA	NA	NA	NA
PRPH2 (NM_000322.5)	c.440dup	p.(Gly148Trpfs*29)	Pathogenic	NF	NA	5,8699	NA	NA
	c.647C>A	p.(Pro216His)	Likely pathogenic	NF	12 of 13	5,0999	NA	NA
RHO (NM_000539.3)	c.670G>A	p.(Gly224Arg)	Likely pathogenic	0,0000159	12 of 13	5,0399	NA	NA
RPGR (NM_001034853.2)	c.1366del	p.(Gln456Lysfs*20)	Pathogenic	NF	NA	4,73	NA	NA
USH2A (NM_206933.4)	c.4955C>T	p.(Pro1652Leu)	Likely pathogenic	0,000016	10 of 13	5,21	NA	NA
	c.6957+1G>C	p.?	Pathogenic	NF	NA	5,8099	NA	NA
	c.13894C>T	p.(Pro4632Ser)	Likely pathogenic	0,00000797	8 of 13	5,21	NA	NA

Column "Classification" refers to classification according to AMCG. [a] Pathogenicity Scores from https://varsome.com/ for missense variants (accessed November 2020) (BayesDel_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, MutationAssessor, MutationTaster, PrimateAI, REVEL and SIFT). Represent predictors supporting the pathogenic effect against the total of available predictors. [b] GERP conservation score based on the reduction in the number of substitutions in the multi-species sequence alignment compared to the neutral expectation using the genomes of 35 mammals. Range: -12.3 to 6.17(most conserved).[c]HGMD public version (accessed November 2020). p.?: Unknown protein effect; NF: No Found ; NA: Not Applicable.

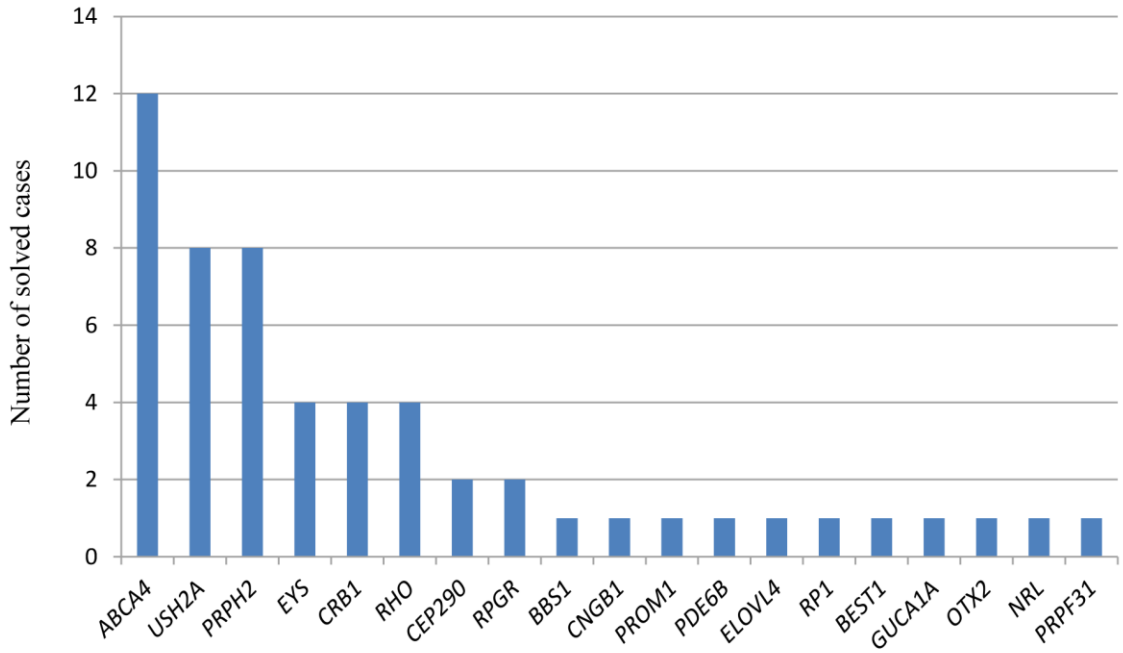


Figure 2. Number of solved cases with mutations in the different disease-causing gene. The X-axis refers to the responsible genes for each solved case, and the Y-axis to the number of solved cases for each disease-causing gene. Only genes responsible for the disease are represented. For patient RPN-670 (FRPN-265), the three different possible responsible genes for the IRD are represented.

Because of the inclusion of deep-intronic regions, we solved two cases who carried deep-intronic pathogenic variants in *ABCA4* and *CEP290* (RPN-750, RPN-734) (Table 1).

Discussion

A total of 92 patients, previously diagnosed clinically with IRD, were analyzed by two IRD-custom panels. These gene panels allowed us to find a genetic diagnosis in 53 patients of 52 different families (Table 1), with a diagnosis ratio of 57.6%, which is within the average of other studies (50-70%) (Ellingford et al., 2016; Di Resta et al., 2018; Wang et al., 2018; Zenteno et al., 2020; Jespersgaard et al., 2019).

In recent years, several deep-intronic mutations have been described as pathogenic due to their effect in the splicing process by leading to the introduction of a pseudoexon (PE) in the coding sequence (Vaché et al., 2012; Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015, 2019; Liquori et al., 2016; Baux et al., 2017; Fadaie et al., 2019; Khan et al., 2019; Sangermano et al., 2019). The inclusion of these regions in present study allowed us

to detect two deep intronic variants and therefore solve the molecular diagnosis in two more families. The variant *CEP290*: c.2991+1655A>G, detected in patient RPN-750, is one of the most prevalent in Leber congenital amaurosis (LCA) associated with *CEP290* (Den Hollander et al., 2006; Coppieters et al., 2010). Coppieters *et al* reported this variant with a frequency of 49% in *CEP290*-associated LCA cases. Also, we identified the deep-intronic variant c.4539+2064C>T in *ABCA4*. Deep-intronic variants in *ABCA4* were reported to be involved in 2.1-17.9% of STGD cohorts (Braun et al., 2013; Zernant et al., 2014; Bauwens et al., 2015; Bax et al., 2015; Zaneveld et al., 2015; Schulz et al., 2017); our finding of 7.1% of cases within STGD patients fits into the observed frequencies. Furthermore, the development of new therapeutical approaches, such as antisense oligonucleotides (AONs), entailed a new advantage for deep-intronic variants correction, which highlights the importance of detecting these changes for future treatments. In this sense, several studies have proven the therapeutic potential of AONs strategy and a phase I/II clinical trial for patients harboring the *CEP290* c.2991+1655A>G mutation using this type of therapy was conducted (Burke et al., 2012; Slijkerman et al., 2016; Duijkers et al., 2018; Cideciyan et al., 2019; Garanto et al., 2019; Sangermano et al., 2019; Xue and MacLaren, 2020).

In our study, *ABCA4* and *USH2A* are the most frequent mutated-genes, similar to other studies (Bernardis et al., 2016; Ezquerra-Inchausti et al., 2018; Jespersgaard et al., 2019). In *ABCA4*, 10% of alleles are reported to be complex (Shroyer et al., 2001; Zhang et al., 2015b), a value that increased to 25% in our results. Moreover, we reported the variant c.3386G>T(p.Arg1129Leu) in *ABCA4* in the 37.5% of resolved patients with mutations in this gene (Table 1), which was not surprising because this variant appears to be very frequent in the Spanish population as we can observe in a study performed by Del Pozo Valero *et al.*(Del Pozo-Valero et al., 2020). On the other hand, variants with an allelic frequency higher than 1% (MAF<0.01) are usually ruled out as the cause of disease in prioritization analyses, however, in some cases, the frequency of some variants, although >1%, is higher in patients than in healthy individuals, suggesting they are causal for the disease. Examples of this have been reported for *ABCA4.* For instance, the variant c.5603A>T (p.Asn1868Ile) was presented in STGD patients four times more frequently than expected (Zernant et al., 2017), and it is a disease-causing variant in the 5% of patients when it is in trans with a severe allele in

ABCA4. This variant was initially considered benign, however, recent studies consider it pathogenic with reduced penetrance (Zernant et al., 2017; Cremers et al., 2018; Runhart et al., 2018). For autosomal dominant cases, *PRPH2* was found to be responsible for 14.8% of the resolved cases (Table 1), whilst a prevalence of 10.3% in *PRPH2* was the highest obtained thus far (Manes et al., 2015).

Concerning to patients who carry mutations in more than one IRD gene (Table 1), It is estimated that 2.7 billion individuals worldwide (36%) are carriers of an IRD disease-causing mutation, whereas 5.5 million are expected to be affected (Hanany et al., 2020). In this study, we diagnosed patient RPN-670, who was carrier of disease-causing variants in *RP1, PRPH2* and *USH2A* (Table 1). Despite the fact that any relative displays any symptoms, we cannot confirm that *PRPH2* and *RP1* variants are *de novo*, as DNA from family members was not available for segregation analysis. Incomplete penetrance can also be suggested, since it has been described for patients carrying pathogenic variants in *PRPH2* and *RP1* (Dietrich, 2002; Boon et al., 2008; Thiadens et al., 2012; Coco-Martin et al., 2020). It is important to remark that the fact of being carrier of pathogenic variants in more than one gene can have a major impact on the reproductive choices for IRD patients, as well as impact their eligibility for gene-specific genetic therapies. This finding outstands the significance of achieving an exhaustive diagnosis.

The *OTX2* gene is associated with syndromic diseases, such as microphthalmia and retinal degeneration with or without pituitary dysfunction (OMIM: 610125; 610125). In this study we also identified a nonsense variant in the *OTX2* gene in a family (FRPN-300) (Table 1) with an autosomal dominant mode of inheritance but only retinal symptoms. Similarly, two autosomal dominant families with heterozygous pathogenic variants in *OTX2* with only retinal degeneration patterns were previously reported (Vincent et al., 2014). Eleven truncating and two missense pathogenic variants have been described in this gene (LOVD accessed 07 April 2021) and both type of variants have been associated with both syndromic and non-syndromic cases (Vincent et al., 2014; Slavotinek et al., 2015; Ellingford et al., 2016; Wang et al., 2016; Bryant et al., 2017; Patel et al., 2018; Sanchez-Navarro et al., 2018). Thus, a correlation between the type of mutation and the clinical diagnosis cannot be assessed.

A clear difference of the use of PV1 or PV2 concerning the solved cases (57.1% with the PV1 and 58.6% with PV2) did not exist. Strikingly, the rate of partially solved cases is almost the half for the PV1 (11.1%) when compared to the PV2 (19.35%). It would be expected that the number of partially solved cases would be lower with a panel that includes all the deep-intronic mutations reported to date for *ABCA4* and *USH2A*. In our opinion, this could be due to the lower sample size analyzed with the PV2.

In general, custom panel designs remain an excellent option for the genetic diagnosis of IRD. However, due to the rapid increase of the amount of genes involved, some groups prefer to use alternative approaches, such as WES (Lee et al., 2015; Riera et al., 2017; Zhang et al., 2018). Both sequencing strategies have pros and cons. A specific panel of genes will allow the study of known IRD at a high time/effectiveness ratio, regardless of the clinical diagnosis, which is an advantage in those cases in which the clinical diagnosis is not well defined or overlaps between different clinical entities. Furthermore, it allows not only the possibility of including a greater number of probes in repetitive regions such as ORF15 of *RPGR* gene, which is highly involved in X-linked IRD cases (Vervoort et al., 2000; Megaw et al., 2015; Chiang et al., 2018; Charng et al., 2019), but also reported deep-intronic pathogenic variants (González-del Pozo et al., 2018; Di Scipio et al., 2020). On the other hand, WES make possible to find mutations in novel IRD-related genes without updating the panel, in low prevalent genes not included for the sake of increasing the depth of coverage or to identify novel IRD genes. The price reduction for high throughput sequencing has made the costs between panels and WES quite even, so, it does not make the difference between panels and WES. Taking this into account, the choice between both depends on the preferences of the clinician/researcher in terms of comprehensiveness, accuracy, and time consumption.

Currently, there is not a significant difference between panels and WES concerning the diagnostic rates, as WES rate did not reach >70% (Lee et al., 2015; Riera et al., 2017; Wang et al., 2018b; Zhang et al., 2018; Liu et al., 2020). Therefore, further studies including introns, other non-coding regions and epigenetics are needed to achieve a comprehensive molecular diagnosis of IRD.

Conflicts of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Author Contributions

Conceptualization, Aller Elena, Millán José-María and García-García Gema; Methodology, García-Bohórquez Belén, Rodríguez-Muñoz Ana; Resources, García-Bohórquez Belén, Rodriguez-Muñoz Ana, Aller Elena, Jaijo Teresa; Writing—Original Draft Preparation, García-Bohórquez Belén; Writing—Review&Editing, García-Bohórquez Belén, Rodriguez-Muñoz Ana, Aller Elena, Jaijo Teresa, García-García Gema, Millán José-María; Supervision, García-García Gema, Millán José-María; Funding Acquisition, Millán José-María. All authors have read and agreed to the published version of the manuscript.

Funding

This study has been funded by the Project PI19/0330. Health Research Institute Carlos III (ISCIII; Spanish Ministry of Health and Innovation) and the Regional Government of the Valencian Community (PROMETEU/2018/135), partially supported the study, as well as partial support from the European Regional Development Fund (ERDF). ARM is recipient of a Rio Hortega contract (CM18/00199) from the ISCIII, BGB is recipient of a predoctoral contract (ACIF/2019/252) from the Government of the Valencian Community and GGG has a postdoctoral contact from CIBERER.

Acknowledgments

We sincerely acknowledge the patients for their voluntary participation and the Association Retina-Comunidad Valenciana.

Additional Information

Supplementary information from this paper can be found at https://doi.org/10.3389/fcell.2021.645600 and in the Appendix section of this thesis document.

CHAPTER II

CHAPTER II

EXPLORING NON-CODING VARIANTS AND EVALUATION OF ANTISENSE OLIGONUCLEOTIDES FOR SPLICING REDIRECTION IN USHER SYNDROME

Belén García-Bohórquez[1,2,3]; Pilar Barberán-Martínez[1,3]; Elena Aller[1,2,3,4]; Teresa Jaijo[1,2,3,4]; Pablo Mín-guez[2,5,6]; Cristina Rodilla[2,5]; Lidia Fernandez-Caballero[2,5]; Fiona Blanco-Kelly[2,5]; Carmen Ayuso[2,5]; Alba Sanchís-Juan[7,8,9]; Sanne Broekman[10]; Erik de Vrieze[10]; Erwin Van Wijk[10]; Gema García-García[1,2,3]; José M. Millán[1,2,3,4].

[1]Molecular, Cellular and Genomics Biomedicine, Health Research Institute La Fe, Valencia, Spain; [2]Center for Biomedical Network Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III, 28029 Madrid, Spain; [3]Joint Unit CIPF-IIS La Fe Molecular, Cellular and Genomic Biomedicine, Valencia, Spain; [4]University and Polytechnic La Fe Hospital of Valencia, Valencia, Spain; [5]Department of Genetics & Genomics, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz University Hospital, Universidad Autónoma de Madrid (IIS-FJD, UAM), 28040 Madrid, Spain; [7]Bioinformatics Unit, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz University Hospital, Universidad Autónoma de Madrid (IIS-FJD, UAM), 28040 Madrid, Spain; [7]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA; [8]Program in Medical and Population Genetics and Stanley Center for Psychiatric Research, Broad Insti-tute of MIT and Harvard, Cambridge, MA, USA; [9]Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA; [10]Department of Otorhinolaryngology, Radboud University Medical Center, Nijmegen, The Nether-lands.

Molecular Therapy: Nucleic Acids – Volume 35, 102374 (2024)

10.1016/j.omtn.2024.102374

(Adapted to meet the format of this document)

Abstract

Exploring non-coding regions is increasingly gaining importance in the diagnosis of inherited retinal dystrophies. Deep-intronic variants causing aberrant splicing have been identified, prompting the development of antisense oligonucleotides (ASOs) to modulate splicing. We performed a screening of five previously described *USH2A* deep-intronic variants among *USH2A* monoallelic patients with Usher syndrome (USH) or isolated retinitis pigmentosa. Sequencing of entire *USH2A* or USH genes was then conducted in unresolved or newly monoallelic cases. The splicing impact of identified variants was assessed using minigene assays, and ASOs were designed to correct splicing. The screening allowed to diagnose the 30.95% of the studied patients. The sequencing of USH genes revealed 16 new variants predicted to affect splicing, with four confirmed to affect splicing through minigene assays. Two of them were unreported deep-intronic variants and predicted to include a pseudoexon in the pre-mRNA, and the other two could alter a regulatory cis-element. ASOs designed for three *USH2A* deep-intronic variants successfully redirected splicing *in vitro*. Our study demonstrates the improvement in genetic characterization of IRDs when analysing non-coding regions, highlighting that deep-intronic variants significantly contribute to *USH2A* pathogenicity. Furthermore, successful splicing modulation through ASOs highlights their therapeutic potential for patients carrying deep-intronic variants.

Introduction

Inherited retinal dystrophies (IRDs) are a group of rare disorders characterized by the progressive degeneration of the retina due to generally progressive photoreceptor or retinal pigment epithelium (RPE) cells death. Although IRDs are considered rare diseases, they can account for 20–25% of the blind working-age population with 2 million people affected worldwide (Heath Jeffery et al., 2021). IRDs are characterized by a high clinical and genetic heterogeneity, since pathogenic variants in 289 genes have been described to be causative (https://web.sph.uth.edu/RetNet/home.htm, Accessed 2nd of June 2024), and each clinical entity can display different levels of progression and severity of the associated clinical symptoms. Within IRDs, retinitis pigmentosa (RP) is the most frequent IRD with a prevalence of 1/4000 worldwide (Verbakel et al., 2018), and is caused by the initial degeneration of rods, followed by cone dysfunction and degeneration in advanced stages of the disease.

Besides being manifested as non-syndromic disorders, IRDs can also present as syndromic conditions, in which besides visual impairment also other clinical symptoms become apparent. Pathogenic variants in more than 200 different genes have been identified to be associated with syndromic forms of IRD (Tatour and Ben-Yosef, 2020). Particularly, RP can be associated with sensorineural hearing loss and balance impairments, known as Usher Syndrome (USH). This syndrome explains more than 50% of hereditary deaf-blindness cases with a prevalence ranging from 4 to 17 individuals per 100,000 (Espinós et al., 1998; Kimberling et al., 2010; Castiglione and Möller, 2022). Typically, Usher syndrome is classified into three types depending on the progression and severity of the symptoms and the age of onset. So far, pathogenic variants in 10 genes are known to underly USH (*USH1C, MYO7A, PCDH15, CDH23, USH1G, CIB2, USH2A, ADGRV1, WHRN* and *CLRN1*), although *CIB2* has been questioned as an USH associated gene (Booth et al., 2018). Recently, a new USH clinical type, named USH4, has been defined and it is related to mutations in the *ARSG* gene (Khateb et al., 2018; Abad-Morales et al., 2020; Fowler et al., 2021; Peter et al., 2021, 2022; Velde et al., 2022). Additionally, other genes have been reported in association deafness-blindness syndromes similar to USH (*CEP78*, *CEP250*), as *USH2A*-phenotype modifier (*PDZD7*) or result in a typical form of digenic inheritance with *ADGRV1* variants. (Ebermann et al., 2010; Khateb et al., 2014a; Tiwari et al., 2016; Fu et al., 2017). Among all the USH genes, pathogenic variants in *USH2A* are the most prevalent explaining 80-90% of USH2

cases (Whatley et al., 2020; Gao et al., 2021; Perea-Romero et al., 2021; Karali et al., 2022) and also the major cause of non-syndromic RP cases (nsRP; RP39) (Toualbi et al., 2020; Perea-Romero et al., 2021).

Traditionally, the study of coding regions through a panel of genes or whole exome sequencing (WES) has provided a diagnosis percentage of around 60% for IRD cases (Shen et al., 2022; Britten-Jones et al., 2023a). Therefore, to achieve the complete characterization for those cases apparently monoallelic for pathogenic variants in recessive genes or not solved is a current goal in genetic diagnosis. In recent years, the analysis of non-coding regions of the genome has allowed to widen the range of variant types with a pathogenic effect that can be identified, thus emphasizing the importance of considering these regions for patients' diagnosis (de Bruijn et al., 2020, 2021; Reurink et al., 2023).

In this line, it is well-known that deep-intronic variants are disease-causing in IRDs (Den Hollander et al., 2006; Frio et al., 2009; Littink et al., 2010; Webb et al., 2012; Braun et al., 2013). These variants promote the inclusion of a pseudoexon (PE) in the mRNA that lead to a truncated protein (Vaché et al., 2012; Liquori et al., 2016; Baux et al., 2017; Khan et al., 2017; Fadaie et al., 2021b; Mansard et al., 2021; Reurink et al., 2023). Remarkably, the deep-intronic variant c.7595-2144A>G in *USH2A* has been reported to be recurrent as it may derive from a common ancestor, similar to the two most common variants in this gene, c.2276G>T and c.2299delG (Vaché et al., 2012; Baux et al., 2014; Slijkerman et al., 2018a).

Since many deep-intronic variants have been described as being pathogenic, antisense oligonucleotides (ASOs) have been introduced as promising future therapeutic molecules to correct aberrant pre-mRNA splicing (Slijkerman et al., 2018a; Reurink et al., 2023). In addition, ASOs have been designed to induce the targeted in frame skipping of (combinations of) frequently mutated exons (Dulla et al., 2021; Schellens et al., 2022). The first ASO, QR-421a/ultevursen, designed to induce the skipping of *USH2A* exon 13, has already reached the clinical phase with promising outcomes.

In the present study, we performed a targeted screening of previously published deep-intronic *USH2A* variants in 42 clinically defined USH/nsRP patients, who were carriers of a single pathogenic variant in the *USH2A* gene. Afterwards, we studied the whole genomic

sequence of *USH2A* and/or all USH genes through a custom panel in monoallelic USH patients. Eventually, ASOs were designed to correct aberrant splicing caused by three identified deep-intronic variants that were shown to result in the inclusion of a pseudoexon. Assessment of the splicing redirection potential of the individual ASOs supported their therapeutic potential.

Results

Screening of five reported deep-intronic variants by Sanger sequencing in 41 patients harbouring a monoallelic *USH2A* variant completed the genetic diagnosis of 13 patients (30.95%) (table 1). Variant c.7595-2144A>G was identified in eight individuals (RP-1741 carried the c.9959-4159A>G and c.7595-2144A>G variants in heterozygosity). Variant c.14134-3169A>G was identified to be present in three patients and the variant c.9959-4159A>G was identified in two additional individuals.

Table 1. Reported *USH2A* deep-intronic variants detected by Sanger sequencing in the cohort.

Patient	Family	Dx	Gene	Allele 1	Sanger screening	Reference
RP-331	-	ARRP	*USH2A*	c.2276G>T; p.(Cys759Phe)	c.7595-2144A>G	(Dreyer et al., 2000) (Vaché et al., 2012)
RP-654	FRP-30	USH	*USH2A*	c.11146C>T; p.(Gln3716*)	c.7595-2144A>G	(Vaché et al., 2012)
RP-1647	FRP-417	ARRP	*USH2A*	c.2276G>T; p.(Cys759Phe)	c.7595-2144A>G	(Dreyer et al., 2000) (Vaché et al., 2012)
RP-1569	FRP-380	USH	*USH2A*	c.2299del; p.(Glu767Serfs*21)	c.7595-2144A>G	(Eudy et al., 1998) (Vaché et al., 2012)
RP-1595	FRP-389	USH	*USH2A*	c.10636G>A; p.(Gly3546Arg)	c.7595-2144A>G	(Vaché et al., 2012)
RP-1596				c.10636G>A; p.(Gly3546Arg)	c.7595-2144A>G	(Garcia-Garcia et al., 2011)
RP-1776	FRP-484	ARRP+ SNHL	*USH2A*	c.2276G>T; p.(Cys759Phe)	c.7595-2144A>G	(Vaché et al., 2012) (Garcia-Garcia et al., 2011)
RP-2055	FRP-619	USH	*USH2A*	c.8435_8438del; p.(Thr2812Metfs*17)	c.7595-2144A>G	(Aller et al., 2006) (Vaché et al., 2012)
RP-1741#	FRP-460	USH	*USH2A*	c.7595-2144A>G; p.(Lys2532Thrfs*56)	c.9959-4159A>G	(Vaché et al., 2012) (Liquori et al., 2016)
RP-1485	FRP-344	USH	*USH2A*	c.1214del; p.(Asn405Ilefs*3)	c.9959-4159A>G	(Bernal et al., 2005) (Liquori et al., 2016)
RP-1472	FRP-341	USH	*USH2A*	c.2299del; p.(Glu767Serfs*21)	c.14134-3169A>G	(Eudy et al., 1998) (Baux et al., 2017)

RP-1494	FRP-348	USH	*USH2A*	c.2299del; p.(Glu767Serfs*21)	c.14134-3169A>G	(Eudy et al., 1998) (Baux et al., 2017)
RP-2224	FRP-722	USH	*USH2A*	c.2431_2432del; p.(Lys811Aspfs*11)	c.14134-3169A>G	(Nájera et al., 2002) (Baux et al., 2017)

RP/RPN: Patient number; FRP/FRPN: Family number; Dx: Diagnosis; ARRP: Autosomal Recessive Retinitis Pigmentosa; USH: Usher Syndrome; SNHL: Sensorineural hearing loss; The "#" symbol refers to the NGS positive control.

Then, 27 patients with a monoallelic (likely) pathogenic variant in a USH gene were selected for targeted genome sequencing of all reported USH genes through a first custom panel (P1) containing the entire sequence of 13 USH genes and 2000 flanking bases of their UTR sequences (Table S1). Subsequently, a second custom panel (P2) that included all the genomic sequence of *USH2A* gene was sequenced in six monoallelic *USH2A* patients (Table S1). The 97% of the bases were sequenced with a depth coverage of ≥20x in both panel designs. Around 8000 variants per patient were identified by P1 and 2500 by P2. The sequencing of P1 allowed us to solve 3 cases and the genetic diagnosis was completed in a fourth case by the analysis of P2.

As a validation of the pipeline and filters applied for the analysis, and described in materials and methods section, the two previously detected deep-intronic variants c.9959-4159A>G and c.7595-2144A>G present in the RP-1741 case were correctly identified.

From the 32 monoallelic analysed cases, we selected a total of 16 variants with pathogenic scores according to splicing prediction tools and the cut-offs defined in materials and methods: 12 in *USH2A*, one in *CDH23*, one in *ADGRV1* and two in *USH1C* (table 2). Out of the 16 novel variants predicted to affect splicing, 11 deep-intronic variants, two intronic variants in close vicinity of an exon and four exonic variants were identified (table 2). The effect of the selected variants on splicing was studied using minigene splice assays and aberrant splicing was observed in four out of 16 variants (table 2): two deep-intronic variants in *USH2A* (c.8681+3960A>G and c.9958+3438A>G), one intronic variant upstream of *USH2A* exon 26 (c.5168-26A>C), and one variant in *USH2A* exon 19 (c.4106C>T) (Figure 1). Among the 16 variants analysed using minigene splice assays, variant c.1234G>A in the *USH1C* gene of patient RP-1222 was classified as variant of unknown significance when it was identified in previous studies. Nonetheless, as a potential splicing effect was predicted by Splice AI (score: 0.31) in this study, we decided to select it for functional assay. However,

the minigene analysis for this variant did not show any aberrant effect in splicing. All products shown in Figure 1 were confirmed by Sanger sequencing.

Concerning the Copy Number Variation (CNV) analysis, four rearrangements were identified (table 3) and two of them were validated using a second technique: one 6.2kb duplication in *USH2A* in patient RP-2159 by MLPA and one 2.5kb duplication in *CDH23* in patient RP-681 by CytoScan HD array. The 2.4kb deletion in *ADGRV1* and the 5kb deletion in *MYO7A* in patients RP-1496 and RP-2213, respectively, turned out to be false positive as results from qPCR showed no alteration in the copy number.

In summary, after these combined studies, a total of 19 index cases were completely characterized, 18 of them having a previous pathogenic variant identified in *USH2A* and one in *CDH23*.

Table 2. Predictions for candidate variants potentially altering splicing

Patient	Gene	Variant identified	regSNP - intron	NNSplice		MaxEnt			SpliceAI	SPiP interp.	SPiP % (rank)	VarSEAK	Minigene Effect
			Predict	WT	MUT	WT	MUT	Var (%)	Score			Class	
RP-2034	***USH2A***	**c.5168-26A>C**	**B**	**-**	**-**	**-**	**-**	**-**	**0.55 (AL)**	**BPA**	**43.04 (35.2 - 51.14)**	**1**	**ES**
RP-1496	*ADGRV1*	c.3443G>A	-	-	-	-	-	-	0.53 (AG)	NEP	03.72 (01.51 - 07.52)	1	NE
RP1950	***USH2A***	**c.4106C>T**	**-**	**-**	**-**	**-**	**-**	**-**	**0.31 (AL)**	**REA**	**54.00 (45.68 - 62.16)**	**1**	**ES + NAS**
		c.11549-5dup	-	-	-	8.57	2.16	-74.8 (AL)	-	NEP	01.85 (00.38 - 05.32)	1	NE
RP-1222	*USH1C*	c.1234G>A	-	-	-	-	-	-	0.31 (AL)/0.34 (DL)	REA	85.91 (79.27 - 91.06)	1	NE
RP-1036	***USH2A***	**c.8681+3960A>G**	**PD**	**-**	**0.99 (AG)**	**1.09**	**9.84**	**802.75 (AG)**	**0.98 (AG)**	**NEP**	**00 (00 - 00.92)**	**5**	**PEC**
RP-1600	*USH2A*	c.15298-1252T>G	B	-	-	-1.63	6.97	527.61 (AG)	-	NEP	00 (00 - 00.92)	1	NE
RP-1943	*USH2A*	c.6049+3895G>A	B	-	0.92 (AG)	2.44	4.93	102.05 (AG)	-	NEP	00 (00 - 00.92)	1	NE
RP-1455	*USH2A*	c.5299-2503A>G	B	-	0.58 (AG)	-3.79	4.96	230.87 (AG)	-	NEP	00.25 (00.01 - 01.39)	1	NE
RP-1994	*USH2A*	c.11389+2566A>G	PD	-	0.73 (AG)	-0.93	7.82	940.86 (AG)	-	NEP	00 (00 - 00.92)	1	NE
		c.11048-2124A>G	PD	-	0.81 (AG)	-2.14	6.61	408.88 (AG)	-	NEP	00 (00 - 00.92)	4	NE
RP-2264	*USH2A*	c.7120+4268A>G	D	-	0.65 (DG)	-4.04	4.14	202.48 (DG)	-	NEP	00 (00 - 00.92)	1	NE
RP-2239	*USH2A*	c.7300+8957A>C	B	0.46	0.74(AG)	6.22	5.44	19.61 (AG)	-	NEP	00 (00 - 00.92)	1	NE
RP-2268	*USH2A*	c.6806-810A>G	D	-	0.92 (DG)	-0.37	7.81	2210.81 (DG)	-	NEP	00.5 (00.06 - 01.78)	3	NE
RP-1815	***USH2A***	**c.9958+3438A>G**	**PD**	**-**	**0.98 (AG)**	**1.66**	**10.41**	**527.11 (AG)**	**0.97 (AG)**	**NEP**	**00 (00 - 00.92)**	**3**	**PEC**
		c.4628-27169C>G	D	-	0.57 (DG)	-1.77	6.49	466.67 (DG)	-	NEP	00 (00 - 00.92)	2	NE

For regSNP-intron, NNSplice and SpliceAI predictors, the scores are given within 0 (benign) to 1 (pathogenic). For VarSEAK the score ranks from 1 (benign) to 5 (pathogenic). Variants highlighted in bold showed an aberrant effect in splicing in the minigene assays. RP: Patient number; NT: Nucleotide; Predict.: Prediction; B: Benign; PD: Probably Damaging; TPR: True Positive Ratio; FPR: False Positive Ratio; WT: Wild Type; MUT: Mutant; AG: Acceptor Gain; DG: Donor Gain; Var: Variation; AL: Acceptor Loss; DL: Donor loss; Interp.: Interpretation; BPA: Branch Point Alteration; NEP: No Effect Predicted; REA: Regulatory Element Alteration; ES: Exon Skipping; NE: No Effect; NAS: New Acceptor Site; PEC: Pseudoexon Creation.

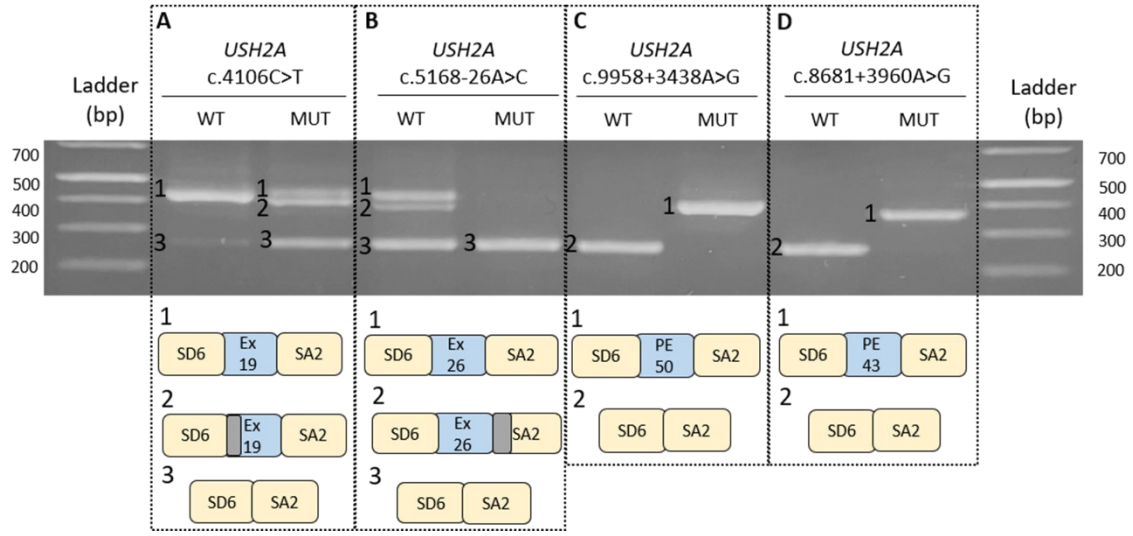


Figure 1. Analyses of potential splicing modulating variants in *USH2A* using minigene splice assays. SD6 and SA2 are the constitutive exons of pSPL3 plasmid and in blue boxes exons (ex) and pseudoex-ons (PEs) are depicted. The first three variants present a(n) (partial) exon-skipping effect, while the last two have included a PE in their sequence. Boxes in grey represents regions that have not been included in the sequence as an alternative splicing has occurred.

Table 3. Copy-number variants identified.

Patient	Family	Gene	Transcript	Genomic region	Location	Type	Validadtion Method
RP-1496	FRP-350	*ADGRV1*	NM_032119	89909918-89912318	int1-int2	DEL	qPCR
RP-2213	FRP-719	*MYO7A*	NM_000260	76862211-76867161	int4-int5	DEL	qPCR
RP-681	FRP-49	*CDH23*	NM_052836	73335893-73360492	int8-int9	DUP	Array HD
RP-2159	FRP-675	*USH2A*	NM_206933	215796232-216596790	int37-int43	DUP	MLPA

"int": intron DEL: deletion; DUP: duplication

USH2A: c.4106C>T

This study allowed us to re-evaluate the pathogenic effect for the variant c.4106C>T, identified in patient RP-1950 monoallelic for the c.2299del (p.Glu767Serfs*21) variant. A score of 0.31 from SpliceAI predicted a splice acceptor loss, supported also by SPiP with a risk of 54% of altering an exonic splicing regulatory element (rank from 45.68% to 62.16%). Assessment of this variant in a minigene splice assay showed that this variant resulted in the (partial) skipping of exon 19 (Figure 1A). In Figure 1A, fragment 1 corresponds to the wildtype situation whereas fragment 2 represents a partial skipping of exon 19 as the first 27 nucleotides are missing since alternative splice acceptor/donor site was used. This event is in frame and no premature stop codon was predicted. Fragment 3 shows the complete

skipping of exon 19, where only constitutive plasmid exons are included in the cDNA. This event is predicted to create a stop codon 17 nucleotides downstream the variant and could generate a truncating protein. However, premature termination codons (PTCs) in USH genes are more likely to cause loss of transcripts due to nonsense mediated decay (NMD). Fragment 1 correspond to approximately the 32.8% of total transcript count, while fragments 2 and 3 account for the 67.2%. Segregation analysis demonstrated that variant c.4106C>T was in *trans* with mutation c.2299del, thus it co-segregates with the disease (Figure S1).

***USH2A*: c.5168-26A>C**

Variant c.5168-26A>C, which flanks exon 26 of USH2A, was identified in patient RP-2034. This patient presented a clinical USH2 diagnosis and was monoallelic for the USH2A c.2299del (p.Glu767Serfs*21) variant. The prediction score from SpliceAI suggested a splice acceptor loss (0.55), while SpiP showed a potential branch point alteration with a score of 43.04 % (35.2% - 51.14%) (table 2). In order to test the effect on splicing, a minigene construct was designed. The cDNA analysis showed a pathogenic effect for the mutant construct as a complete exon 26 skipping effect is observed (Figure 1B). In the wild-type construct, we observe three different bands, with bands 1 and 2 exclusively present in the wild-type context. Fragment 1 corresponds to the recognition of exon 26 and fragment 2 results from the lack of recognition of the first 43 bp of a pSPL3 constitutive exon (Figure 1B). Eventually, fragment 3 corresponds to the coding sequence from the pSPL3 constitutive exons, which means that the exon 26 of USH2A is not recognized. The in silico analysis predicted a premature stop codon to be created 92 nucleotides downstream exon 26 resulting in a truncating protein. This exon skipping event is present in both the wild-type and mutant constructs, but the exon skipping band accounts for 100% of the total transcript in the mutant minigene, compared to 34.6% for the wild-type minigene. Segregation analysis confirmed that this variant is present in trans with the c.2299del and co-segregates with the disease (Figure S1).

***USH2A*: c.9958+3438A>G (PE50)**

The variant c.9958+3438A>G was identified in patient RP-1815, who was diagnosed of USH2 and monoallelic for the c.908G>A (p.Arg303His) variant in the *USH2A* gene. The splicing predictors' scores supported the variant pathogenicity as a result of an acceptor site gain ("tgcAGtga"). Alternative downstream donor sites were evaluated with NNSplice and SpliceAI 500, so we could identify a potential candidate donor site "caagGTaatg" (NNSplice: 0.98; SpliceAI 500: 0.85), which would lead to the inclusion of a PE of 144bp. A fragment of 734bp including the potential PE with ~250 nucleotides of flanking up- and downstream intronic sequences was cloned into the pSPL3 plasmid. cDNA analysis after expression study showed a clear splicing effect where the predicted 144bp pseudoexon was introduced in all of the detected transcripts, in the absence of any additional fragments (Figure 1C). Segregation analysis confirmed that both variants (c.9958+3438A>G and c.908G>A) are located on different alleles (Figure S1), thus supporting their pathogenicity as it co-segregates with the disease.

***USH2A*: c.8681+3960A>G (PE43)**

Deep-intronic variant c.8681+3960A>G was identified in patient RP-1036, who was clinically diagnosed with USH. RP-1036 harboured variant c.2809+1G>A in heterozygosity in *USH2A*. All splicing analysis tools revealed pathogenic scores (NNSplice: 0.99; MaxEnt: 802.75% variation; SpliceAI: 0.98; VarSeak: 5), and all of them concurred with an acceptor site gain effect. Additionally, NNSplice and SpliceAI 500 tools were used to search for alternative donor sites that could promote the inclusion of a PE in the cDNA. Both predictors revealed the alternative donor site "gaggGTaaga" (NNSplice: 1.00; SpliceAI 500: 0.82), which was 111 nucleotides away from the new acceptor site. Considering this region as a possible PE, a minigene was designed including 738 bp of *USH2A* intron 43. Results from minigene assay revealed a full splicing effect in the mutant construct with the insertion of 112 intronic bp in the coding sequence (Figure 1D). The inclusion of this PE would promote the creation of a premature stop codon in the mRNA. DNA from relatives was not available for segregation analysis, so it remains unclear if the c.8681+3960A>G variant resides on the same allele as the previously identified canonical splice site variant c.2809+1G>A.

ASOs splicing redirection assay

The PE inclusion caused by the two deep-intronic variants identified in this study (c.9958+3438A>G and c.8681+3960A>G), and the previously described variant in intron 64 (c.14134-3169A>G), drove us to design ASOs with the aim of redirecting aberrant splicing thereby preventing the PE inclusion in the coding sequence. Two ASOs were designed targeting the different PE regions, except for PE43 in which only one matched all requirements to be design, and their efficacity was evaluated using minigene splice assays (table S2). The 2 nucleotides mismatch ASO (mmASO) used as control indicate the specificity of the ASOs as it is not able to redirect splicing.

Related to PE64, at a dose of 20 nM, ASO1-PE64 redirected the aberrant splicing, and it can also be observed how from 10 to 20 nM the fragments intensities show that the minimum efficient dose is 20 nM (Figure 2a). For ASO2-PE64, 5 nM is still effective for splicing redirecting (Figure 2a). Concerning PE50, caused by c.9958+3438A>G, ASO1-PE50 was capable of redirecting the splicing at a dose of 100 nM (Figure 2b), while a combination of ASO1-PE50 and ASO7-PE50 was efficient enough at 20 nM (10 nM of each ASO), showing a synergic effect (Figure 2b). Results in PE43, due to c.8681+3960A>G variant, ASO1-PE43 showed great redirection of the splicing process with a minimum efficient dose of 50 nM (Figure 2c). The results obtained from the two mismatch ASO used as a control (mmASO), showed a full specificity of all designed ASOs for each PE (Figure 2).

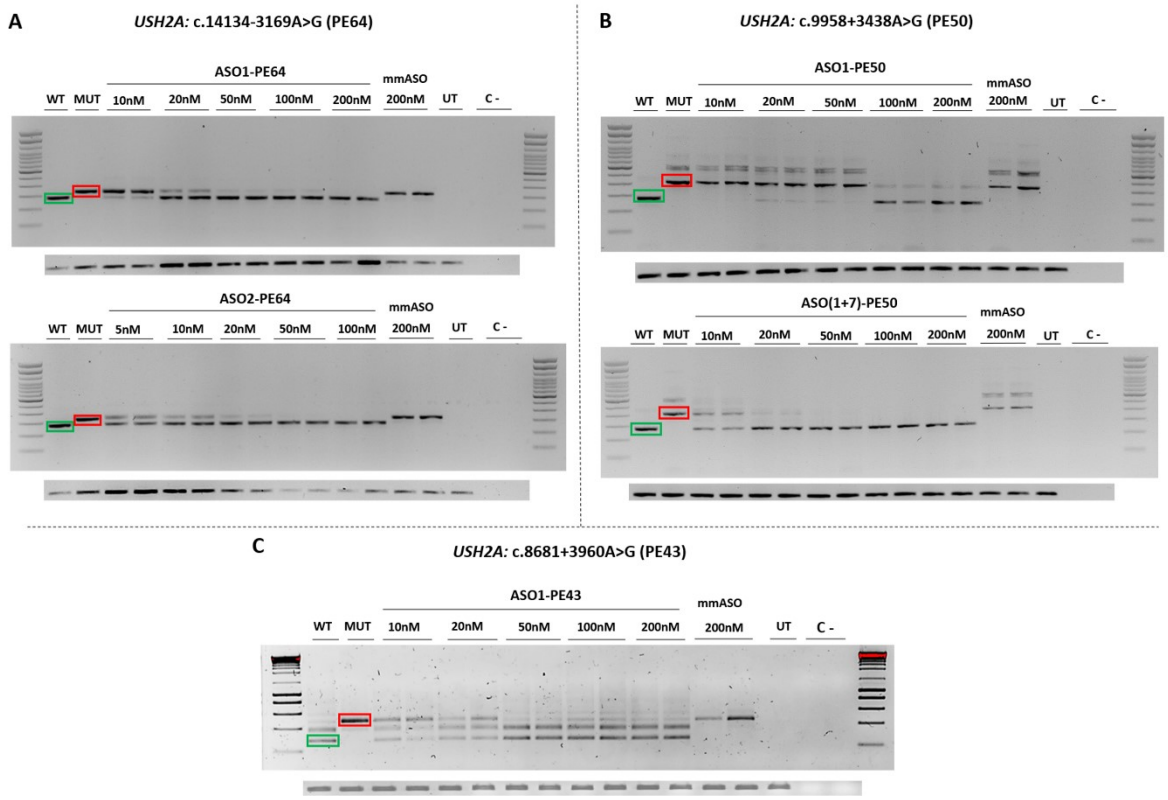


Figure 2. ASO-induced redirection of aberrant splicing caused by deep-intronic variants. Each section represents WT (green) and MUT (red) fragments and the different treatment dose. The lower panels in every section are the GAPDH loading control in each experiment. For pseudoexon 64 (A), two different ASO were tested separately. For PE50 (B), ASO7-PE50 was tested together with ASO1-PE50 due to the lower efficiency it showed individually and the concentrations shown for ASO(1+7)-PE50 refer to the final concentration of both ASO. For PE43 (C), only ASO1-PE43 was tested as the sequence did not agree with all the requirements. Differences between wildtype and mutant fragments of PE50 and PE43 constructs in Figure 1 compared to Figure 2 can be observed due to the use of different cloning splice vector for each experiment WT: Wild type; MUT: Mutant; UT: Untreated; C-: Negative control.

Discussion

The diagnosis of both IRD and USH patients has commonly been accomplished by targeted NGS through custom panels and whole exome sequencing (WES). Nevertheless, these approaches are focused on the coding sequence, which means that the role of regulatory and deep-intronic regions in the pathogenicity of these diseases is overlooked. Lately, more studies progressively emphasize the importance of non-coding regions when searching for pathogenic variants (Chen et al., 2014; Coppieters et al., 2015; de Sousa Dias et al., 2015; Radziwon et al., 2017; Varela et al., 2022; Reurink et al., 2023; Yang et al., 2023). This has

already resulted in the identification of several deep-intronic variants in *USH2A* and *CLRN1* that result in the inclusion of a pseudoexon (PE) in the mRNA (Vaché et al., 2012; Liquori et al., 2016; Baux et al., 2017; Khan et al., 2017; Reurink et al., 2023).

After the study of our USH patients by either custom gene panels or WES, 10-20% of analysed individuals appeared to be monoallelic for a pathogenic variant in one of the USH genes, in the absence of a second pathogenic variant (Aparisi et al., 2014; García-garcía et al., 2014; Fuster-García et al., 2018, 2019; Rodríguez-Muñoz et al., 2020; García Bohórquez et al., 2021). Motivated by this, we designed a custom panel including the whole genomic sequence of the USH genes in order to identify the second causative variant in non-coding regions and so unravelling the genetic diagnosis in these cases.

It is remarkable how just the analysis of five deep-intronic previously reported variants in patients with monoallelic variants in the *USH2A* gene, allowed us to complete the diagnosis in over 30% of the patients studied (table 1). As expected, since it is one of the most frequent pathogenic *USH2A* variants found in the Mediterranean region, the intronic variant c.7595-2144A>G was also common in our cohort (Steele-Stallard et al., 2013; Baux et al., 2014; Krawitz et al., 2014; Slijkerman et al., 2016), found in 61.5% of the solved patients of the screening. Even more, we identified a patient carrying two different deep-intronic *USH2A* variants (c.7595-2144A>G and c.9959-4159A>G) both in heterozygosity, highlighting the importance of screening the non-coding regions, as classical approaches would have completely missed these variants.

The combination of whole USH genes sequencing along with *in silico* and *in vitro* studies, through minigene assays due to the low expression of USH genes in peripheral blood, allowed us to confirm aberrant splicing induced by four different variants.

On one hand, it has allowed us to identify two new deep-intronic variants in two different patients and a PE inclusion was shown in minigene assays (c.8681+3960A>G and c.9958+3438A>G). On the other hand, an effect in cDNA was validated in two variants located in splicing regulatory elements of *USH2A* (c.5168-26A>C; c.4106C>T). Variant c.4106C>T in *USH2A* was previously described (Cremers et al., 2006), but there was no evidence of its impact in splicing. In addition to the splicing predictions reported in this

study, our minigene assay showed an exon skipping together with the creation of a new acceptor site. Nevertheless, wildtype fragment is still present in the mutant context at 32.8%. Thus, we cannot confirm that this variant is clearly pathogenic as 20% of wildtype *USH2A* transcript has been reported to be enough for correct protein function (Dulla et al., 2021). It should be taken into account that the outcomes observed by minigenes may differ from the *in vivo* situation since minigene assay is an *in vitro* model that present some limitations. Minigenes constructs incorporate only a part of the genomic sequence, which does not capture the full complexity of regulatory elements. Moreover, splicing can be different in different cell types due to the presence of cell-type specific splice factors. In this line, complementary studies using patient-derived cells, such as photoreceptor precursor cells differentiated from patient-derived iPSCs, are crucial for obtaining a more accurate understanding of splicing events and their pathogenic consequences (Buskin et al., 2018; Zhang et al., 2020; Reurink et al., 2023). Despite their limitations, minigene studies remain a very useful and practical tool for assessing potential splicing alterations in pathologies where the genes have low or no expression in accessible tissues.

Predicting the effect of nucleotide changes on splicing is challenging because this is a complex process regulated by numerous factors. Various studies have analysed the sensitivity and specificity of different splicing predictors and cut-off values (Jaganathan et al., 2019; Riepe et al., 2021; Rowlands et al., 2021). Recently, Reurink and colleagues suggested to use a SpliceAI score of 0.1 for deep intronic and canonical splice site variants, and a more restrictive cut-off of ≥ 0.2 for exonic changes in non-canonical positions (Reurink et al., 2023). In the current study, we selected variants with a score ≥ 0.2 for SpliceAI (Rowlands et al., 2021; Walker et al., 2023) and/or with a variation score >10% for MaxEnt (Liquori et al., 2016). However, only four out of the 16 selected candidate variants (25%) in our study showed a deleterious effect on splicing, which led us to recommend a more stringent pipeline. Of the four variants whose splicing effect was functionally validated, all were predicted by SpliceAI. However, two of them would have been missed based solely on MaxEnt predictions. Among the 12 selected variants where an effect was not confirmed in the minigene assay, the majority were selected based on MaxEnt criteria. If we had only considered variants with a SpliceAI score ≥ 0.2, only 6 variants would have been selected for validation, and functional aberrant splicing effects would have been confirmed in 4 of

them (67%). These data suggest that SpliceAI is consolidating itself as one of the best tools for predicting potential aberrant effects on splicing. In contrast, MaxEnt predictions alone yielded several false positives, so we would recommend using it in combination with other more accurate predictors.

Variants in UTR regions have been already described in many genes with different effects on gene expression. However, predicting the effects of alterations in these regions remains challenging and cannot be done in a simple and automated way (Fishilevich et al., 2017; Cherry et al., 2020). Additionally, functional assays are still necessary to assess the impact of these variants. Recently, Dueñas Rey *et al*. have developed a strategy for the prioritization and evaluation of 5'UTR variants in combination with funtional studies (Dueñas Rey et al., 2024). They identified several variants in 5'UTR predicted to have different functional consequences, highlighting the contribution of non-coding regions in IRDs. Considering the transcriptional complexity of the retina, more studies using Chip-seq or RNA-seq techniques are needed to a better identification and definition of the regulatory regions (Liu et al., 2021; Nishi et al., 2024; Santana et al., 2024; Zhang et al., 2024).

Several USH genes contain repetitive regions, which resulted in a lower capture efficiency. Therefore, we cannot rule out that variants in these poorly covered regions might have been missed due to technical limitations. Currently, a proper alternative would be whole genome sequencing (WGS), as it helps overcoming these issues associated with sequence capture and provides more uniform sequencing (Mazzarotto et al., 2020; Reurink et al., 2023). In a study carried out by Reurink *et al*. in 2023, 49% of patients that previously underwent WES analysis (Reurink et al., 2023), were solved by the identification of either a deep-intronic variant or SVs (Structural Variants) through WGS. This study also remarks the relevance of SVs in IRDs' patients (Carss et al., 2017; Van Schil et al., 2018; de Bruijn et al., 2020). In our study, four putative CNVs were identified using a specific bioinformatic pipeline, and only two of them were validated by different approaches. However, the complexity of some types of SVs makes their alterations difficult to detect using short reads sequencing technologies. To solve this limitation, long-reads sequencing approaches or optical genome mapping are yielding promising results in the identification of SV and other complex alterations such short repeat tandem (Fadaie et al., 2021a; de Bruijn et al., 2023).

In order to redirect aberrant splicing caused by identified variants and prevent the inclusion of PEs in the coding sequence, ASOs were designed for the *USH2A* deep-intronic variants c.9958+3438A>G and c.8681+3960A>G identified in this study, as well as for the previously identified pathogenic *USH2A* variant c.14134-3169A>G (Baux et al., 2017). We obtained minimum efficient doses of 20nM (ASO1-PE64) and 5nM (ASO2-PE64) for c.14134-3169A>G; 100nM (ASO1-PE50) and 20nM (ASO(1+7)-PE50) for c.9958+3438A>G; 50nM (ASO1-PE43) for c.8681+3960A>G. This results match previous data from deep-intronic variants in *USH2A* (Slijkerman et al., 2016; Reurink et al., 2023), and highlight the effectiveness of ASOs to modulate splicing through PE inclusion blocking. In total, every minimum efficient dose was below 200nM, which was the maximum dose established for each experiment and which reassures their therapeutic potential.

Antisense oligonucleotides have a high potential to modulate gene expression (Stephenson and Zamecnik, 1978; McClements et al., 2019; Merkle et al., 2019). Genetic approaches based on ASOs present certain advantages compared to other genetic intervention strategies such as gene augmentation: they can only temporarily interfere in the mRNA (so there is not the risk of permanent off-target effect at genomic level), endogenous gene expression levels are not modified and do not alter the transcript and protein isoform landscape of the target genes. Moreover, the small size of ASOs allow the intravitreal delivery with an effective reach of all retinal cells with low inflammatory effects instead of the use of AAVs that requires surgical subretinal techniques for delivery and only a fraction of the retina will be reached resulting in a lower efficacy. Particularly for splicing correction, ASOs do not rely on preclinical animal testing, which is a requisite for exon-skipping approaches in which the resulted proteins function should be proofed, for instance "ultervursen" for *USH2A* exon 13 skipping (Dulla et al., 2021; Girach et al., 2022). This does not excuse the need of a dose finding study for ASOs modulating splicing at least with an *in vitro* model such as 3D organoids, and also toxicity assays are required mostly in vertebrates (sometimes in non-human primates) (Kurzawa-Akanbi et al., 2024). Furthermore, strategies improving the ASOs targeting of specific tissues and cell types has also been described (Havens and Hastings, 2016; Khvorova and Watts, 2017; Bennett, 2019; Seth et al., 2019).

To overcome the instability *in vivo* due to nuclease activity, in the second generation ASOs some improvements have been done, such as including a phosphorothioate backbone and a sugar modification (2'-O-methoxyethyl; MOE) (Bennett and Swayze, 2010; Yu et al., 2013). It has been reported that second generation ASOs allow an increased *in vivo* half-life times, being around 200 days (Dulla et al., 2018; Girach et al., 2022). Despite this, some disadvantages involve the need of recurrent intravitreal injections. The use of AAV could be a good solution as its potential has been previously shown in cellular and animal models of Leber Congenital Amaurosis associated with a deep-intronic *CEP290* mutation (Garanto et al., 2016). However, since viral vectors do not produce and deliver second generation ASOs, the specificity and efficacy of the treatments would be affected. An alternative for this limitation is an strategy based on the enclosure of ASOs in a U7snRNA complex followed by an AAV-based delivery, which turned out to improve the long-term efficacy of the treatment (Aupy et al., 2020).

Regarding this, clinical trials based on the use of ASOs for treating IRD caused by a deep-intronic variant in *CEP290* as well as by variants harboured in exon 13 of *USH2A* were initiated (ClinicalTrials.gov: NCT03780257, NCT03140969, NCT03913143) (Cideciyan et al., 2019). However, after initially being discontinued by ProQR Therapeutics, Théa (https://www.laboratoires-thea.com) is currently preparing phase 3 clinical trials for both programs. In addition, an ASO-based allele specific mRNA knockdown/degradation approach for dominantly inherited RP caused by pathogenic variants in *RHO* is underway in a clinical trial (NCT04123626). Also two different approved treatments for SMA (Spinal Muscular Atrophy) and DMD (Duchenne muscular dystrophy) based on ASOs that modulate the splicing process (Finkel et al., 2017; Li et al., 2018). Although ASO-based splicing modulation is mostly a mutation-specific approach, mutational hotspots could also be a great target to focus on.

In summary, this study demonstrates the importance of the analysis of non-coding regions in order to solve some of the cases that are not diagnosed after gene-panels, clinical exome or WES. The molecular diagnosis is essential for the eligibility of receiving a gene- or mutation-based treatments. To date between 30-40% of patients still do not receive a conclusive genetic diagnosis, which in part could be explained by the presence of

pathogenic variants in regions that are (technically) difficult to sequence (for instance ORF15 in *RPGR*). However, the presence of variants in non-coding regions (introns or cis-regulatory elements), the insertion of mobile elements and even epigenetic modifications could underlie the lack of diagnosis in the major part of these unexplained cases. Identification of (deep-)intronic variants that affect pre-mRNA splicing opens up future treatment options using antisense technology.

Materials and methods

Patient selection

Fifty-nine patients from 58 different families (table S1) harbouring a pathogenic variant in a USH gene and in whom the coding regions of the genes of interest had been previously studied, were selected (Aparisi et al., 2014; Fuster-García et al., 2018, 2019; Rodríguez-Muñoz et al., 2020; García Bohórquez et al., 2021) (Data not published). Their diagnosis varied from any of the USH subtypes (42/59) to autosomal recessive nsRP (16/59) (table S1). DNA from peripheral blood was isolated from all of them by automatic extraction with QIAsymphony (QIAGEN).

Different ophthalmologic tests were carried out in each patient, such as OCT, eye fundus, ERG, evoked potentials. The Hospital La Fe Ethics and Fundacion Jimenez Diaz-University Hospital Ethics Committees approved this study in agreement with the Declaration of Helsinki. Informed consent was signed by all patients and relatives who participated in the study.

Sanger screening of *USH2A* deep-intronic variants

We performed a Sanger screening of the five deep-intronic variants (c.7595-2144A>G; c.5573-834A>G; c.8845+628C>T; c.9959-4159A>G; c.14134-3169A>G) that were already described as pathogenic in *USH2A* gene in 41 carriers of one heterozygous mutation in this gene (Vaché et al., 2012; Liquori et al., 2016; Baux et al., 2017). We did not accomplish the screening in patients RP-2264 and RPN-803, who were previously sequenced with a custom

panel containing these 5 variants. The five regions were analysed as previously described (Vaché et al., 2012; Liquori et al., 2016; Baux et al., 2017).

Panels design and high-throughput sequencing

A first custom panel (P1) was designed including the whole genomic sequence of the 13 USH genes and 2000 flanking bases of UTR regions of all of them *(MYO7A, CDH23, PCDH15, USH1G, USH1C, CIB2, USH2A, ADGRV1, WHRN, CLRN1, HARS, CEP250, PDZD7*). The panel size encompasses 3,295.139 kb The library preparation was performed following the Kapa HyperPlus Workflow (Roche) and it was sequenced in a NextSeq platform (Illumina, San Diego, CA) in 300 cycles of 2x150 base pairs reads. The P1 was sequenced in 27 patients, of which 17 had a mutation in *USH2A*, three in *ADGRV1*, two in *CDH23*, two in *MYO7A*, two in *USH1C* and one in *PCDH15* (table S1). Patient RP-1741 was included as an internal control for panel sequencing. This case carried two heterozygous deep-intronic variants in *USH2A* gene (c.9959-4159A>G and c.14134-3169A>G).

A second panel (P2) was designed including the whole genome sequence of *USH2A*, since all remaining USH patients harboured one mutation in *USH2A*. Thus, a total of 779.928 kb was sequenced in six patients. The library was prepared following the SureSelectXT Target Enrichment System (Agilent Technologies) and sequenced in a MiSeq platform (Illumina, San Diego, CA) in 600 cycles of 2x300bp. The P2 was sequenced in six patients, all of them carrying a mutation in *USH2A* gene (table S1).

Sequencing data analysis

Quality control of reads was performed using FastQC (v0.11.4, https://github.com/s-andrews/FastQC) and samtools (v1.3.1) (Danecek et al., 2021). The reads were aligned against the GRCh38 human genome of reference using BWA aligner (v0.7.15-r1140) (Li and Durbin, 2009), and Single Nucleotide Variants (SNVs) and small insertions and deletions (indels) were called using strelka (v2.9.10) (Kim et al., 2018). Variants were annotated with Variant Effect Predictor (ensembl-vep v99.2) (McLaren et al., 2016), using MaxEnt and SpliceAI (v1.3, https://github.com/Illumina/SpliceAI).

For variant filtering, we selected variants with a MAF≤0.01 (Minor Allele Frequency) and those located in the target gene. Subsequently, variants with either a SpliceAI score ≥0.2 or a MaxEnt variation score >10% and/or a value score ≥3 for acceptor/donor gain predictions. Additional *in silico* analysis was performed using the splicing predictors NNSplice (https://www.fruitfly.org/seq_tools/splice.html), VarSeak (https://varseak.bio/), regSNP-intron (https://regsnps-intron.ccbb.iupui.edu/), and SPiP (available in https://mobidetails.iurc.montp.inserm.fr/MD/) to predict alterations of natural splice sites. Variants predicted to create a new splicing site were further investigated using SpliceAI 500 (available in https://mobidetails.iurc.montp.inserm.fr/MD/), in order to trace the presence of alternative splicing sites which can promote a PE inclusion. Variants supposed to alter either a branch point or an enhancer motif were studied with different predictors such as ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), SVM-BPfinder (http://regulatorygenomics.upf.edu/Software/SVM_BP/) and LaBranchoR (available in https://genome.ucsc.edu/). Alternative open reading frames (ORFs) caused by premature stop codons were analysed for every variant in which an alteration in splicing was predicted (https://www.bioinformatics.org/sms/orf_find.html).

Additionally, all coding regions were re-analysed following the filters already described in previous studies (Rodríguez-Muñoz et al., 2020; García Bohórquez et al., 2021).

Copy number variation analysis

Analysis of CNV has been assessed by a bioinformatic pipeline (https://github.com/TBLabFJD/NextVariantFJD) including three different CNV detection programs: CoNVaDING (Johansson et al., 2016); ExomeDepth (Plagnol et al., 2012); and panelcn.MOPS (Povysil et al., 2017). Programs were run using a bed file defining windows of 150 base pairs within the captured regions (Johansson et al., 2016) in order to facilitate the performance of the algorithms, all based on sequenced depth comparison. Analysis batches were defined by gene panels and sequencing runs to avoid different coverages due to technical reasons. Variants were annotated using AnnotSV (Geoffroy et al., 2023). We prioritized variants for further validation based on their presence in genes of interest, pathogenicity predictions and number of programs that detected them. In addition, for cases with no variants identified the analysis was extended to the rest of genes.

Once variants were prioritized, they were validated by different techniques relying on the region to be analysed: Multiplex Ligation dependent Probe Amplification (MLPA; MRC Holland; probemixes P361 and P362 for USH2A), CytoScan HD Array (Thermofischer sc., USA) or qPCR.

Validation and segregation of candidate variants

Validation of candidate SNV (Single Nucleotide Variant) was assessed by Sanger sequencing as previously described (García Bohórquez et al., 2021). Similarly, segregation analysis was performed in 4 families out of the 21 solved patients (Figure S1), since DNA from all relatives was not available.

Functional validation of the identified deep-intronic variants

Splicing effects were validated by minigene assays. DNA was amplified by the Fidelity Phusion polymerase (Thermo Fisher Scientific Waltham, MA, USA). The products were purified and digested with either XhoI or NdeI and NheI. Afterwards, digested products were cloned between restriction sites in pSPL3 plasmid (Dr. I. Botillo and Dr. S. Tuffery-Giraud), by using T4 ligase. The different primers of the regions amplified and cloned as well as the different minigene construct design are detailed in supplemental material (table S3 and Figure S2). Transformation of *Escherichia coli* occurred by electroporation. Later, 1 ug of each clone was transfected in duplicate into HEK293 cells (LipofectamineTM 3000 reagents (Thermo Fisher Scientific) grown in a six-well plate. After 24 hours, RNA was isolated with RNeasy mini kit and reverse transcription was assessed with the PrimeScript RT Reagent Kit (TaKaRa, Kusatsu, Japan). Constitutive exons primers from pSPL3 were used to amplify the final product in a 2% agarose gel (Bottillo et al., 2007), from which the different bands were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Eventually, the products were sequenced by Sanger and analysed by the chromatogram viewer FinchTV (https://digitalworldbiology.com/FinchTV) with the purpose of identifying any modification due to an alteration in the splicing process. Calculating the transcript counts resulting from minigene assays was performed with the ImageJ (https://imagej.net/ij/), an image processing program. First, we determined the intensity of

the fragment of interest in relation to the background and then we calculated the relative intensity of this fragment respecting the rest of bands observed.

In order to test the splicing redirection potential of several ASOs, minigenes constructions were built again as previously described (Sangermano et al., 2016). The pCI-neo plasmid, which contains exons of *RHO* allowing the study of splicing, was cloned by gateway cloning approach. In this line, ASOs assay could also matched described guidelines and efficiency observed in recent work (Aartsma-Rus, 2012; Slijkerman et al., 2018b; Reurink et al., 2023).

Antisense oligonucleotides design and testing

As mentioned in the section above, the designing of the ASOs was carried out following published guidelines (Aartsma-Rus, 2012; Slijkerman et al., 2018b), and purchased through Eurogentec (Seraing, Belgium). The ASOs were ordered with 2'-O-MOE modifications and a fully phosphorothiorate backbone. Two different ASOs overlapping the 5' and 3' boundaries between PE and intron were designed for PE50 (ASO1-PE50; ASO7-PE50) y PE64 (ASO1-PE64; ASO2-PE64), except for PE43 (ASO1-PE43), whose secondary structure made it complicated to target more than one region. For PE50 and PE64, ASOs were tested both separately and together by adjusting the final concentration in order to see a possible synergic effect. The relative position of each ASO on the different PE sequence is depicted in Figure 3.

In order to ensure a specific binding of the ASO, unspecific targets were searched in NCBI blast. The ASO reconstitution was done in PBS 1x to a concentration of 1mM and later aliquoted and diluted with PBS 1x to a concentration of 0.1mM. Different volumes were co-transfected in duplicate with the pCI-neo-based minigenes constructs with FuGENE® HD transfection reagent (Promega) to test different doses of ASOs. Twenty-four hours later, cells were harvested and the RNA was analysed. The ASOs were firstly tested at 20nM and 200nM both separately and together (for PE50 and PE64), which lead to further studies in which the minimum effective doses were established. A two nucleotides mismatch ASO (mmASO) was used as control for every experiment and it was transfected at 200nM and in duplicate. Since it is not able to redirect splicing, this control ASO is an indicator of the specificity of the ASOs for each PE.

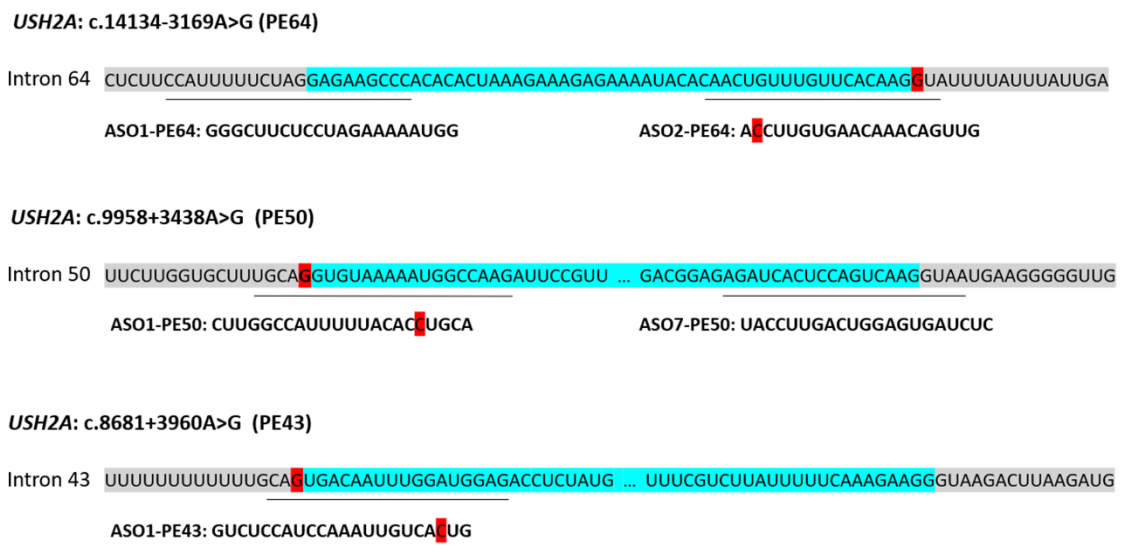


Figure 3. Position of the designed ASOs respecting the different PEs. Sequences in grey represent the intronic region whereas the blue nucleotides correspond to the PEs. Variants are depicted in red. The lines below the different intron indicate the specific sequence for each ASO, which are also empathized right under them. PE50 and PE43 are disrupted by dots to make all sequences equal in length.

Data and code availability

Genomic data will be available upon request. In addition, all sequencing data are deposited to a public repository under the dataset code EGAD50000000687 (European Genome-Phenome Archive).

Acknowledgements

J.M.M. received two grants from Instituto de Salud Carlos III (ISCIII), “PI22/00213” and “AC21_2/00022.” G.G.-G. acknowledges two grants from Instituto de Salud Carlos III (ISCIII), “CP22/00028” and “PI22/01371,” co-funded by the European Union. G.G.-G. has also a grant funded by the European Union in the HORIZON programme HORIZON-HLTH-2023-TOOL-05-04 (BETTER, 101136262). P.B.-M. received a grant from Ministerio de Universidades “FPU20/04736”, co-funded by the European Union. This study was also supported by Stichting Ushersyndroom, de Gelderse Blindenstichting, and the Foundation Fighting Blindness USA (grant no. PPA-0517-0717-RAD to E.V.W.). C.A. received a grant from Instituto de Salud Carlos III (ISCIII), “PI22/00321.”.

Author contributions

Conceptualization and supervision, J.M.M., G.G.-G., E.V.W., and E.d.V.; methodology, B.G.-B., P.B.-M., S.B., and E.d.V.; resources, B.G.-B., P.B.-M., E.A., C.R., L.F.-C., S.B., and E.d.V.; bioinformatic analysis and data curation, P.M. and A.S.-J.; writing – original draft, B.G.-B.; writing – review & editing, B.G.-B., P.M., A.S.-J., C.A., E.V.W., G.G.-G., and J.M.M.; funding acquisition, J.M.M. and G.G.-G. All authors have read and agreed to the published version of the manuscript.

Declaration of interests

The authors declare to not have any conflicts of interests.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102374.

Keywords

Inherited Retinal Dystrophies; Usher syndrome; USH2A; non-coding regions; deep-intronic; splicing; pseudoexon; minigene; antisense oligonucleotides.

CHAPTER III

CHAPTER III

Two novel mutations in TBC1D32 add complexity to the Oro-Facial-Digital syndrome

Belén García-Bohórquez[1,2]; Purificación Marín-Reina[3]; Elena Aller[1,2]; Pilar Barberán-Martínez[1]; Miguel Armengot[4]; Roberto Llorens-Salvador[5]; Inmaculada Concepción Almor-Palacios[6]; José M. Millán[1,2]; Gema García-García[1,2].

[1]Molecular, Cellular and Genomics Biomedicine, Health Research Institute La Fe. 46026 Valencia, Spain; 2Center for Biomedical Network Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III, 28029 Madrid, Spain. 3Division of Neonatology, University and Polytechnic Hospital La Fe. 46026 Valencia, Spain. 4Department of Otorhinolaryngology, and Primary Ciliary Dyskinesia Unit, University and Polytechnic Hospital La Fe. 46026 Valencia, Spain. 5Clinical Imaging Area; University and Polytechnic Hospital La Fe. 46026 Valencia, Spain. 6Department of Ophthalmology, University and Polytechnic La Fe Hospital. 46026 Valencia, Spain. Clinical imaging Area; Hospital Universitario y Politénico La Fe, Valencia, Spain

Pending submission
(Adapted to meet the format of this document)

Abstract

Ciliopathies are characterized by the dysfunction of cilia and, therefore, they encompass a broad group of diseases in which several tissues can be affected. Inherited retinal dystrophies (IRD), which are included in the group of sensory ciliopathies, are characterized by the death of retinal pigment epithelial cells (RPE) and photoreceptors, leading to vision loss. The most common IRD is retinitis pigmentosa (RP), some of whose responsible genes are involved in specific ciliary functions, such as the formation or regulation of cilia length. On the other hand, oro-facial-digital syndrome (OFD) is characterized by the association of facial cleft, microphthalmia/anophthalmia, coloboma, and polydactyly due to mutations in genes responsible for cilia formation. Recently, RP has been associated with mutations in the *TBC1D32* gene, which is responsible for OFD in several families also affected by RP. For this reason, the *TBC1D32* gene has been redefined as responsible for OFD type IX (OFD IX), which also associates RP. In this study, a clinical exome analysis was conducted on a patient presenting a syndromic clinical picture compatible with OFD IX, who also had moderate bilateral sensorineural hearing loss (SNHL). After a thorough review of the clinical data collected since the patient's birth and the identification of two variants in the *TBC1D32* gene, this study could contribute to expand the clinical spectrum associated with this gene by including sensorineural hearing loss among the associated symptoms, thus contributing to clinical and genetic diagnosis in patients with a similar clinical depiction.

Introduction

Ciliopathies are a wide group of diseases characterized by the dysfunction of the cilia. Ciliary disorders are categorized as first- and second-order ciliopathy depending on whether they are caused by the disturbance of ciliary proteins (first-order) or they result from the disturbance of non-ciliary proteins which are essential for ciliary function (second-order) (Reiter and Leroux, 2017).

Inherited retinal dystrophies (IRDs) are one of the so-called sensory ciliopathies (Wheway et al., 2014). IRDs are a group of retinal rare diseases characterized by the degeneration of the retinal pigmentary epithelium (RPE) or photoreceptors cells (Sundaram et al., 2012; Hu et al., 2021; Malvasi et al., 2023; Brar et al., 2024). This eventually cause vision loss, usually being progressive. IRDs can also be manifested as a part of a syndrome that associates other clinical symptoms, for which more than 200 genes have described to be causative (Tatour and Ben-Yosef, 2020). Many functions have been associated to IRD genes since the proteins encoded by them can be localized in the outer and inner segment, the nucleus and the synaptic region, the connecting cilium and the apical cilium of the photoreceptors (Hamel, 2014; May-Simera et al., 2018; Verbakel et al., 2018; Bachmann-Gagescu and Neuhauss, 2019). Specifically, some IRD-causing genes are also in charge of cilium formation and its regulation in length (Reiter and Leroux, 2017; Malvasi et al., 2023). Interestingly, not only some IRD genes have a function related to the primary cilium, but also some of them regulate the RPE maturation (May-Simera et al., 2018), which shows the interrelated functions proteins of many different nature involved in retina development and maintenance.

Within this protein network, *TBC1D32* was reported as a ciliary gene (Ishikawa et al., 2012) and as a gene causing Oro-Facial-Digital syndrome type IX (OFD IX) (Adly et al., 2014). This syndrome is caused by defects in proteins responsible for cilia formation (Lopes et al., 2011; Adly et al., 2014), and it is clinically associated with facial cleft, microphthalmia/anophthalmia, coloboma, polydactyly and retinopathies (Franco et al., 1993; Gurrieri et al., 2007). Several studies have contributed to the molecular spectrum of OFD type IX by the identification of patients harbouring *TBC1D32* variants (May-Simera et al., 2018; Monies et al., 2019; Alsahan and Alkuraya, 2020). It was not until 2020 that

Hietamäki et al. identified *TBC1D32* variants in two siblings with OFD type IX, one also showing RP (ophthalmologic data was only available for one sibling) (Hietamäki et al., 2020). This was followed by a study in 2023 reporting retinal dystrophy in an OFD type IX case with mutations in this gene (Harris et al., 2023). Recently, *TBC1D32* has also been associated with isolated RP in four patients from three families (Bocquet et al., 2023). Nevertheless, given the numerous tissues in where ciliary genes play a role (Reiter and Leroux, 2017), the clinical data around ciliopathies turns out to be very complex. Such is the case that clinical features observed in OFD type IX patients regarding ocular alterations and the variability observed for the congenital hypopituarism, suggest a remarkable variable expressivity around *TBC1D32* (Adly et al., 2014; Hietamäki et al., 2020).

In the present work, we performed a clinical exome analysis in a patient who displayed a possible ciliopathy with features related to a non-filiated dysmorphic syndrome, including IRD and hearing loss symptoms. After the analysis, we identified two compound heterozygous likely pathogenic variants in *TBC1D32* gene that were not previously reported (c.2073del and c.769+5G>A). For c.769+5G>A, functional studies have been carried out in order to confirm its pathogenic effect in splicing. Moreover, this is the first study in which variants in *TBC1D32* have been identified in a patient displaying hearing loss. This way, our results help to expand the clinical spectrum of this human ciliopathy associated to the *TBC1D32* gene, since tissues such as inner ear in which the TBC1D32 protein is expressed and where cilia are present, could also be affected.

Materials and methods

Clinical evaluation

The patient underwent physical exploration, bone series, hormonal studies, renal function tests, abdominal ultrasound, brain MRI (Magnetic Resonance Image), maxillofacial exploration, audiograms and ophthalmological tests such as OCT (Optic Coherence Tomography), ERG (electroretinography), eye fundus, BCVA (visual acuity measure), evoked potentials and visual fields. All the clinical studies were performed in the Hospital La Fe of Valencia, Spain. Moreover, the familiar and physiological background of the pregnancy was evaluated.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples following standard procedures.

The genes *RNU4ATAC* and *MYCN*, which cause Roiffman syndrome and Opitz-GBBB, respectively, were studied by amplifying their sequence by conventional PCR and posterior sequencing by Sanger. In addition, a genomic array was performed (Affymetrix CytoScan 750 array). Several genetics tests were also performed to study other syndromes such as Aarskog and Robinow, as well as for Hypochondroplasia (*FGFR3*) and short stature (*SHOX* deletions) by MLPA.

A study has been carried out using massive sequencing techniques consisting of the analysis equivalent to a clinical exome sequencing (CES). This includes the coding region and flanking intronic regions of 3204 genes that were analysed by next generation sequencing (NGS). Among the genes studied, the gene responsible for frontometaphyseal dysplasia 1 (*FLNA*) was included, along with more than 700 genes related to other neurodevelopmental disorders. For the sequencing of this DNA sample, the ClearSeq Inherited Disease Panel (Agilent Technologies) was used with an average coverage greater than 200x. The capture and reading processes of sequences have been carried out in the Genomics Platform of the IIS La Fe. Furthermore, given that a second gene responsible for frontometaphyseal dysplasia has recently been described (*MAP3K7*) (Wade et al., 2016), not included in this panel, the region where the recurrent mutation p.P485L is located was analysed by conventional Sanger sequencing using specific primers.

Particularly, a virtual gene panel containing 207 genes associated to retinal dystrophies were analysed by NGS using the aforementioned ClearSeq Inherited Disease panel data.

Afterwards, an updated version of CES including 5227 genes (Custom Constitutional Panel 17 MB; Agilent Technologies) was sequenced in the patient. The library preparation was carried out according to the Bravo NGS SureSelect QXT Automated Target Enrichment protocol (Agilent Technologies) for Illumina Multiplexed Sequencing. The captured libraries were sequenced on NextSeq500 (Illumina) in a paired-end mode to generate a median raw target coverage of 100x. The obtained sequences were aligned against the genome reference sequence (GRCh37/hg19) to perform the variant calling with the Alissa Clinical

Informatics Platform (Agilent Technologies). The annotated variants were initially filtered according to a minor allele frequency (MAF) value ≤0.01. The frequency of the variants was explored in the Exome Aggregation Consortium database/gnomAD (gnomad.broadinstitute.org/) and 1,000 genomes (internationalgenome.org/). As a first approach, variants reported in RetNet (https://web.sph.uth.edu/RetNet/home.htm) to be IRD-causing were analysed first. Subsequently, variants harboured in genes reported in the bibliography in association with IRD that were not present in RetNet were examined in detail. Similarly, all genes described and reported in the Deafness Variation Database to cause hearing loss (https://deafnessvariationdatabase.org/), were analysed. Several databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), HGMD (https://www.hgmd.cf.ac.uk/ac/index.php) and LOVD (https://www.lovd.nl/) were reviewed. Moreover, a deeper *in silico* analysis was carried out by using bioinformatic tools such as Varsome (https://varsome.com/), Franklin (https://franklin.genoox.com/clinical-db/home) and MobiDetails (https://mobidetails.iurc.montp.inserm.fr/MD/). A possible effect in splicing was also evaluated with NNSplice and MaxEnt (https://www.fruitfly.org/seq_tools/splice.html). Eventually, the predictor ORF Finder was used for prediction of Open Reading Frames (ORF Finder (bioinformatics.org). Afterward, filtered DNA variants were classified according to the American College of Medical Genetics guidelines (Richards et al., 2015).

Validation of candidate variants identified and segregation analysis in the DNA from available relatives were performed using Sanger Sequencing.

Functional assays

For the minigene analysis, a protocol developed in previous works was applied (Fuster-García et al., 2018; Rodriguez-Muñoz et al., 2022). Fidelity Phusion polymerase (Thermo Fisher Scientific Waltham, MA, USA) was used to amplify the target region, which was, subsequently, digested with XhoI and NheI restriction enzymes. The purified digested product was cloned into pSPL3 plasmid (Dr. I. Botillo and Dr. S. Tuffery-Giraud) with T4 ligase. Afterwards, *Escherichia coli* was transformed by electroporation, from which a mutant and a wild-type clone were obtained. Later, HEK293 cells were transfected in duplicate with 1µg of each clone (LipofectamineTM 3000 reagents (Thermo Fisher Scientific). Twenty-four

hours later, the RNA was isolated and purified in order to perform the reverse transcription with PrimeScript RT Reagent Kit (TaKaRa, Kusatsu, Japan) and the cDNA was amplified from the constitutive pSPL3 exons to be, ultimately, sequenced by Sanger.

Results

Clinical findings

A 10-month-old boy was referred for evaluation for suspected skeletal dysplasia. This was the first child of healthy non-consanguineous parents, who was born after a controlled pregnancy without incident.

On physical examination, the patient presented hypertelorism; asymmetry between both eyes; long, narrow palpebral fissures; prominent forehead; sunken nose bridge; sublingual frenulum; hamartomatous lesions on the inner surface of the lip; wide root and nasal tip; low set ears; thick hair; short neck; narrow chest; muscle hypertrophy; anteversion of the shoulders; clavicle narrowing; small nipple; brachydactyly in four limbs; impression of distal phalanx shortening; absence of the first interphalangeal fold in the second and third fingers of both hands; clinodactyly of fifth fingers of the hand; and bilateral single palmar crease (table 1).

In his growth, the patient maintains a height percentile at p3, with a head circumference at p90 and a breaststroke height reduced compared to his height. Learning difficulties and signs of ADHD (Attention-Deficit/Hyperactivity Disorder) at eight years of age was observed. This syndromic condition seemed to be highly suspected of genetic origin (figure 1). Furthermore, at the age of 12, he was diagnosed with retinopathy, which was classified as a suspected diagnosis of rod dystrophy or retinitis pigmentosa (RP) (figure 1a).Complementary exploration by MRI also showed absent of septum pellucidum with mid-line fusion of both fornices and an abnormal sulcation of both hippocampi (figure 1b), while bone series, hormonal studies, renal function and abdominal ultrasound did not exhibit any anomaly (table 1). The patient also suffers from bilateral mixed hearing loss, with a transmissive component due to secretory otitis media, probably related to Eustachian tube dysfunction secondary to polymalformation syndrome. Likewise, he displays a moderate bilateral sensorineural hearing loss at medium and high frequencies (figure 1c), along with a malformation of the internal auditory canal through which the statoacoustic and facial nerves circulate.

Table 1. Genotypes and phenotype of the *TBC1D32* cases reported in the bibliography

Reference	Allele 1	Allele 2	Original patient code	Kinship	Brain MRI	Ocular manifestation	Other features
(Adly et al., 2014)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	Index 1	-	ABS PG; seizures; agenesis of the corpus callosum	Right MTM and left ATM; bilateral coloboma	CLs; hands alteration; small occipiofrontal circumference; MIC; severe midline cleft; HPT; severe choanal stenosis; left hand post-axial PDL; ambiguous genitalia; patent ductus arteriosus and atrial septal defect
(Monies et al., 2019)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	17-0889#	-	Agenesia of the corpus callosum	Cyclops	Holoprosencephaly; CL, ventricular septal defect; sandal gap
(Hietamäki et al., 2020)	c.1165_1166dup; p.(Gln390Phefs*32)	c.2151del; p.(Lys717Asnfs*29)	I.3	S	ABS anterior and ECT posterior PG	NA	Craniofacial dysmorphism; HPT; upturned nose; communicating hydrocephalus; global DD; abnormal genitalia; chronic secretory otitis media
	c.1165_1166dup; p.(Gln390Phefs*32)	c.2151del p.(Lys717Asnfs*29)	I.5		ABS anterior and ECT posterior PG	Retinal dystrophy; reduced visual acuity	Craniofacial dysmorphism; abnormal dentition; HPT; upturned nose; SDL; sandal gap; lower limb length; motoric DD; neuromuscular scoliosis; chronic secretory otitis media
	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	II.8	S	Anterior pituitary HPP; agenesis of corpus callosum; cerebral vermis HPP	Reduced visual acuity	Tongue alteration; cleft palate; HPT; upturned nose; choanal stenosis; PDL; global DD; chronic secretory otitis media
	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	c.1372+1G>T; p.(Arg411_Gly458delinsSer)	II.9#		?	?	Midline CL; intestinal malrotation
(Alsahan and Alkuraya, 2020)	c.3724C > T; p.Arg1242*	c.3724C > T; p.Arg1242*	REQ19-3468	-	Pituitary HPP?	ATM	Hydrocephaly; CHA?
(Harris et al., 2023)	c.3182G>C; p.(Gly1061Ala)	c.2134A>G; p.(Asn712Asp)	Proband 1	-	-	DSE	Brain and spinal cor MRI altered; MIC; brachycephaly; DD; prominent nose; long philtrum; thin upper lip; MIC; VTM; dandy walker; SDL; mild restriction of joints; global DD
	c.2481+4A>T; p.¿?	c.3572A>G; p.(Tyr1191Cys)	Proband 2	-	ECT PG	Strabismus; heterochromia iridis	CHA; median central incisor; midfase HPP; MAC; VTM; brachydactyly; global DD; short long bones, VTM
	c.2650C>T; p.(Arg884Trp)	c.2650C>T; p.(Arg884Trp)	Proband 3	-	ABS sella turcica; abnormal vermis	Bilateral microphthalmia; retinal dysplasia; fused optic nerve	Single fused nostril; long philtrum; micrognathia; enlarged cranium; lobar holoprosencephaly; VTM; fused thalami, dysplastic hypothalami; flexed wrists; rocker bottom feet; AVSD; adrenal HPP; multiple anomalies during pregnancy

	c.2650C>T; p.(Arg884Trp)		c.2650C>T; p.(Arg884Trp)	Proband 4	-	ABS sella turcica; abnormal vermis	Retinal dysplasia; fused optic nerve; bitaleral MTM	High positioned nose and partially fused nares; low set ears; mildface HPP; lobar holoprosencephaly; VTM; fused thalami; dysplastic hypothalami; short digits; Short, curved ulnea; renal and adrenal HPP; micropenis; multiple anomalies fetal growth restriction
	c.3325_3326del; p.(Ser1109Leufs*4)		c.3325_3326del; p.(Ser1109Leufs*4)	Proband 5	S	ABS sella turcica	Bilateral ATM; ABS optic nerve	Nose ABS; low set ears; midface HPP; enlarged cranium; syndanctyly; sandal gap; dextro-cardia; gastrointestinal omphalocele; situs inversus; FGA
	c.3325_3326del; p.(Ser1109Leufs*4)		c.3325_3326del; p.(Ser1109Leufs*4)	Proband 6#		-	-	Midline facial cleft; anencephaly; unilateral postaxial; sandal gap; gastrointestinal omphalocele; FGA
	c.3527A>G; p.(His1176Arg)		c.3527A>G; p.(His1176Arg)	Proband 7	-	-	DSE	HPT; coarse facies; ABS 5th toe phalange; short long bones; ID
(Bocquet et al., 2023)	c.317+5G>A;	p.¿?	c.846delTCCTA; p.(Asn282Lysfs*7)	Patient 1	-	Mild anomalies and atrophy of superior vermis	Mild retinal dystrophy; plausible RPE loss	-
	c.18_27del; p.(Ser6Argfs*8)		c.1141-1G>A;	p.¿?	Patient 2	-	Mild anomalies and atrophy of superior vermis	Retinal dystrophy; plausible RPE loss	Nonproteinuric chronic kidney disease
	c.1267G>T; p.(Glu423*)		c.3513G>T; p.(Trp1171Cys)	Patient 3	S	-	Late onset retinal dystrophy; plausible RPE loss	-
	c.1267G>T; p.(Glu423*)		c.3513G>T; p.(Trp1171Cys)	Patient 4		-	Late onset retinal dystrophy; plausible RPE loss	-
This study	c.2073del; p.(Gly693Aspfs*15)		c.769+5G>A; p.(Asp231Leufs*21)	II:1	-	ABS of septum pellucidum; Sella turcica HPP	Eyes asymmetry; Retinal dystrophy	HPT; long, narrow palpebral fissures; prominent forehead; wide root and nasal tip; low set ears; short neck; narrow chest; muscle hypertrophy; clavicle narrowing; small nipples; brachydactyly; clinodactyly; bilateral single palmar crease; moderate bilateral sensorineural hearing loss.

#: Induced pregnancy termination; PG: Pituitary gland; AVSD: Atrioventricular septal defect; S: Siblings; DSE: Deep set eyes; ATM: Anophthalmia; MTM: Microphtalmia; ABS: Absent; HPT: Hypertelorism; ECT: Ectopic; CL: Cleft lips; MIC: Microcephaly; MAC: Macrocephaly; DL: Developmental delay; PDL: Polydactyly; SDL: Syndactyly; ID: Intellectual disability; VTM: Ventriculomegaly; CHA: Choanal Atresia; HPP: Hypoplasia; FGA: Fetal growth alterations; RPE: Retinal Pigment Epithelium; NA: Not available; "?": Unclear.

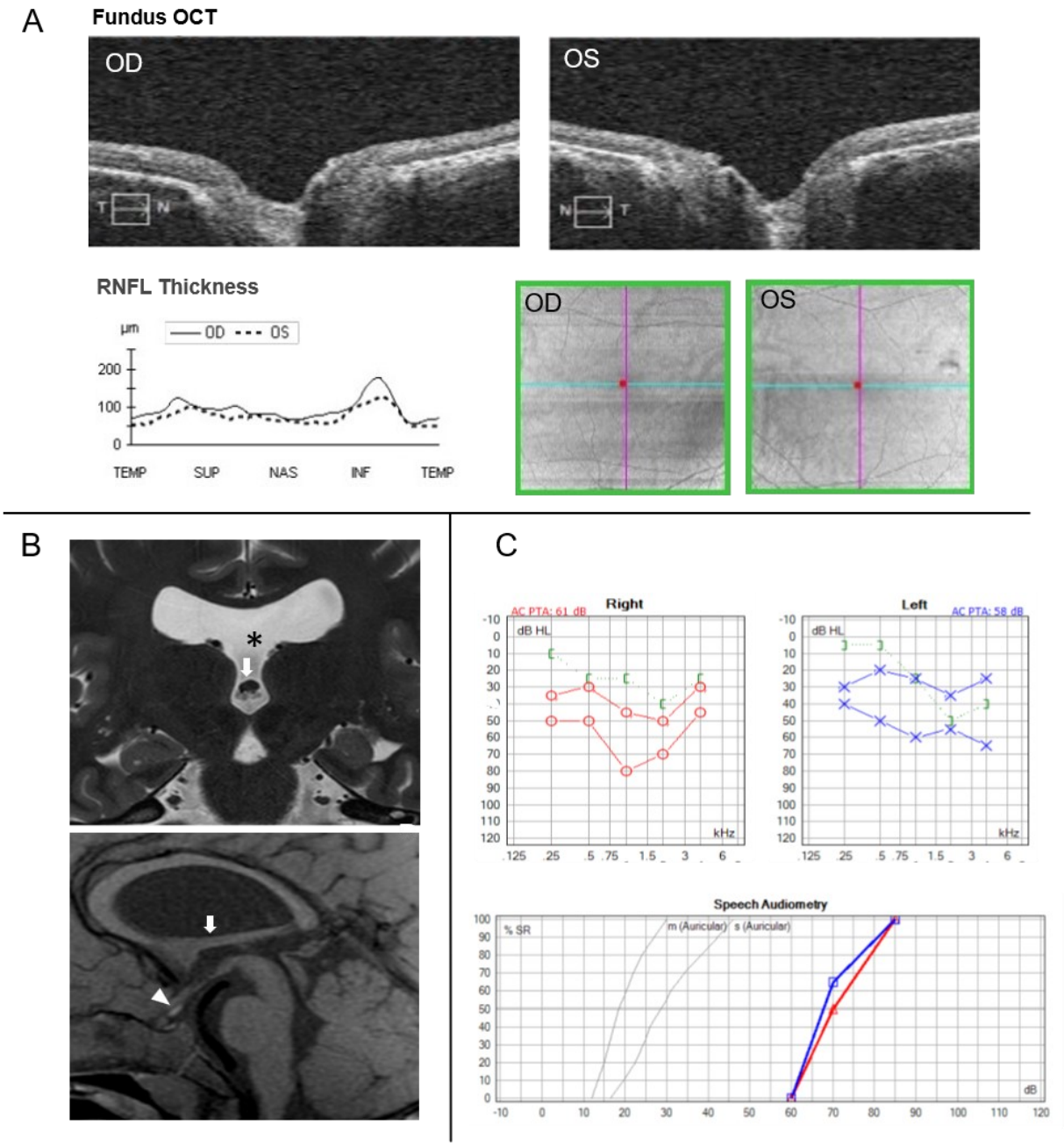


Figure 1. Clinical findings in index patient. (A) OCT of both eyes. (B) Brain MRI. The upper section shows coronal T2 weighted MRI with complete absence of the septum pellucidum (*) and mid-line fusion of both fornices (arrow). Abnormal sulcation of both hippocampi is also noted. The lower section shows sagittal T1 weighted of the mid-line brain: abnormal thickenned fornix (arrow) and ectopic neurohypophisis (arrowhead). Hypoplastic sella turcica. (C) Audiograms of both ears confirm a moderate bilateral SNHL in medium and high frequencies. The speech audiometry shows a 50% of understanding achieved at 50 decibels and 100% at 80 decibels bilaterally.

Molecular findings

Any pathological result was obtained from the analysis of Opitz-GBBB, Roiffman, Aarskog and Robinow syndromes, hypochondroplasia, short stature, neurodevelopment and retinal dystrophy genes (between years 2016 and 2020) (see Materials and Methods section).

Finally, in 2023 a CES updated version was performed. After sequencing the clinical exome in the patient, the results showed a 79% of reads aligned against the target region. Coverage data also showed an average depth in targeted region of 107x with a 90% of targeted bases at 20x coverage. At the moment of the study, we found no variants that could explain the patient's phenotype in the study of genes present in RetNet. Next, we analyzed variants in genes reported in the literature to impact the retina, but not considered as candidates in RetNet. We identified two variants in heterozygous state, c.2073del in exon 18 and c.769+5G>A in intron six, both in *TBC1D32* (figure 2). Both variants were classified as likely pathogenic. We identified no likely pathogenic variants in hearing loss-causing genes. We confirmed the presence of the *TBC1D32* variants in the patient through Sanger sequencing and performed segregation analysis in the DNA of available relatives (figure 3a). This revealed that the variants were in trans, c.769+5G>A inherited from the mother while c.2073del was inherited from the father, which contributed to confirming the genetic diagnosis.

We performed functional validation for the intronic variant c.769+5G>A in *TBC1D32*. *In silico* predictors suggested this variant affects splicing by potentially causing the loss of a donor splice site, indicated by a MaxEnt variation of -36.98% (a variation greater than 15%) and a SpliceAI score of 0.67 (on a scale of 0-1, with thresholds of ≥ 0.2|0.5|0.8 indicating impact). Based on these predictions, exon six of *TBC1D32* would be skipped. These predictions were validated by minigene assays (figure 3b), and the resulted products were sequenced by Sanger to confirm the exon skipping effect (figure 3c). Additionally, the creation of a new stop codon in exon seven was predicted by ORF Finder in the mutated sequence.

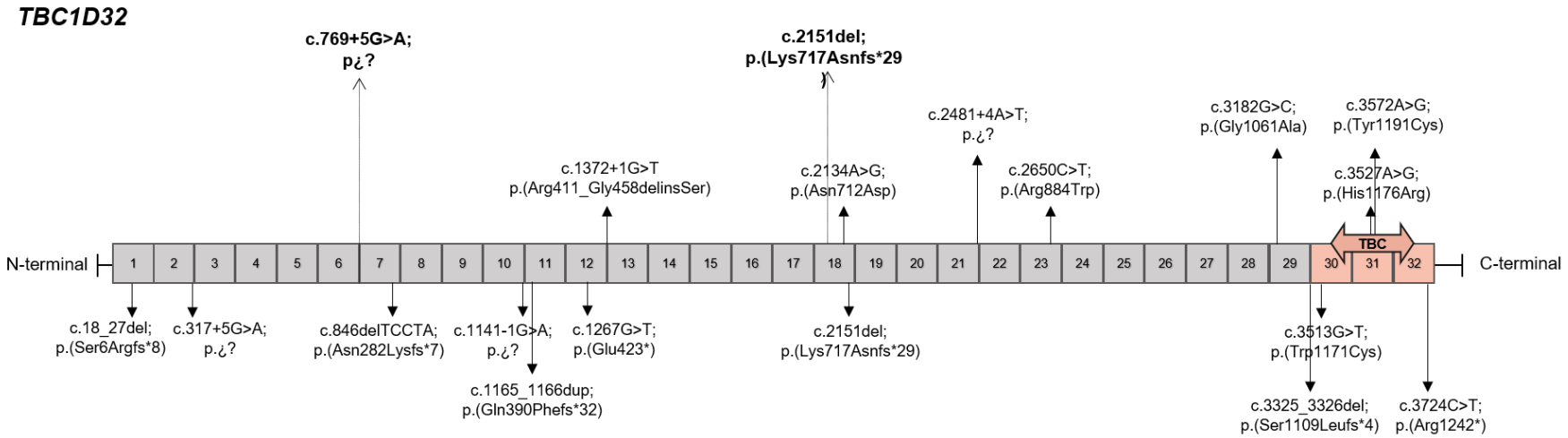


Figure 2. Scheme of *TBC1D32* cDNA sequence (NM_152730) and the localization of mutations described so far. Numbering from 1 to 32 represents all the exons contained in the cDNA of this gene. TBC domain is depicted by an orange arrow as well as the exons that codify it. Black variants highlighted in bold are those identified in our patient.

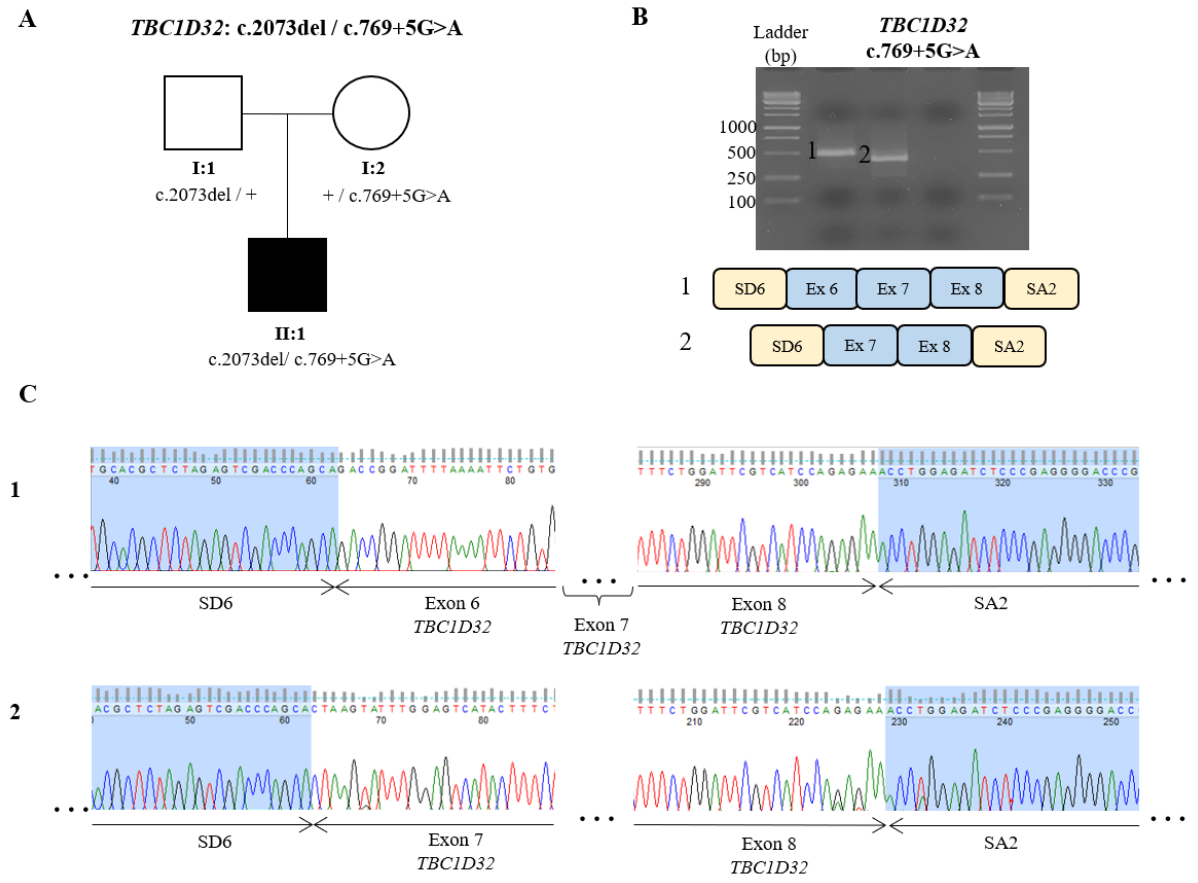


Figure 3. Segregation analysis of identified variants and minigene assay performed for variant *TBC1D32* (NM_152730): c.769+5G>A. (A) Segregation analysis performed in the parents confirming their co-segregation with the disease. (B) Amplification of the target region in the cDNA both wild-type and mutant as a result of the functional assay by minigene of variant c.769+5G>A. SD6 and SA2 represent the constitutive exons of the plasmid pSPL3. Wild-type and mutant contexts are depicted by bands 1 and 2, respectively, where band 2 depicts the exon 6 skipping; (C) cDNA sequence by Sanger of bands 1 and 2 observed in the gel of section B.

Discussion

OFD-proteins are classified into three different groups relying on wither they are involved in centriole elongation, functions carried out in the transition zone, and intraflagellar transport (Bruel et al., 2017; Wang et al., 2018a). *TBC1D32* was reported as a ciliary gene in 2012 (Ishikawa et al., 2012) and the encoded protein is included in the group responsible for centriole elongation. TBC1D32 protein regulates the structure of the primary cilium in neural tube in Zebrafish *tbc1d32* morphants and in a murine *TBC1D32* knockout model (Ko et al., 2010). Moreover, a recent study in a *Xenopus* model showed that *tbc1d32* knockdown

altered the differentiation of RPE and photoreceptors, as well as caused defects in the ciliogenesis (Bocquet et al., 2023). Additionally, analyses in fibroblasts cells from a patient harbouring mutations in *TBC1D32* confirmed that the protein is located in the centrosome of primary cilium and the mutations provoke cilium elongation defects (Bocquet et al., 2023).

Initially, *TBC1D32* was reported as a gene causing OFD type IX, a heterogenous syndromic disorder involving oro-facial, ocular and extremities anomalies (Adly et al., 2014) (table 1). Despite that, this gene is not yet associated to any pathology in OMIM. Later, other clinical signs have been appearing in patients harbouring mutations in *TBC1D32*, including cases with isolated inherited retinal dystrophy (Adly et al., 2014; Monies et al., 2019; Alsahan and Alkuraya, 2020; Hietamäki et al., 2020; Bocquet et al., 2023; Harris et al., 2023) (table 1). This allowed to establish a clear association of IRD to the OFD IX phenotype and leads us to suggest the inclusion of *TBC1D32* as an IRD-causing gene in RetNet (https://web.sph.uth.edu/RetNet/home.htm). However, TBC1D32 has been recently included in RetNet owing its association to IRD to the finding from Bocquet *et al* (Bocquet et al., 2023). Our patient presents a OFD IX phenotype, with an altered brain MRI and several oral-facial features that confirm the clinical diagnosis. In addition, at the age of 10, audiograms showed moderate bilateral sensorineural hearing loss and, at 12 years old, he was diagnosed with IRD.

To date, no patient with mutations in *TBC1D32*, who manifested sensorineural hearing loss, had been described. We cannot completely rule out the possibility that this clinical sign is caused by alterations in a gene not previously linked to hearing impairment, or even associated to non-genetic causes. However, as no variant in a known gene responsible for hearing loss was identified in our patient, and considering the multitude of ciliopathies associated with defects in inner ear cilia and reporting hearing loss (Fuster-García et al., 2021; Leventea et al., 2021; Shi et al., 2022; Goutaki et al., 2023), it is tempting to expeculate that alterations in the *TBC1D32* may also involve hearing impairment. Particularly, mutations in *TBC1D24*, a TBC1 domain family gene, were identified in three family displaying non-syndromic recessive hearing loss (Rehman et al., 2014). Additionally, in 2020, it was observed that a mouse model with ciliopathy, knockout for *Bromi* gene (homologue of *TBC1D32*),

presented cochlear shortening with a decrease in hair cells that also were developed prematurely, and an impaired sonic hedgehog pathway signalling as a consequence of defects in the primary cilium ciliogenesis (Moon et al., 2020), among others. Similarly, Bocquet et al., detected expression of *tbc1d32* in the otic vesicle of their *Xenopus* model (Bocquet et al., 2023). Moreover, the polarity of hair bundle was also altered. Thus, it is tempting to correlate OFD IX with hearing loss and this study could potentially broaden the phenotype associated with *TBC1D32*.

Mutations in *TBC1D32* have been described all along the gene. Our findings raise the total number of pathogenic variants described in *TBC1D32* to 19 so far (five of them in non-coding regions) (figure 3). Functional assays that confirm the pathogenic effect due to intronic variants have only been carried out for variant c.1372+1G>A, showing a deleterious effect (p.Arg411_Gly458del) (Hietamäki et al., 2020). Effect in splicing was also confirmed for c.317+5G>A through patient fibroblasts (Bocquet et al., 2023). Nevertheless, no assay has been performed for variant c.2481+4A>T in *TBC1D32* located in non-canonical intronic regions. This way, our study is the first in which minigene assay has been done to confirm the effect of a variant located in a non-canonical splice position. Our results show the importance of these assays, and highlights the necessity of carrying out functional assays that confirm the effect in splicing of variants that do not alter any canonical splice site.

We tried to establish a preliminary genotype-phenotype correlation, but no clear evidence was identified. We observed that most of patients harbouring mutations located to the TBC domain present ocular malformation, though eye manifestations are very variable among patients. The lack of information about the domains and TBC1D32 protein's structure makes it difficult to predict how different variants could affect its function. To date, the TBC domain, located at C-terminal region, is the only defined in its sequence so far. This domain is involved in the interaction of TBC1D32 protein with CCRK and CFAP20, proteins involved in the intraflagellar transport (IFT) machinery of primary cilia, (Shaheen et al., 2019; Noguchi et al., 2021; Satoda et al., 2022). However, its involvement in IFT was questioned in a recently published study (Bocquet et al., 2023). Differences in the severity of the protein disruption and the residual protein function have been proposed as a possible explanation

for the wide clinical variability observed in patients harbouring alterations in this gene (Bocquet et al., 2023).

In conclusion, the study of the *TBC1D32* gene, combined with functional studies and a thorough clinical evaluation, has allowed us to open the possibility of a new phenotype associated with this gene that includes hearing loss as a clinical sign. The wide clinical spectrum of variants in *TBC1D32* highlights the complexity of ciliopathies and the importance of comprehensive genetic and clinical evaluations in this group of disorders.

ACKNOWLEDGEMENTS

JMM received two grants from Instituto de Salud Carlos III (ISCIII), “PI22/00213” and “AC21_2/00022” co-funded by the European Union, and the grant CIPROM/2023/26 from the Generalitat Valenciana. GGG acknowledges two grants from Instituto de Salud Carlos III (ISCIII), “CP22/00028” and “PI22/01371”, co-funded by the European Union. GG-G has also a grant funded by the European Union in the HORIZON programme HORIZON-HLTH-2023-TOOL-05-04 (BETTER, 101136262). PB-M received a grant from Ministerio de Universidades “FPU20/04736”.”, co-funded by the European Union.

DECLARATION OF INTERESTS

The authors declare no competing interests.

GENERAL DISCUSSION

GENERAL DISCUSSION

Inherited retinal dystrophies are a group of diseases that individually are considered as rare due to its low prevalence. This represents a problem when it comes to financing and resources, which in turn hinders the development of new therapies and slows down their study. In addition, the complexity of IRDs poses a challenge when it comes to their diagnosis and the development of new therapies. This includes the high clinical and genetic heterogeneity that hinders the identification of the genetic cause.

With the aim of overcoming these challenges, this thesis is focused on the evaluation of the efficacy of a gene panel to improve the diagnosis of IRD patients. In order to increase the percentage of solved patients we explored non-coding regions of IRD and, more specifically, USH genes. The outcome of these studies enabled the application of ASOs for the development of therapeutic approaches, which demonstrated their potential in splicing modulation and its applicability in gene therapy. Eventually, this thesis highlighted the importance of a thorough assessment of clinical data together with a proper genetic evaluation for complex syndromic cases.

GENETIC DIAGNOSIS IN IRD

Two different panels containing IRD-associated genes were designed and sequenced in a total of 92 patients clinically diagnosed with IRD. This allowed us to identify the genetic cause in 57.6% of patients. These results align with those reported in other targeted sequencing studies in which the percentage of diagnosis in cohorts of patients with all types of IRD did not exceed 62% (Jespersgaard et al., 2019; Zenteno et al., 2020; Britten-Jones et al., 2023a). This value is reduced when the study is aimed at diagnosing patients with the same clinical entity (Britten-Jones et al., 2023a). Targeted sequencing approaches face some limitations in discovering new genes, which can be overcome with WES or WGS. Surprisingly, the diagnosis ratio after analysing the whole exome remains at 64% (Britten-Jones et al., 2023a), although we would expect to identify the genetic cause in a greater number of patients. The reasons behind this may not only lie in the challenges of discovering new genes related to the disease (which sometimes requires well-curated

databases providing information on RNA and protein expression and robust functional studies) but also in the identification of a high number of variants classified as variants of unknown significance (VUS). Many of these VUS may eventually be reclassified as (probably) benign or (probably) pathogenic after further studies in the coming years.

In our cohort, we observed the high prevalence of pathogenic variants in the *ABCA4* and *USH2A* genes, accounting for 22% and 15% out of the solved patients, respectively. Mutations in *USH2A* cause nsRP and Usher syndrome type 2, for which it is the most frequently mutated gene. It has been described that between 12-25% of patients with nsRP carry mutations in *USH2A* (Verbakel et al., 2018; Ordoñez-Labastida et al., 2023), being 19.1% in the Spanish population (Perea-Romero et al., 2021). Of them all, the most frequent nsRP-causing variant is c.2276G>T (p.Cys759Phe), accounting for around 8.4% of nsRP cases solved in the Spanish population (Perea-Romero et al., 2021), and followed by the variant c.2299delG (Ordoñez-Labastida et al., 2023). This can also be reflected in our results, where the frequency of this variant respecting all *USH2A* alleles is 12.5%. Truncating variants in *USH2A*, either in compound heterozygosity or in homozygosity, are responsible for Usher syndrome. However, the c.2299delG variant turns out to be the second most frequent cause of nsRP, when found in *trans* with a non-truncating allele (Ordoñez-Labastida et al., 2023). Interestingly, this variant is distributed in the European population following a gradient from highest to lowest frequency from north to south of the continent (Dreyer et al., 2001; Aller et al., 2010).

On the other hand, mutations in *ABCA4* are responsible for 30% of autosomal recessive IRD cases, which is predicted to correspond to around 1.4 million people affected worldwide (Hanany et al., 2020). In autosomal recessive non-RP Spanish families, mutations in *ABCA4* explain 82.7% of cases (Perea-Romero et al., 2021). Specifically, for cases of CRD and MD, the frequencies vary around 38% and 14%, respectively (Del Pozo-Valero et al., 2022). It was described in the Spanish population that the most frequent variant described in this gene is c.3386G>T, constituting the 5% of the total alleles described in this study and explaining the diagnosis in 21.5% of solved autosomal recessive non-RP families (Del Pozo-Valero et al., 2020; Perea-Romero et al., 2021). Similarly, the c.5882G>A variant was reported with a fairly high frequency in several populations (Perea-Romero et al., 2021).

The previously reported worldwide carrier frequency showed that *ABCA4* has the highest genetic prevalence, followed by *USH2A* and *EYS* in autosomal recessive cases (Hanany et al., 2020). This matched our results, except for *EYS* that was the fourth most prevalent mutated gene in our cohort. In patients with an autosomal dominant inheritance pattern, we observed that *PRPH2* is the most prevalent mutated gene, as reported in the literature in the Spanish population (Perea-Romero et al., 2021). Particularly, this gene was the third most prevalent mutated gene among all patients from out cohort. However, it is worthy to remark that genes prevalences can differ among cohorts when they are not large enough and their predominant clinical entities may be the result of biases on the part of ophthalmologists and the study population itself.

Hypomorphic variants and complex alleles in ABCA4

In STGD patients with a late-onset phenotype or with milder clinical signs, hypomorphic variants in *ABCA4* are described (Cornelis et al., 2017; Kaway et al., 2017; Zernant et al., 2017, 2018; Collison et al., 2019). These variants are characterized by presenting incomplete penetrance, since they only contribute to the phenotype when they are found in *trans* with a deleterious variant. This is why many of them have a high population frequency as they are present in many healthy individuals who are either monoallelic, homozygous for one of these variants or carriers of two hypomorphic variants in compound heterozygosity. In our cohort, patients RPN-544 and RPN-666 carry the c.5882G>A (p.Gly1961Glu) variant, which was described as a hypomorphic related to late-onset of symptoms (Salles et al., 2018). This aligns with our findings, as both patients exhibited their first clinical symptoms during the third decade of life. Another example of a hypomorphic variant described in the literature could be the case of c.5603A>T (Zernant et al., 2017; Cremers et al., 2018; Runhart et al., 2018). This variant has a high frequency in STGD patients and it is a disease-causing variant in 5% of cases when found in *trans* with a deleterious variant.

Regarding complex alleles, in patient RPN-679 diagnosed with STGD disease we identified the c.5714+5G>A variant in *ABCA4*, previously described as hypomorphic (Cornelis et al., 2017), along with c.4880del (p. Leu1627Argfs*35) and c.2953G>A (p.Gly985Arg) in this same gene. Neither of these last two variants were previously described. However, since we were not able to perform the segregation analysis, we cannot conclude whether the

c.5714+5G>A variant is causing the phenotype (in case the other two variants were on the same allele), or the clinical signs observed are due to the variants c.4880del (p.Leu1627Argfs*35) and c.2953G>A (p.Gly985Arg). Similarly, patient RPN-734 carries the variants c.3113C>T (p.Ala1038Val), c.4539+2064C>T and c.1364T>A (p.Leu455Gln) in *ABCA4*. Despite the unknown allelic distribution of these variants, the c.3113C>T allele has already been described in *cis* with c.1364T>A in the literature (Salles et al., 2018). In cases in which the c.1364T>A variant is found in trans with another deleterious variant, a late onset was observed (Del Pozo-Valero et al., 2020). This suggests that this variant could also be considered hypomorphic. In addition to this case, we found RPN-749, in which three variants were also identified and whose results reaffirm the high prevalence of complex alleles associated with *ABCA4*.

Given the wide range of phenotypic variability and age of onset observed in patients with mutations in the same gene, it is not be surprising that, as occurs with *ABCA4*, there are other genes in which hypomorphic variants alter the patient phenotype, or even complex alleles that contribute to the variability in the clinical signs (Farag et al., 2024; Thuma et al., 2024; Tsui et al., 2024; Waldock et al., 2024).

Carriers of mutations in more than one IRD gene

Around 2.7 billion individuals are described to be carriers of a pathogenic variant in a AR gene (Hanany et al., 2020). Thus, it is not unusual to identify patients that carry pathogenic variants in more than one AR gene, and even cases in which variants in more than one gene can be influencing the phenotype. In fact, in our study, we identified five families in which one of these situations occur (RPN-670; RPN-717; RPN-726; RPN-735; RPN-745) (Chapter I, Table 1).

Particularly, RPN-670 (FRPN-265) was diagnosed with CD before the age of 20. We identified variants c.3157del (p.Tyr1053Thrfs*4) in *RP1*, c.623G>A (p.Gly208Asp) in *PRPH2* and c.6957+ 1G>C (p.?) and c.4955C>T (p.Pro1652Leu) in *USH2A*, all of them in heterozygosity (Chapter I, Table 1). Although the patient was diagnosed with CD, any of these genes may be influencing the phenotype. At the time of the study the family segregation analysis could not be carried out, but after the publication of the data we disposed of DNA from the

parents, both asymptomatic. Thus, we confirmed that the *RP1* variant c.3157del and variant c.6957+1G>C in *USH2A* were inherited from the mother, while variants c.623G>A in *PRPH2* and c.4955C>T in *USH2A* from the father. Both *PRPH2* and *RP1* follow an AD mode of inheritance, and it has been described incomplete penetrance associated to both genes (Dietrich, 2002; Soucy et al., 2023; Hitti-Malin et al., 2024). After these studies, we considered the variant identified in *PRPH2* as IRD-causing in this case. Eventually, although *USH2A* and *RP1* cause RP, it cannot be ruled out that they can have an implication in the phenotype throughout the course of the disease. This reflects the relevance of carrying out a segregation analysis in complex cases such as RPN-670, as well as the significance of incomplete penetrance in the diagnosis of IRD (Rodríguez-Muñoz et al., 2021)

These results underscore the importance of performing an exhaustive genetic analysis to identify all variants and genes that are responsible for the disease and can influence the patient's phenotype. This is crucial for ensuring proper monitoring of the patient, providing correct genetic counselling and enabling participation in gene-specific clinical trials or treatments.

IMPACT OF NON-CODING REGIONS ON THE GENETIC DIAGNOSIS OF IRD AND USH

Variants in non-coding regions, such as deep-intronic and those localized to untranslated regions (UTRs), can significantly impact gene regulation and splicing. In the context of IRDs, these variants may alter RNA processing, leading to aberrant splicing or misregulation of gene expression, which eventually contributes to retinal degeneration.

Deep-intronic variants

Deep intronic mutations have been identified in numerous genes associated to IRD and the number of this type of pathogenic variants has grown rapidly in the past three years (Fadaie et al., 2021b; Corradi et al., 2023; Reurink et al., 2023; Yang et al., 2023; Sangermano et al., 2024; Zeuli et al., 2024). This has been possible, in many cases, thanks to WGS approaches, which allow the analysis of non-coding regions. This type of variants can alter transcription regulatory motifs in the sequence giving rise to different splicing effects, such as the inclusion of a PE in the coding region.

As mentioned, the identification of deep-intronic variants is booming with the analysis of the whole genome. It is worth highlighting the numerous deep-intronic variants identified in the *ABCA4* gene (more than 30) accounting for 15% of the disease-causing *ABCA4* variants reported in the Human Gene Mutation Database (HGMD, accessed on 20th of November) (De Angeli et al., 2024). Several hot spots of deep-intronic variants are reported in *ABCA4*, with many variants identified within specific clusters (Zernant et al., 2014; Albert et al., 2018; Runhart et al., 2018; De Angeli et al., 2024). These regions are described to be more susceptible to accumulate deep-intronic variants. Since the inclusion of a PE can be caused by the alteration of either an acceptor/donor splice site or an enhancer/silencer motif, it is not surprising that variants in the vicinity can induce a similar effect in the pre-mRNA. This is the case of variants c.5196+1137G>A and c.5196+1216C>A, both of which caused the same PE inclusion (Braun et al., 2013). In terms of therapy, this could mean an advantage as a single ASO could correct the splicing aberrant effect caused by several closely deep-intronic variants (Albert et al., 2018). Similarly to *ABCA4*, more than 10 deep-intronic variants have already been identified in *USH2A* (LOVD and HGMD, November 2024), making it one of the genes associated with RP that harbours the highest number of reported deep-intronic variants (Vaché et al., 2012; Liquori et al., 2016; González-del Pozo et al., 2018; Fadaie et al., 2021b; Mansard et al., 2021; Reurink et al., 2023).

Given this exponential increase in deep-intronic findings, it is tempting to speculate that the contribution of this type of variants in these genes is even higher than what is described so far, encouraging to keep focusing in the non-coding region analysis.

Deep-intronic variants previously described can be studied by including those regions in customized gene panels or by Sanger sequencing. In this thesis, following these two strategies we completed the genetic diagnosis in a total of 15 patients. The two IRD gene panels including non-coding regions allowed us to identify the second disease-causing variant in two patients. In patient RPN-750 the change c.2991+1655A>G was identified in homozygosity in *CEP290*, reported to be responsible for around half of the LCA cases (Den Hollander et al., 2006; Coppieters et al., 2010; Valkenburg et al., 2018; Girach et al., 2022; Cideciyan et al., 2023). In patient RPN-734, c.4539+2064C>T was identified in *ABCA4* (Zernant et al., 2014). The study of five deep-intronic *USH2A* variants previously described

in the nsRP/USH monoallelic cases allowed to complete the genetic diagnosis in 13 patients. Particularly, variant c.7595-2144A>G was identified in 61.5% of the solved patients, which is consistent with the frequency reported in the literature (Krawitz et al., 2014; Slijkerman et al., 2016). This analysis was specially determinant in patient RP-1741 as variant c.7595-2144A>G was identified in compound heterozygosity with c.9959-4159A>G, also previously described (Liquori et al., 2016).

Despite this, gene panels are usually limited in identification of new non-coding variants. Following this premise, we carried out the study of the whole non-coding regions of USH genes in both monoallelic USH patients and nsRP carrying one variant in *USH2A*. This analysis let the identification of two new deep-intronic variants in *USH2A*, c.8681+3960A>G and c.9958+3438A>G.

Nonetheless, studying non-coding genomic regions presents significant complexity due to their structure and composition. Many of these regions contain highly repetitive sequences, making them difficult to map and avoiding a correct variant calling (Lord and Baralle, 2021). These are often GC-rich regions, which can create secondary structures that hinder sequencing and computational analysis (Lord and Baralle, 2021). These features pose difficulties for analysing and understanding the functional impact of the variants harboured in those regions. Furthermore, splicing prediction is also challenging. This is why many studies have evaluated the cut-off values in order to assess the sensitivity of different tools that would help to analyse these variants (Jaganathan et al., 2019; Riepe et al., 2021; Rowlands et al., 2021). In spite of this, SpliceAI is considered as one of the best tools for splicing prediction. It is a matching learning-based tool for splicing prediction that has shown an accuracy of above 90% for deep-intronic variants when compared with other splicing tools individually (Jaganathan et al., 2019; Rowlands et al., 2019; Wai et al., 2020). Related to this, it is interesting to remark the identification of variants c.4106C>T and c.5168-26A>C in *USH2A* patients RP-1950 and RP-2034, respectively (Chapter II, Table 3) (García-Bohórquez et al., 2024). These two variants were probably detected in previous analysis, but they were not considered because a comprehensive splicing prediction analysis was not performed, or the prediction algorithms available at the time were not sufficiently optimized (Jaganathan et al., 2019; Wai et al., 2020). Based on a score ≥ 0.2 for

SpliceAI and a score >10% for MaxEnt, we detected 16 variants candidate variants, from which four showed an effect in splicing after the functional analysis by minigenes. The accuracy showed by SpliceAI is evident in our results, as if we would had relied solely on SpliceAI, only two of six selected variants (12.5%) would have been false positives. Both variants c.4106C>T and c.5168-26A>C were non-canonical exonic variants, and it is already reported that for this type of variants the cut-offs should be more stringent than those for deep-intronic (Reurink et al., 2023). However, the SpliceAI predicting potential turns out to be more accurate when used in combination with other prediction tools as it does not give information of enhancers and silencers disruption (Rowlands et al., 2021). Eventually, the prediction capacity of this tool can also depend on the gene of interest (Lord and Baralle, 2021).

Since many regulatory elements are not fully characterized, functional studies are needed to validate the predicted effect *in silico*, for instance by minigene assays. It is important to have in mind the limitations associated to this approach. Minigenes are *in vitro* models that do not always quantitatively reproduce the *in vivo* situation as, for instance, it only includes part of the genomic sequence. Moreover, the splicing events are different among tissues and, particularly in retina, the specific splicing factors determine the functional significance of the distinct splicing products (Wen et al., 2010; Albert et al., 2018; Matalkah et al., 2022). Despite this, when affected tissues are inaccessible and the target genes are not expressed in other tissues, minigenes and midigenes assays are very useful as they are considered a standardized approach for preliminary variant pathogenicity classification (Bauwens et al., 2019; Rodriguez-Muñoz et al., 2022). Midigenes provide the possibility of including a larger sequence without disturbing any regulatory element in the vicinity of the variant in order to slightly better resemble the *in vivo* situation. This has been shown in a study carried out by Sangermano *et al*. in 2018, in which they confirmed the capacity of midigenes to show a natural skipping of several exons in *ABCA4* (Sangermano et al., 2018). Moreover, as almost all introns size do not exceed 11kb (Sakharkar et al., 2004), midigenes are very suitable for assessing the splicing effect of many deep-intronic variants. Despite this, in our study, we were able to provide new information about variants affecting splicing by functional studies by minigenes, which can bring some light for future genomic analysis and therapeutic approaches.

An alternative that provides more evidence about what happens *in vivo* is the use of patient-derived cells that can be differentiated to photoreceptors precursor cells (Koolen et al., 2022; Reurink et al., 2023). Nevertheless, different effects between *in vivo* and *in vitro* can occasionally be observed, such as in the inclusion of alternative PEs or in the proportion of mutant and wild-type transcripts. (Khan et al., 2020a; Reurink et al., 2023; De Angeli et al., 2024). Other studies carried out in *ABCA4* variants showed equivalent results in retinal cells differentiated from patient-derived cells (Parfitt et al., 2016; Albert et al., 2018).

Untranslated regions (UTR)

Studying UTRs is crucial in the genetic diagnosis of hereditary diseases such as IRDs, as these regions can regulate gene expression. Variants in UTRs may alter mRNA stability or translation, contributing to pathogenicity in many diseases, making them key in understanding genetic disorders (Whiffin et al., 2020; Ruberto et al., 2021; Trizuljak et al., 2024).

In the design of the panel containing the genomic sequence of USH genes whose study has been carried out in this thesis, UTRs have been taken into account as areas likely involved in the pathology of the patients. However, the scope of its analysis has not allowed us to obtain conclusive results.

In clinical practice, many variants are missed within UTRs due to limitations both in sequencing and in the annotation and interpretation of these variants. It is estimated that only 3 to 20% of the 5'UTR region of the IRD-causing genes are completely covered with WES, and the percentage can vary between 7-39% depending on the kit used (Dueñas Rey et al., 2024). Although the use of WGS does allow the identification of these variants, their interpretation and the lack of *in silico* predictors remain a drawback. In addition, just as we have placed special emphasis on the importance of reanalysis of deep-intronic regions due to the continuous improvement of prediction tools, UTRs should also be reanalysed to avoid overlooking relevant changes.

The information from databases that collets variants identified so far is greatly important, especially due to the difficulty in defining the regulatory elements located in UTR regions

that require ChIP-seq or RNA-seq type analysis (Nishi et al., 2024; Santana et al., 2024; Zhang et al., 2024). Indeed, in a recent retrospective analysis, 58% of 5'UTR variants identified in IRD genes that were submitted in ClinVar were classified as VUS and many others have been overlooked by bioinformatic pipelines and were not reflected in databases. (Dueñas Rey et al., 2024). Currently, *in silico* prediction tools are not capable to predict the pathogenicity of all variants located in the 5'UTR region (Steri et al., 2018), being the most frequently used those which predict uORF cis-elements alterations (Zhang et al., 2021; Filatova et al., 2023). However, the development of an adequate pipeline for correct annotation and prioritization allowed the identification of three variants in 5'UTR in the *MERTK*, *PRPF31* and *RDH12* genes in a single study (Dueñas Rey et al., 2024). Likewise, the annotation of variants in the 5'UTR based on each transcript isoform is very important. In fact, the reanalysis of transcript expression data at a retinal level showed higher levels of expression of non-canonical isoforms of IRD genes (Dueñas Rey et al., 2024). Additionally, a recent study has assessed the prediction capacity of seven different algorithms for non-coding variant interpretation in comparison with UTR variants, which showed less accuracy in UTR variant prediction (Li et al., 2024). This led them to develop two new models in order to predict 5'UTR and 3'UTR variants with higher efficiency. Variants in 3'UTR are also reported in the bibliography (Brewster et al., 2012; Devanna et al., 2018). Despite this, further investigation is needed to develop robust prediction tools as the ability of determining the clinical impact of these variants is still inaccurate (Romo et al., 2024).

Concerning the necessity of validating these variants, some drawbacks to take into account also refers to the need for functional studies, such as luciferase assays (preferably in retinal cell lines) or RNA analysis of patient tissue (Coppieters et al., 2015; Ruberto et al., 2021; Daich Varela et al., 2023; Dueñas Rey et al., 2024). Besides, neither all tissues are equally accessible nor all genes are expressed in peripheral blood, which further limits this study. Though the variants in UTR regions were sequenced and analysed in this thesis, due to the complexity and limitations that we have just mentioned, we cannot rule out that some patients carry variants that were overlooked. In this way, these regions must to be reanalysed in the future.

UNSOLVED CASES

Despite the advancements in the genetic diagnosis of IRD, the percentages of unsolved patients that we observed are 42.4% and 67.2% for the custom IRD genes panels and the USH genes panel, respectively. These remarkable numbers of unresolved cases suggest that there are other factors involved in the pathogenicity of these diseases. These may have been overlooked or not taken into account in the design of these strategies.

At a clinical level, the existence of phenocopies could be intervening in some uncharacterized patients. This occurs when a clinical profile caused by mutations in a certain gene is indistinguishable from other clinical entities. Phenocopies have been described in certain IRD genes, as well as cases of mosaicism due to mutations in genes that mimic other diseases (Strubbe et al., 2021; Abdolrahimzadeh et al., 2022; Tan et al., 2023). Particularly, several genes such as *CEP250*, *CEP78*, *ESPN*, *ARSG*, among others (Nolen et al., 2020), present a clinical picture similar to USH. Nonetheless, none of them fit into the three USH subtypes, since there are some differences in the age of onset or the severity of the symptoms. Due to these discrepancies, they were classified under the atypical or even USH-like group. Moreover, in some cases RP and SNHL could be inherited independently simulating an USH case (Neuhaus et al., 2017). This second scenario is not unlikely given the high prevalence of carriers for a variant in an autosomal recessive IRD gene as well as for nonsyndromic hearing loss (Nishiguchi and Rivolta, 2012; Shearer and Smith, 2012). In the same way, other syndromes, such as PHARC, combine RP and hearing loss with other possible clinical signs and can be mistaken for USH. (Wawrocka et al., 2024). Thus, it would not be surprising that some uncharacterized IRD patients present other clinical features that have not been reflected in the patient anamnesis of unsolved cases.

An example of this could be the *TBC1D32* gene, which is described in the bibliography as causing Oro-Facial-Digital Syndrome (OFD) type IX and it has recently been associated with RP in three patients diagnosed with OFD IX from two different families. (Hietamäki et al., 2020; Harris et al., 2023). Lastly, mutations in *TBC1D32* have been identified in three families that showed isolated RP (Bocquet et al., 2023), which is a great finding that could contribute to classifying this gene as a candidate for RP. In this thesis, we have presented a *TBC1D32*-related complex ciliopathy case, who associated RP and SNHL. We identified two variants in trans in *TBC1D32* after the thorough analysis of genes included in the clinical

exome. After these results, it is tempting to suggest that *TBC1D32* clinical spectrum has been extended to hearing impairment, particularly, to bilateral SNHL. This is not surprising as many ciliopathies associate inner ear cilia problems (Shi et al., 2022; Goutaki et al., 2023). This reinforces the need of performing a detailed clinical anamnesis and an exhaustive search of genes recently described in the literature.

Besides, the identification of new IRD genes is increasing (Jurkute et al., 2022; Millo et al., 2022; Stallworth et al., 2023), whose number comes to 316 in RetNet (https://retnet.org/home.htm, accessed December, 2024) so far. Given this situation, it is not unreasonable to suggest that the genetic cause of some uncharacterized patients is harboured in a new unidentified gene that is not yet included in the designed panels. In line with this, not all genes reported to cause IRD are reflected in databases such as HPO (https://hpo.jax.org/) and OMIM (https://www.omim.org/), among others. This is essential for genetic diagnosis since many genes could be overlooked if databases are not updated.

It is plausible that the genetic cause of some of our unsolved patients may be behind other reasons. For instance, it is reasonable that some of the VUS that have been identified are actually causing the phenotype in some unsolved patients in our cohort. However, using the bioinformatics tools available now, we cannot classify them as pathogenic. To overcome these issues, additional functional studies as RNA and protein expression assays and different animal and cellular models as well as organoids could be very useful (Zeitz et al., 2024). This is especially convenient when the expression of the gene in question is restricted to the retina or other inaccessible tissues, or even when no functional data are available (Zhou et al., 2020; Moran et al., 2023; Esteve-Garcia et al., 2024). As an example, CRISPR editing tools together with transcriptomics have been used to validate the function of VUS highlighting the importance of these studies in patients who suffer from rare diseases (Fear et al., 2022). In this line, Huynh *et al*., remark the need of assessing the consequences in protein function of VUS, as it can also be essential for ruling out incorrect clinical diagnosis and administrating a correct treatment to patients when applicable (Huynh et al., 2024). On account of all this, robust prediction tools and different integrative approaches should be developed in the near future. Nevertheless, except for the patients analysed with the panel containing the entire sequence of USH genes, non-coding regions are not included in the

IRD gene panels, so it is possible that some unsolved patients harbour the disease-causing mutations in those regions.

Eventually, regions such as UTR, would need a deeper analysis and well curated tools in order to unravel pathogenic variants localised to those regions. Otherwise, analysing SVs, STRs, TEs and other complex genomic rearrangements by these approaches remain challenging. To overcome these limitations, in the following section we discuss some strategies to broaden our study that can help with some of these issues.

New Strategies to Improve the Diagnosis of IRDs

As it was discussed above, non-coding variants play an important role in IRD genetic diagnosis. Regarding this, WGS has meant a promising approach to dig into non-coding regions. The diagnostic capacity of this type of strategies in combination with an accurate pipeline and an exhaustive interpretation of the variants turns out to be greater than with WES or customized gene panels (Reurink et al., 2023). In a recent study, a 93% of solved IRD patients was achieved after the analysis of a smMIPs-based panel in combination with WGS (Basharat et al., 2024). The cohort encompasses consanguineous families, which certainly eases the analysis. However, this diagnostic percentage could also insinuate the importance of a further study such as WGS to increase the diagnostic ratio as well as the reanalysis of samples that had been previously studied. Even though this is an exceptional high diagnostic rate, it is still outstanding the greater number of solved patients for WGS rather than WES (Fadaie et al., 2021b; Hussain et al., 2023; Wen et al., 2023). Thus, WGS provides a new diagnostic approach that allows the detection of some SV, non-coding variants and even those located in GC-rich regions (Dockery et al., 2021; González-del Pozo et al., 2022). However, some progress needs to be done with data storage and accurate protocols for non-coding variants interpretation.

Despite the advantages of short-read WGS, this technology has some drawbacks due to the size of the sequences, since it does not detect complex SVs, STRs, transposable elements (TEs), among others. All these events can contribute to the genetic characterization of the unsolved IRD patients, as it has been described that SVs contribute to the IRD mutational landscape from 5 to 15% (Van Schil et al., 2018; Nikopoulos et al., 2019; Zampaglione et al.,

2020; Biswas et al., 2021; de Bruijn et al., 2023). This has been possible due to the development of strategies based on long-read sequencing that are able to precisely identify SVs (Beyter et al., 2021). Different technologies are now being applied for long-read sequencing, such as Oxford Nanopore and Pacific Biosciences (PacBio). Long-read sequencing also entails several limitations related to the high costs, low throughput, computational overload and the lack of information reflected in the databases (Mastrorosa et al., 2023). In general, long-reads sequencing is quite efficient in reducing the percentage of off-target and provides good results in sequence coverage, as they allow real-time DNA analysis without the need of specific sample preparation and amplification (Loose et al., 2016; Nakamichi et al., 2023; Wojcik et al., 2023).

Optical Genome Mapping has emerged as another cytogenetics-based strategy that allows identification of large SVs by analysing ultra-long DNA fragments. This approach has shown that, in combination with reanalysis of short-read data, the diagnosis rate can be increased, which highlights the importance of not overlooking SVs (de Bruijn et al., 2023).

As a future perspective, patients who remain undiagnosed after our initial analysis will be subjected to further studies incorporating these advanced diagnostic approaches. The combination of WGS, long-read sequencing technologies and Optical Genome Mapping, aims to maximize diagnostic yield and uncover the genetic cause of IRD patients that may have been missed in prior approaches. Integrating these technologies will enhance the likelihood of achieving a comprehensive genetic diagnosis for these patients.

ANTISENSE OLIGONUCLEOTIDES AS A PROSPECTIVE THERAPY FOR IRDs

Recent advances in therapy for IRDs, including viral vector-based approaches, gene editing technologies and cell therapy strategies have shown promising results (Liu et al., 2024c; Yalla and Kuriyan, 2024). However, deep-intronic variants, which can disrupt gene function, present unique challenges. One emerging therapeutic option for these variants is the use of ASOs, which can modulate splicing and restore normal gene expression with many advantages (Girach et al., 2022).

Thus, the thorough study of intronic regions has made the splicing modulation one of the strategies of most interest so far. Particularly, PEs blocking is one of the approaches that is being assessed given its high efficacy and the continuous increase in the number of deep-intronic disease-causing variants that are being identified (Garanto et al., 2016; Garanto and Collin, 2018; Reurink et al., 2023; Yamada et al., 2024). For this reason, a splicing modulation strategy was designed in this thesis in three *USH2A* deep-intronic variants by using ASOs in order to block the inclusion of PEs. We obtained good results of minimum efficient doses, which are comparable with other studies and would be sufficient to continue with further studies, so that a promising therapy would be developed (Lentz et al., 2013; Dulla et al., 2021; Schellens et al., 2023). Assays carried out in PE50 in *USH2A* gene also showed that problematic regions, such as GC-rich regions, can interfere negatively in the efficiency of ASOs. In fact, we obtained the higher minimum efficient dose in PE50 assays, requiring the synergistic use of two different ASOs to increase the effectiveness in PE50 blocking. Even so, our results are encouraging for continuing with *in vitro/in vivo* studies and toxicity assays.

ASOs offer a series of advantages comparing to strategies such as gene augmentation. Firstly, they do not modify the amount of RNA or protein as they directly and temporarily interfere with the pre-mRNA, also reducing off-targets. Due to its small size, its release can be carried out intravitreally and is capable of reaching all cell types of the retina. Furthermore, the use of ASOs to modulate splicing does not necessarily involve preclinical animal testing (which does not exempt the need to establish minimum doses and toxicity tests) (Girach et al., 2022).

On the other hand, to overcome the limitations that the therapeutic use of ASOs entails, such as stability and half-life *in vivo,* different studies have been carried out. Second-generation ASOs, which include 2'-O-MOE modifications, have helped improve these aspects by increasing the binding affinity and conferring more nuclease resistance (Bennett and Swayze, 2010; Yu et al., 2013). This advantage also carries a drawback, since systems such as AAVs neither produce nor release second-generation ASOs. To this end, the use of U7snRNA complexes followed by AAV-mediated release may provide a solution that also contributes to improve the half-life and reduce the number of intravitreal injections (Aupy

et al., 2020). This complex protects the ASO from degradation promoting its accumulation in the nucleus for splicing correction. Furthermore, to improve their ability of reaching different cell types and tissues, various chemical modifications have been studied. These strategies include those focused on enhancing nuclease stability and affinity to RNA and protein, or those that help cross several barriers in target tissues. (Seth et al., 2019). The evaluation in efficacy of three different ASOs modifications has also been assessed in retina (Vázquez-Domínguez et al., 2024). Particularly, they have observed a higher splicing modulation efficacy with 2'O-methoxyethyl-phosphoriate (MOE/PS) ASOs with no external alterations in *in vivo* analysis performed in mouse retina (Vázquez-Domínguez et al., 2024).

Several trials based on ASOs have been developed for hereditary conditions, including IRDs. Particularly, there are three trials ongoing for *CEP290* and *RHO*-associated IRDs and *USH2A*-associated USH cases under the name of Sepofarsen/QR-110, QR-1123 and Ultevursen/QR-421a, respectively (Girach et al., 2022) using different strategies related to ASOs, which involve exon-skipping, knockdown of mutant mRNA and base RNA editing by changing specific sites (Collin and Garanto, 2017; Xue and MacLaren, 2020; Kuijper et al., 2021).

Regarding PEs blocking, Sepofarsen (QR-110) is a clinical trial for splicing modulation of the frequent deep-intronic variant c.2991+1655A>G in *CEP290*, which is currently in charge of Théa Laboratoires (https://www.laboratoires-thea.com). As visual advances and long durability of the treatment was shown after a single injection, the study was extended to both eyes in patients included in the trial. Findings showed an enhanced sensitivity of foveal cones after injection and they suggest that posterior intravitreal injections should be spaced at least two years apart to minimize toxicity (Cideciyan et al., 2023). As it was remarked before, this deep-intronic variant was identified in patient RPN-750, standing out the implication of our studies by providing this patient a new future therapeutic option. More studies are focused on this strategy, such as the case of the c.1245-521A>G variant in *CHM* (Zhai et al., 2023).

ASOs also have the ability of skipping constitutive exons of a gene, so that these regions are not included in the protein sequence. This has been very useful in the case of Ultevursen (QR-421a). The target of this trial is the skipping of exon 13 of *USH2A* gene. This strategy is very interesting as this is the most frequently mutated gene in USH and one of the most

prevalent genes in nsRP. Moreover, exon 13 in this gene harbours the two variants with the highest frequency among *USH2A*-associated USH and nsRP patients (Lin et al., 2024b, 2024a). At this moment, this therapeutic strategy is being studied by Théa Laboratoires (Dulla et al., 2021). Intravitreal injection of Ultevursen in adults showed significantly improved retinal sensitivity in the treated eyes compared to controls as well as restoration of usherin expression and good responses from ERG (Girach et al., 2022). Eventually, further studies for efficacy and safety evaluation had been initiated (Girach et al., 2022). These findings have driven the researchers to continue studying exon-skipping based strategies in this gene (Schellens et al., 2023).

Eventually, the QR-1123 trial based on mRNA allele-specific degradation through ASOs in RP autosomal dominant cases due to a mutation in *RHO* is being evaluated (NCT04123626).

The outcomes from the effort in studying the therapeutic potential of ASOs are perfectly represented by the approved treatment for SMA (Spinal Muscular Atrophy). Additionally, for DMD (Duchenne Muscular Dystrophy), four ASOs strategies based on the pre-mRNA splicing modulation have been conditionally approved by FDA (Finkel et al., 2017; Li et al., 2018; Wilton-Clark and Yokota, 2023). In addition, many other trials are been developed for this condition (Wilton-Clark and Yokota, 2023).

Although the use of ASOs for specific mutations do not represent an attractive approach in terms of number of patients to be treated, there are some exceptions that should not be underestimated. This is the case of variants such as c.7595-2144A>G in *USH2A* and c.2991+1655A>G in *CEP290* that have turned out to be very frequent, or those ASO-based strategies that are being evaluated for treating deep-intronic variants in close vicinity in *ABCA4*. In this line, we should not rush into estimating the frequency of new deep-intronic variants in order to be being a future target for ASO-based approaches, as well as the final scope of many of these strategies.

CONCLUSIONS

CONCLUSIONS

1. The use of gene panels targeting inherited retinal dystrophies-associated genes allowed us to identify the genetic cause in 57.6% of cases, aligning with global diagnostic rates achieved through targeted sequencing and WES.

2. The most frequently mutated genes in our inherited retinal dystrophies cohort are *ABCA4, PRPH2* and *USH2A*, which account for the 53% of the solved cases.

3. In 9% (five cases) of the solved patients, we identified pathogenic variants in more than one gene, and in two of them, more than one gene could be influencing the patient's phenotype. This highlights the importance of a thorough analysis in order to provide a proper genetic counselling and clinical follow-up.

4. The analysis of deep-intronic variants previously described in Usher syndrome and/or inherited retinal dystrophies-associated genes allowed us to complete the genetic diagnosis in a total of 15 patients. Thirteen of them carried the second disease-causing variant in *USH2A,* one in *ABCA4* and one in *CEP290*. These results underscore the importance of analysing non-coding regions in clinical practice to increase the diagnostic yield.

5. The analysis of the panel containing the whole genome sequence of Usher syndrome genes revealed two new deep-intronic variants in *USH2A* and allowed us to complete the genetic diagnosis in two patients. In this regard, the progressive application of whole genome sequencing in unsolved patients will facilitate the identification of additional pathogenic variants in non-coding regions in IRD-related genes.

6. The re-analysis of exon-flanking or missense variants detected two variants that alter a splicing regulatory element and were missed in previous studies. This exposes the necessity of assessing *in silico* splicing analysis for all types of variants, as well

as the importance of re-evaluating previously identified variants using updated splicing prediction tools.

7. Functional assays using minigenes enabled us to validate a deleterious effect on the splicing process in four out of the 16 candidate variants. This is a robust validation technique that provides valuable insights into potential splicing alterations.

8. In this study, we demonstrated the efficacy of antisense oligonucleotides in redirecting aberrant splicing caused by three deep-intronic *USH2A* variants: c.9958+3438A>G, c.8681+3960A>G and c.14134-3169A>G. The results demonstrated successful PE blocking and restoration of normal splicing patterns in vitro for these three variants.

9. Despite analysing the untranslated regions of Usher syndrome genes, we did not find any candidate variants. Nevertheless, predicting their functional effects in those regions remains challenging and variants located in these regions should be re-evaluated in the future.

10. The findings in the patient harbouring *TBC1D32* mutations, illustrate the importance of performing a proper anamnesis and comprehensive genetic approaches. This would represent the first reported *TBC1D32*-related case involving retinitis pigmentosa and sensorineural hearing loss, expanding its clinical spectrum to include hearing impairment.

APPENDIX

APPENDIX

Supplemental material from Chapter I

Table S1. Genes included in PV1.

ABCA4	*CA4*	*CNGB3*	*GDF6*	*KIZ*	*OTX2*	*PRPF6*	*RHO*	*SLC7A14*
ADAM9	*CABP4*	*CNNM4*	*GNAT2*	*KLHL7*	*PDE6A*	*PRPF8*	*RIMS1*	*SNRNP200*
ADAMTS18	*CACNA1F*	*CRB1*	*GUCA1A*	*LCA5*	*PDE6B*	*PRPH2*	*RLBP1*	*SPATA7*
AIPL1	*CACNA2D4*	*CRX*	*GUCA1B*	*LRAT*	*PDE6C*	*RAB28*	*ROM1*	*TIMP3*
ARL2BP	*CDH3*	*DHDDS*	*GUCY2D*	*MAK*	*PDE6G*	*RAX2*	*RP1*	*TOPORS*
ARL6	*CDHR1*	*DRAM2*	*HK1*	*MERTK*	*PDE6H*	*RBP3*	*RP1L1*	*TTC8*
BBS1	*CEP290*	*DTHD1*	*IDH3B*	*MVK*	*PITPNM3*	*RBP4*	*RP2*	*TTLL5*
BBS2	*CERKL*	*EFEMP1*	*IMPDH1*	*NEK2*	*POC1B*	*RD3*	*RP9*	*TULP1*
BEST1	*CHM*	*ELOVL4*	*IMPG1*	*NEUROD1*	*PRCD*	*RDH12*	*RPE65*	*UNC119*
C1QTNF5	*CLRN1*	*EYS*	*IMPG2*	*NMNAT1*	*PROM1*	*RDH5*	*RPGR*	*USH1C*
C21orf2	*CNGA1*	*FAM161A*	*IQCB1*	*NR2E3*	*PRPF3*	*RGR*	*RPGRIP1*	*USH2A*
PCARE	*CNGA3*	*FLVCR1*	*KCNJ13*	*NRL*	*PRPF31*	*RGS9*	*SAG*	*ZNF408*
C8orf37	*CNGB1*	*FSCN2*	*KCNV2*	*OFD1*	*PRPF4*	*RGS9BP*	*SEMA4A*	*ZNF513*
chr1:216064520-216064560 (*USH2A*)			chr1:94492980-94493020 (*ABCA4*)			chr12:88494940-88494980 (*CEP290*)		
chr19:54633379-54633419 (*PRPF31*)				chrX:13770172-13770212 (*ODF1*)				

Genomic hg19 coordinates correspond to deep-intronic regions in which variants have been previously described as pathogenic.

Table S2. Genes included in PV2.

ABCA4	*CDHR1*	*GNAT2*	*MAK*	*PRPF31*	*RP1L1*	chr1:216064520-216064560 (*USH2A*)
ADAM9	*CEP290*	*GUCA1A*	*MERTK*	*PRPF4*	*RP2*	chr1:215967733-215967833 (*USH2A*)
ADAMTS18	*CERKL*	*GUCA1B*	*NEK2*	*PRPF6*	*RPE65*	chr1:215827262-215827362 (*USH2A*)
AIPL1	*CHM*	*GUCY2D*	*MFSD8*	*PRPF8*	*RPGR*	chr1:94492980-94493020 (*ABCA4*)
ARL2BP	*CNGA1*	*HK1*	*NMNAT1*	*PRPH2*	*RPGRIP1*	chr1:94549552-94549652 (*ABCA4*)
ARL3	*CNGA3*	*IDH3B*	*NR2E3*	*RAB28*	*SAMD11*	chr1:94549731-94549831 (*ABCA4*)
ARL6	*CNGB1*	*IFT140*	*NRL*	*RAX2*	*SAG*	chr1:94526884-94526984 (*ABCA4*)
BBS1	*CNGB3*	*IFT172*	*OFD1*	*RBP3*	*SEMA4A*	chr1:94546730-94546864 (*ABCA4*)
BBS2	*CNNM4*	*IMPDH1*	*OTX2*	*RBP4*	*SLC7A14*	chr1:94527648-94527748 (*ABCA4*)
BEST1	*CRB1*	*IMPG1*	*PDE6A*	*RD3*	*SNRNP200*	chr1:94511076-94511176 (*ABCA4*)
C1QTNF5	*CRX*	*IMPG2*	*PDE6B*	*RDH12*	*SPATA7*	chr1:94509749-94509849 (*ABCA4*)
C21orf2	*CTNNA1*	*IQCB1*	*PDE6C*	*RDH5*	*TOPORS*	chr1:94496459-94496559 (*ABCA4*)
PCARE	*DHDDS*	*KCNJ13*	*PDE6G*	*REEP6*	*TTC8*	chr1:94494092-94494192 (*ABCA4*)
C8orf37	*DRAM2*	*KCNV2*	*PDE6H*	*RGR*	*TTLL5*	chr1:94493851-94493951 (*ABCA4*)

CA4	*ELOVL4*	*KIAA1549*	*PITPNM3*	*RHO*	*TULP1*	chr1:94492886-94493050 (*ABCA4*)
CABP4	*EYS*	*KIZ*	*POC1B*	*RIMS1*	*UNC119*	chr1:94484032-94484132 (*ABCA4*)
CACNA1F	*FAM161A*	*KLHL7*	*PRCD*	*RLBP1*	*USH2A*	chr1:94483872-94484052 (*ABCA4*)
CACNA2D4	*PRDM3*	*LCA5*	*PROM1*	*ROM1*	*ZNF408*	chr1:94481917-94482017 (*ABCA4*)
CDH3	*GDF6*	*LRAT*	*PRPF3*	*RP1*	*ZNF513*	chr12:88494940-88494980 (*CEP290*)
chr1:216039671-216039771 (*USH2A*)						chr19:54633379-54633419 (*PRPF31*)
chr1:216247426-216247526 (*USH2A*)						chrX:13770172-13770212 (*ODF1*)

Genomic hg19 coordinates correspond to deep-intronic regions already present in PV1 and those in which new variants have been described as pathogenic post-PV1.

Table S3. Deep-intronic variants described in *USH2A* and *ABCA4* as pathogenic.

Gene	Nucleotide change	Reference
USH2A (NM_206933.2)	c.7595-2144A>G	(Vaché et al., 2012)
	c.5573-843A>G c.8845+628C>T c.9959-4159A>G	(Liquori et al., 2016)
	c.14134-3169A>G	(Baux et al., 2017)
ABCA4 (NM_000350.2)	c.5196+1056A>G c.5196+1216C>A	(Braun et al., 2013)
	c.1938−619A>G c.4539+2064C>T	(Zernant et al., 2014)
	c.4539+2001G>A	(Bauwens et al., 2015)
	c.769-605C>T c.4539+859C>T c.4539+2065C>G	(Khan et al., 2019)
	c.2919−826T>A c.3050+370C>T	(Fadaie et al., 2019)
	c.769–784T>C c.4253+43G>A c.859-506G>C c.1937+435C>G c.4539+1100A>G	(Sangermano et al., 2019)
	c.859-540C>G c.5197-557G>T	(Bauwens et al., 2019)

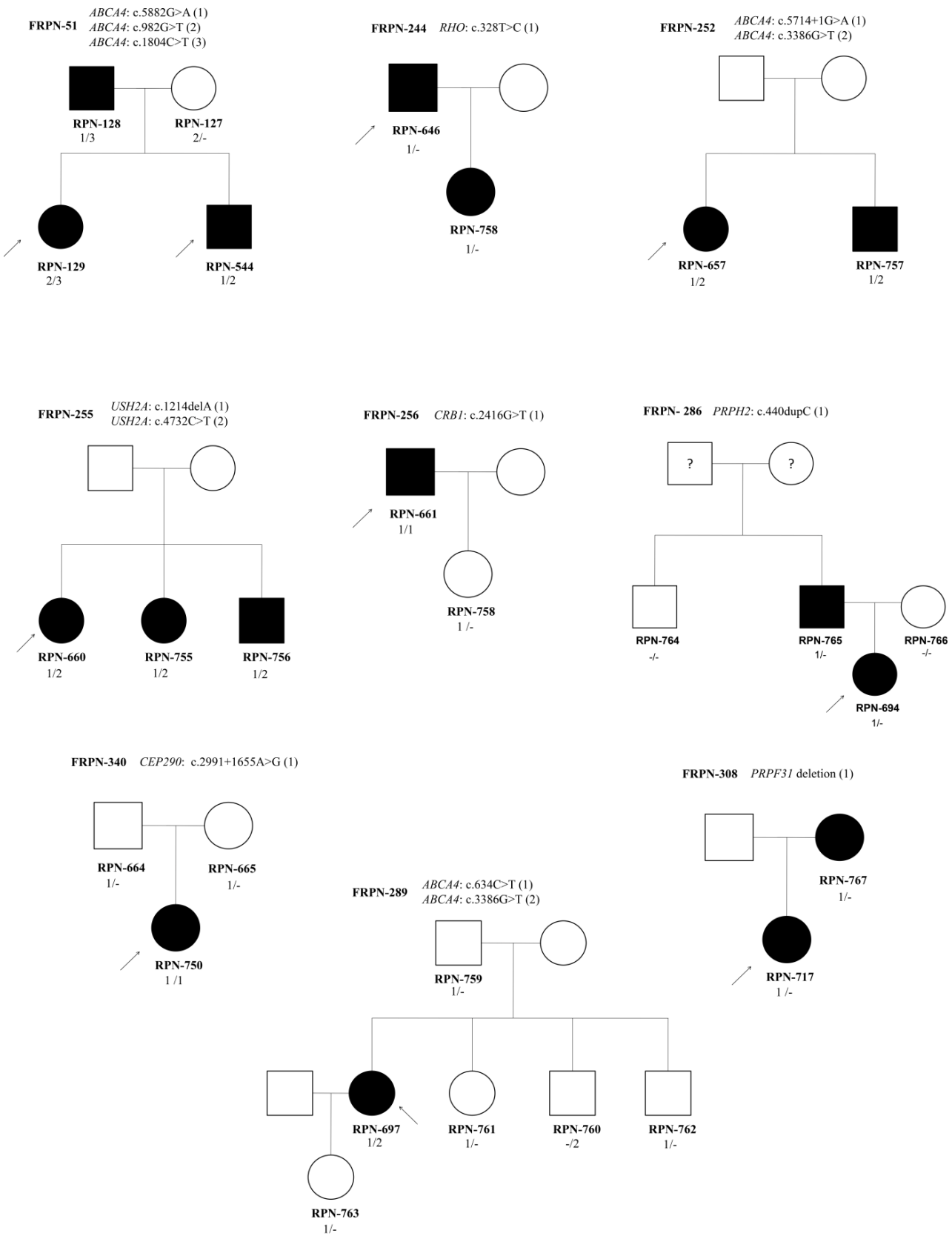


Figure S1. Family pedigrees in which segregation analysis was carried out. Arrows indicates proband members in each family. FRPN: Family number; RPN: Patient number; ?: Unknown clinic.

Supplemental material from Chapter II

Table S1. Studies performed in the selected patients.

Patient	Family	Gene	Dx	Allele 1	SS	NGS	SEG	Reference
RP-331	**-**	***USH2A***	**ARRP**	**c.2276G>T; p.(Cys759Phe)**	**SP**	**NP**	**NA**	(Dreyer et al., 2000)
RPN-645	FRPN-243	*USH2A* *CEP290*	ARRP	c.2276G>T ; p.(Cys759Phe) c.7394_7395del; p.(Glu2465Valfs*2)	SP	P1	NA	(Dreyer et al., 2000) (Nishiguchi and Rivolta, 2012)
RP-654	**FRP-30**	***USH2A***	**USH**	**c.11146C>T; p.(Gln3716*)**	**SP**	**NP**	**NA**	(Vaché et al., 2012)
RP-681	FRP-49	*CDH23*	USH	c.7279del; p.(Thr2427Leufs*47)	NP	P1	NA	(Fuster-García et al., 2018)
RPN-803	FRPN-372	*USH2A* *ZNF408*	ARRP	c.2276G>T; p.(Cys759Phe) c.1534dup; p.(Arg512Profs*27)	NP	P1	NA	(Dreyer et al., 2000) This study
RP-903	FRP-57	*USH2A*	USH	c.5039A>G; p.(Lys1680Arg)	NP	P2	NA	(Costa et al., 2017)
RP-904	FRP-58	*USH2A*	USH	c.9799T>C; p.(Cys3267Arg)	SP	NP	NA	(Nájera et al., 2002)
RP-925	-	*USH2A*	USH	c.2276G>T; p.(Cys759Phe)	SP	NP	NA	(Dreyer et al., 2000)
RP-963	FRP-140	*USH2A*	USH	c.2276G>T; p.(Cys759Phe)	SP	NP	NA	(Dreyer et al., 2000)
RP-1036	FRP-163	*USH2A*	USH	c.2809+1G>A; p.(?)	NP	P1	CC	(Baux et al., 2014)
RP-1222	FRP-228	*USH1C*	USH	c.1234G>A; p.(Asp412Asn)	NP	P1	NA	(Fuster-García et al., 2018)
RP-1360	FRP-283	*USH2A*	USH	c.2276G>T; p.(Cys759Phe)	SP	P1	NA	(Dreyer et al., 2000)
RP-1387	FRP-320	*PCDH15*	USH	c.3817C>A; p.(Arg1273Ser)	NP	P1	NA	This study
RP-1455	FRP-337	*USH2A*	USH	c.5666A>G; p.(Asp1889Gly)	SP	P1	NA	(Perea-Romero et al., 2021)
RP-1472	**FRP-341**	***USH2A***	**USH**	**c.2299del; p.(Glu767Serfs*21)**	**SP**	**NP**	**NA**	(Eudy et al., 1998)
RP-1485	**FRP-344**	***USH2A***	**USH**	**c.1214del; p.(Asn405Ilefs*3)**	**SP**	**NP**	**NA**	(Bernal et al., 2005)
RP-1494	**FRP-348**	***USH2A***	**USH**	**c.2299del; p.(Glu767Serfs*21)**	**SP**	**NP**	**CC**	(Eudy et al., 1998)
RP-1496	FRP-350	*ADGRV1*	USH	c.3443G>A; p.(Gly1148Asp)	NP	P1	NA	(Wang et al., 2014)
RP-1541	FRP-367	*USH2A*	USH	c.12067-2A>G; p.?	SP	NP	NA	(Rivolta et al., 2000)
RP-1569	**FRP-380**	***USH2A***	**USH**	**c.2299del; p.(Glu767Serfs*21)**	**SP**	**NP**	**NA**	(Eudy et al., 1998)
RP-1595 **RP-1596**	**FRP-389**	***USH2A***	**USH**	**c.10636G>A; p.(Gly3546Arg)** **c.10636G>A; p.(Gly3546Arg)**	**SP** **SP**	**NP** **NP**	**NA** **NA**	(Garcia-Garcia et al., 2011)
RP-1600	FRP-391	*USH2A*	USH	c.1144G>A; p.(Gln5796*)	SP	P1	NA	(Garcia-Garcia et al., 2011)
RP-1634	FRP-410	*ADGRV1*	USH	c.17386C>T; p.(Gln5796*)	NP	P1	NA	(García-García et al., 2013)
RP-1635	FRP-411	*USH2A*	USH	c.2276G>T; p.(Cys759Phe)	SP	NP	NA	(Dreyer et al., 2000)

RP-1647	**FRP-417**	***USH2A***	**ARRP**	**c.2276G>T; p.(Cys759Phe)**	**SP**	**NP**	**NA**	(Dreyer et al., 2000)
RP-1716	FRP-437	*USH2A*	USH	c.5858C>G; p.(Ala1953Gly)	SP	P1	NA	(McGee et al., 2010)
RP-1726	FRP-448	*USH2A*	USH	c.13514A>G; p.(Tyr4505Cys)	SP	NP	NA	(Zampaglione et al., 2020)
RP-1727	FRP-449	*USH2A*	USH	c.1214del; p.(Asn405Ilefs*3)	SP	NP	NA	(Bernal et al., 2005)
RP-1728	FRP-446	*USH2A*	USH	c.13514A>G; p.(Tyr4505Cys)	SP	NP	NA	(Zampaglione et al., 2020)
RP-1736	FRP-455	*USH2A*	USH	c.2299del; p.(Glu767Serfs*21)	SP	NP	NA	(Eudy et al., 1998)
RP-1741#	**FRP-460**	***USH2A***	**USH**	**c.7595-2144A>G; p.(Lys2532Thrfs*56)**	**SP**	**P1**	**NA**	(Vaché et al., 2012)
RP-1776	**FRP-484**	***USH2A***	**ARRP + SNHL**	**c.2276G>T; p.(Cys759Phe)**	**SP**	**NP**	**NA**	(Dreyer et al., 2000)
RP-1815	FRP-505	*USH2A*	USH	c.908G>A; p.(Arg303His)	NP	P2	CC	(Yan et al., 2009)
RP-1943	FRP-566	*USH2A*	ARRP	c.2276G>T; p.(Cys759Phe)	SP	P1	NA	(Dreyer et al., 2000)
RP-1950	FRP-568	*USH2A*	USH	c.2299del; p.(Glu767Serfs*21)	SP	P1	NA	(Eudy et al., 1998)
RP-1953	FRP-570	*USH1C*	USH	c.1859G>T; p.(Arg620Leu)	NP	P1	NA	(Bonnet et al., 2016)
RP-1994	FRP-585	*USH2A*	ARRP	c.2299del; p.(Glu767Serfs*21)	SP	P1	NA	(Eudy et al., 1998)
RP-2034	FRP-614	*USH2A*	USH	c.2299del; p.(Glu767Serfs*21)	SP	P1	CC	(Eudy et al., 1998)
RP-2055	**FRP-619**	***USH2A***	**USH**	**c.8435_8438del; p.(Thr2812Metfs*17)**	**SP**	**NP**	**NA**	(Aller et al., 2006)
RP-2106	FRP-645	*USH2A*	USH	c.5933C>T; p.(Pro1978Leu)	SP	NP	NA	This study
RP-2131	FRP-667	*MYO7A*	USH	c.3G>A; p.(?)	NP	P1	NA	(Fuster-García et al., 2018)
RP-2140	FRP-676	*CDH23*	USH	c.6393del; p.(Ile2132Serfs*11)	NP	P1	NA	(Oshima et al., 2008)
RP-2159	FRP-675	*USH2A*	USH	c.3637_3642dup; p.(Phe1213_Ala1214dup)	SP	P1	NA	This study
RP-2213	FRP-719	*MYO7A*	ARRP	c.640G>A; p.(Gly214Arg)	NP	P1	NA	(Adato et al., 1997)
RP-2214	FRP-720	*USH2A*	ARRP	c.2431_2432del; p.(Lys811Aspfs*11)	NP	P1	NA	(Nájera et al., 2002)
RP-2224	**FRP-722**	***USH2A***	**USH**	**c.2431_2432del; p.(Lys811Aspfs*11)**	**SP**	**NP**	**NA**	(Nájera et al., 2002)
RP-2232	FRP-722	*USH2A*	USH	c.1898C>A; p.(Ser633*)	SP	P1	NA	(Baux et al., 2014)
RP-2237	FRP-724	*USH2A*	ARRP	c.12575G>A; p.(Arg4192His)	SP	NP	NA	(Ávila-Fernández et al., 2010)
RP-2238	FRP-725	*USH2A* *PDE6H*	ARRP	c.1724G>A; p.(Cys575Tyr) c.134+2T>C; p.(?)	SP	NP	NA	(Bonnet et al., 2011) This study
RP-2239	FRP-726	*USH2A*	ARRP	c.7061G>A; p.(Arg2354His)	SP	P1	NA	This study
RP-2241	FRP-728	*USH2A*	ARRP	c.2276G>T; p.(Cys759Phe)	SP	NP	NA	(Dreyer et al., 2000)
RP-2262	FRP-730	*ADGRV1*	USH	c.1875G>A; p.(Asp6252Asn)	NP	P1	NA	This study
RP-2264	FRP-732	*USH2A*	USH	c.1055C>T; p.(Thr352Ile)	SP	P2	NA	(Baux et al., 2007)

RP-2266	FRP-734	*USH2A*	USH	c.9799T>C; p.(Cys3267Arg)	SP	NP	NA	(Nájera et al., 2002)
RP-2268	FRP-736	*USH2A*	ARRP	c.10073G>A; p.(Cys3358Tyr)	NP	P2	NA	(McGee et al., 2010)
RP-2269	FRP-737	*USH2A*	ARRP	c.1214del; p.(Asn405Ilefs*3)	NP	P2	NA	(Bernal et al., 2005)
RP-2270	FRP-738	*USH2A*	ARRP	c.12574C>T; p.(Arg4192Cys)	NP	P2	NA	(Corton et al., 2013)
RP-2275	FRP-740	*USH2A*	USH	c.5776+1G>A; p.(?)	SP	P1	NA	(Sandberg et al., 2008)

Homozygous and heterozygous patients for deep-intronic variants in USH2A are highlighted in bold. RP/RPN: Patient number; "-": Unkown; RP/FRPN: Family number; Dx: Diagnosis; ARRP: Autosomal Recessive Retinitis Pigmentosa; USH: Usher Syndrome; SS: Sanger Screening; SP: Screening Performed; NP: Not Performed; P1: Panel design one; P2: Panel design two; SEG: Segregation analysis. The "#" symbol refers to the NGS positive control; CC: Cosegregation confirmed.

Table S2. Sequence of antisense oligonucleotides tested for the aberrant splicing modulation caused by three deep-intronic variants.

Variant	Oligonucleotide	Sequence (3'-5')	G-C%
USH2A: c.14134-3169A>G	AON1-PE64	CCAUUUUUCUAGGAGAAGCCC	47.6
	AON2-PE64	CAACUGUUUGUUCACAAGGU	40
USH2A: c.9958+3438A>G	AON1-PE50	UGCAGGUGUAAAAAUGGCCAAG	45.5
	AON7-PE50	UACCUUGACUGGAGUGAUCUC	47.6
USH2A: c.8681+3960A>G	AON1-PE43	CAGUGACAAUUUGGAUGGAGAC	45.5

G-C: Guanine-Cytosine content.

Table S3. Primer sequences used for the minigene assays

Variant	Restriction enzyme	Rectriction site and tails	Primer sequence (5'-3')
USH2A: c.4106C>T	XhoI	AAGAATCTCGAG	gggaaatccagtacatcacc
	NheI	AAGAATGCTAGC	caatatcaactgagtgtggac
USH2A: c.5168-26A>C	XhoI	AAGAATCTCGAG	cactgcctcctatatttacag
	NheI	AAGAATGCTAGC	catctgaaaggagatggaaac
USH2A: c.9958+3438A>G	XhoI	AAGAATCTCGAG	cctgtactatgactaaccag
	NheI	AAGAATGCTAGC	ggtgttctgacttctctttc
USH2A: c.8681+3960A>G	NdeI	AAGAATCATATG	gaagctgccgaagtgagtc
	NheI	AAGAATGCTAGC	gaaacacgtgtcatggaactg

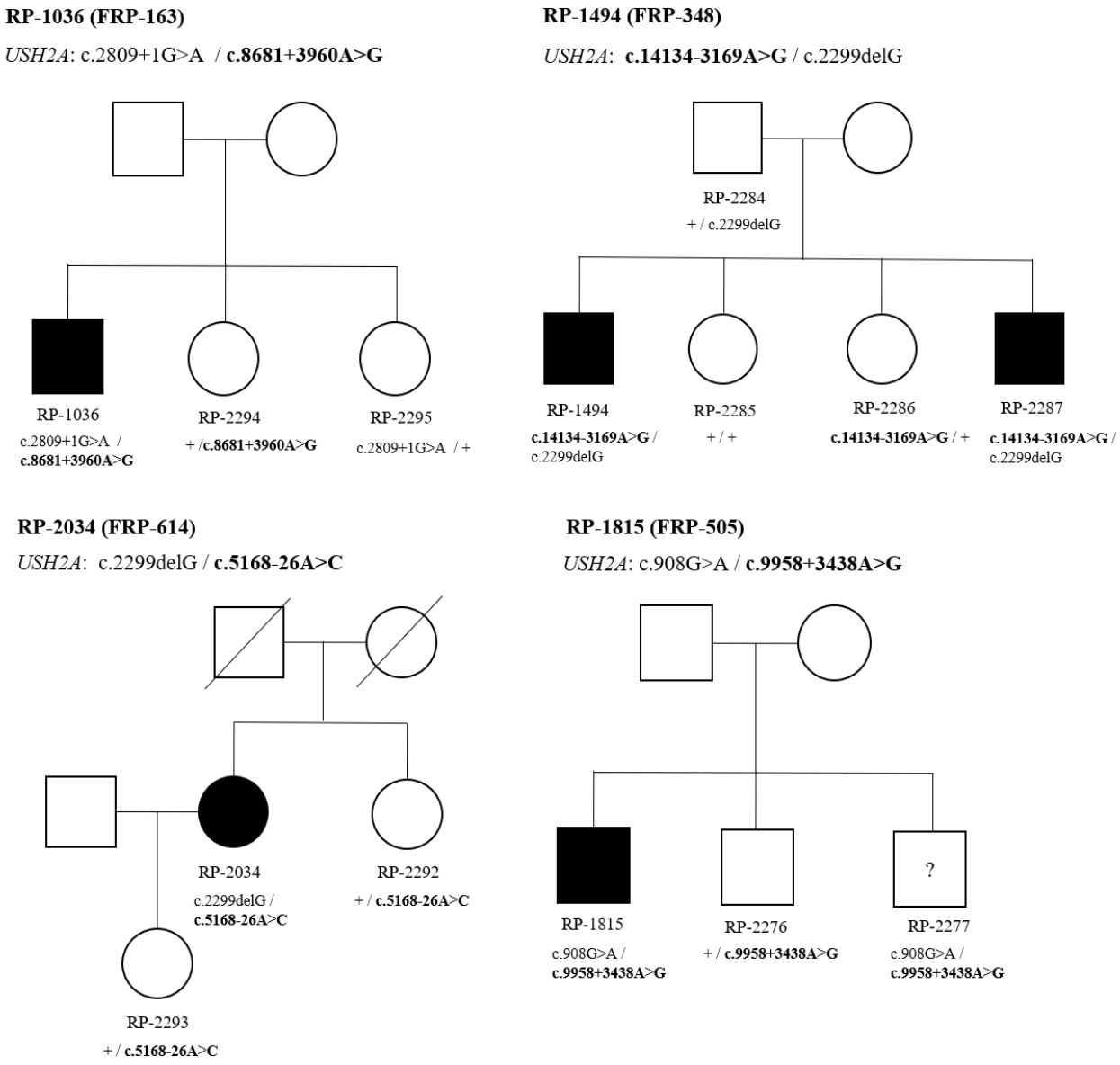


Figure S1. Family pedigrees in which segregation analysis were assessed. Variants identified in this study are highlighted in bold.

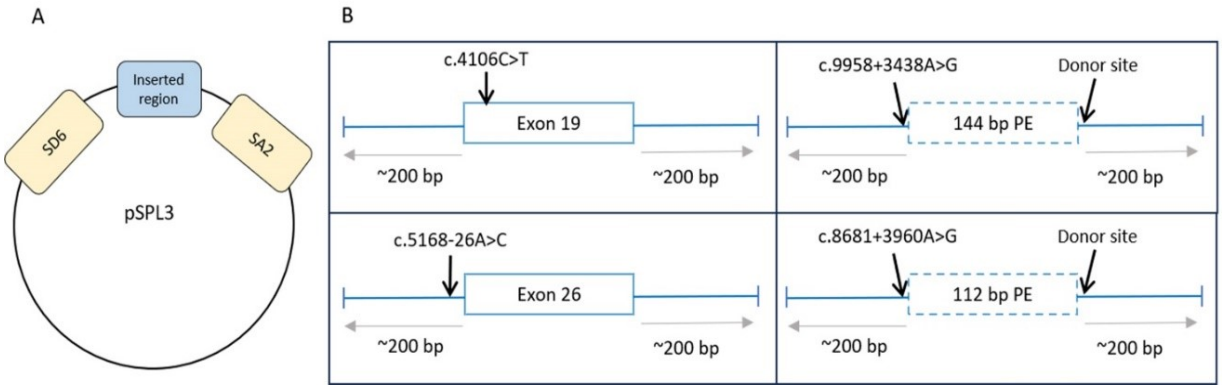


Figure S2. Minigene constructs for the analysis of *USH2A* variants. Regions inserted are depicted in blue in both sections A and B. In section A, constitutive exons are represented in yellow (SD6 and SA2). Each square in section B represents designs performed for each variant. PE: Pseudoexon; bp: base pair.

REFERENCES

REFERENCES

Aarem, A. Van, Wagenaar, M., Pinckers, A. J. L. G., Huygen, P. L. M., Bleeker-wagemakers, E. M., Kimberling, B. J., et al. (1995). Ophthalmologic findings in Usher syndrome type 2A. *Ophthalmic Genet.* 16, 151–158. doi: 10.3109/13816819509057856

Aartsma-Rus, A. (2012). "Overview on AON Design," 117–129. doi: 10.1007/978-1-61779-767-5_8

Abad-Morales, V., Navarro, R., Burés-Jelstrup, A., and Pomares, E. (2020). Identification of a novel homozygous ARSG mutation as the second cause of Usher syndrome type 4. *Am. J. Ophthalmol. Case Reports* 19, 100736. doi: 10.1016/j.ajoc.2020.100736

Abadie, C., Blanchet, C., Baux, D., Larrieu, L., Besnard, T., Ravel, P., et al. (2012). Audiological findings in 100 USH2 patients. *Clin. Genet.* 82, 433–438. doi: 10.1111/j.1399-0004.2011.01772.x

Abdolrahimzadeh, S., Formisano, M., Di Pippo, M., Lodesani, M., and Lotery, A. J. (2022). The Role of the Choroid in Stargardt Disease. *Int. J. Mol. Sci.* 23, 7607. doi: 10.3390/ijms23147607

Aboshiha, J., Dubis, A. M., Carroll, J., Hardcastle, A. J., and Michaelides, M. (2016). The cone dysfunction syndromes. *Br. J. Ophthalmol.* 100, 115–121. doi: 10.1136/bjophthalmol-2014-306505

Adato, A., Vreugde, S., Joensuu, T., Avidan, N., Hamalainen, R., Belenkiy, O., et al. (2002). USH3A transcripts encode clarin-1, a four-transmembrane-domain protein with a possible role in sensory synapses. *Eur. J. Hum. Genet.* 10, 339–350. doi: 10.1038/sj.ejhg.5200831

Adato, A., Weiv, D., Kalinski, H., Ayadi, H., Petit, C., and Korostishevsky, M. (1997). Mutation Profile of All 49 Exons of the Human Myosin VIIA Gene , and Haplotype Analysis , in Usher 1 B Families from Diverse Origins. 813–821.

Adly, N., Alhashem, A., Ammari, A., and Alkuraya, F. S. (2014). Ciliary Genes TBC1D32 / C6orf170 and SCLT1 are Mutated in Patients with OFD Type IX. *Hum. Mutat.* 35, 36–40. doi: 10.1002/humu.22477

Ahmed, Z. M., Jaworek, T. J., Sarangdhar, G. N., Zheng, L., Gul, K., Khan, S. N., et al. (2018). Inframe deletion of human ESPN is associated with deafness, vestibulopathy and vision impairment. *J. Med. Genet.* 55, 479–488. doi: 10.1136/jmedgenet-2017-105221

Ahmed, Z. M., Riazuddin, S., Ahmad, J., Bernstein, S. L., Guo, Y., Sabar, M. F., et al. (2003). PCDH15 is expressed in the neurosensory epithelium of the eye and ear and mutant alleles are responsible for both USH1F and DFNB23. *Hum. Mol. Genet.* 12, 3215–23. doi: 10.1093/hmg/ddg358

Ahmed, Z. M., Smith, T. N., Riazuddin, S., Makishima, T., Ghosh, M., Bokhari, S., et al. (2002). Nonsyndromic recessive deafness DFNB18 and Usher syndrome type IC are allelic mutations of USHIC. *Hum. Genet.* 110, 527–531. doi: 10.1007/s00439-002-0732-4

Albert, S., Garanto, A., Sangermano, R., Khan, M., Bax, N. M., Hoyng, C. B., et al. (2018). Identification and Rescue of Splice Defects Caused by Two Neighboring Deep-Intronic ABCA4 Mutations Underlying Stargardt Disease. *Am. J. Hum. Genet.* 102, 517–527. doi: 10.1016/j.ajhg.2018.02.008

Aller, E., Jaijo, T., Beneyto, M., Na, C., Oltra, S., Ayuso, C., et al. (2006). Identification of 14 novel mutations in the long isoform of. 1–5. doi: 10.1136/jmg.2006.041764

Aller, E., Larrieu, L., Jaijo, T., Baux, D., Espinós, C., González-Candelas, F., et al. (2010). The USH2A c.2299delG mutation: dating its common origin in a Southern European population. *Eur. J. Hum. Genet.* 18, 788–793. doi: 10.1038/ejhg.2010.14

Allikmets, R., Singh, N., Sun, H., Shroyer, N. F., Hutchinson, A., Chidambaram, A., et al. (1997). A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Starqardt macular dystrophy. *Nat. Genet.* 15, 236–246. doi: 10.1038/ng0397-236

Alsahan, N., and Alkuraya, F. S. (2020). Confirming <scp> *TBC1D32* </scp> -related ciliopathy in humans. *Am. J. Med. Genet. Part A* 182, 1985–1987. doi: 10.1002/ajmg.a.61717

Amorim, A. M., Ramada, A. B., Lopes, A. C., Lemos, J., and Ribeiro, J. C. (2024). Balance Control Impairments in Usher Syndrome. *Ear Hear.* doi: 10.1097/AUD.0000000000001558

Andreev, D. E., Loughran, G., Fedorova, A. D., Mikhaylova, M. S., Shatsky, I. N., and Baranov, P. V. (2022). Non-AUG translation initiation in mammals. *Genome Biol.* 23, 111. doi: 10.1186/s13059-022-02674-2

Ankala, A., Jain, N., Hubbard, B., Alexander, J. J., and Shankar, S. P. (2018). Is exon 8 the most critical or the only dispensable exon of the VCAN gene? Insights into VCAN variants and clinical spectrum of Wagner syndrome. *Am. J. Med. Genet. Part A* 176, 1778–1783. doi: 10.1002/ajmg.a.38855

Anna, A., and Monika, G. (2018). Splicing mutations in human genetic disorders: examples, detection,

and confirmation. *J. Appl. Genet.* 59, 253–268. doi: 10.1007/s13353-018-0444-7

Antiñolo, G., Sánchez, B., Borrego, S., Rueda, T., Chaparro, P., and Cabeza, J. C. (1994). Identification of a new mutation at codon 171 of rhodopsin gene causing autosomal dominant retinitis pigmentosa. *Hum. Mol. Genet.* 3, 1421–1421. doi: 10.1093/hmg/3.8.1421

Aoun, M., Passerini, I., Chiurazzi, P., Karali, M., De Rienzo, I., Sartor, G., et al. (2021). Inherited Retinal Diseases Due to RPE65 Variants: From Genetic Diagnostic Management to Therapy. *Int. J. Mol. Sci.* 22, 7207. doi: 10.3390/ijms22137207

Aparisi, M. J., Aller, E., Fuster-garcía, C., García-garcía, G., Rodrigo, R., Vázquez-manrique, R. P., et al. (2014). Targeted next generation sequencing for molecular diagnosis of Usher syndrome. doi: 10.1186/s13023-014-0168-7

Araujo, P. R., Yoon, K., Ko, D., Smith, A. D., Qiao, M., Suresh, U., et al. (2012). Before It Gets Started: Regulating Translation at the 5′ UTR. *Comp. Funct. Genomics* 2012, 1–8. doi: 10.1155/2012/475731

Ashkenazy, N., Sengillo, J. D., Iyer, P. G., Negron, C. I., Yannuzzi, N. A., and Berrocal, A. M. (2023). Phenotypic expansion of KCNJ13- associated snowflake vitreoretinal degeneration. *Ophthalmic Genet.* 44, 505–508. doi: 10.1080/13816810.2022.2149816

Astuti, G. D. N., Bertelsen, M., Preising, M. N., Ajmal, M., Lorenz, B., Faradz, S. M. H., et al. (2016). Comprehensive genotyping reveals RPE65 as the most frequently mutated gene in Leber congenital amaurosis in Denmark. *Eur. J. Hum. Genet.* 24, 1071–1079. doi: 10.1038/ejhg.2015.241

Athanasiou, D., Aguila, M., Bellingham, J., Li, W., McCulley, C., Reeves, P. J., et al. (2018). The molecular and cellular basis of rhodopsin retinitis pigmentosa reveals potential strategies for therapy. *Prog. Retin. Eye Res.* 62, 1–23. doi: 10.1016/j.preteyeres.2017.10.002

Aupy, P., Zarrouki, F., Sandro, Q., Gastaldi, C., Buclez, P.-O., Mamchaoui, K., et al. (2020). Long-Term Efficacy of AAV9-U7snRNA-Mediated Exon 51 Skipping in mdx52 Mice. *Mol. Ther. - Methods Clin. Dev.* 17, 1037–1047. doi: 10.1016/j.omtm.2020.04.025

Ávila-Fernández, A., Cantalapiedra, D., Aller, E., Vallespín, E., Aguirre-Lambán, J., Blanco-Kelly, F., et al. (2010). Mutation analysis of 272 Spanish families affected by autosomal recessive retinitis pigmentosa using a genotyping microarray. *Mol. Vis.* 16, 2550–2558. doi: 272 [pii]

Ayuso, C., and Millan, J. M. (2010). Retinitis pigmentosa and allied conditions today: a paradigm of translational research. *Genome Med.* 2, 34. doi: 10.1186/gm155

Bachmann-Gagescu, R., and Neuhauss, S. C. (2019). The photoreceptor cilium and its diseases. *Curr. Opin. Genet. Dev.* 56, 22–33. doi: 10.1016/j.gde.2019.05.004

Baralle, M. (2003). Identification of a mutation that perturbs NF1 agene splicing using genomic DNA samples and a minigene assay. *J. Med. Genet.* 40, 220–222. doi: 10.1136/jmg.40.3.220

Basharat, R., de Bruijn, S. E., Zahid, M., Rodenburg, K., Hitti-Malin, R. J., Rodríguez-Hidalgo, M., et al. (2024). Next-generation sequencing to genetically diagnose a diverse range of inherited eye disorders in 15 consanguineous families from Pakistan. *Exp. Eye Res.* 244, 109945. doi: 10.1016/j.exer.2024.109945

Battu, R., Ratra, D., and Gopal, L. (2022). Newer therapeutic options for inherited retinal diseases: Gene and cell replacement therapy. *Indian J. Ophthalmol.* 70, 2316. doi: 10.4103/ijo.IJO_82_22

Bauwens, M., De Zaeytijd, J., Weisschuh, N., Kohl, S., Meire, F., Dahan, K., et al. (2015). An Augmented ABCA4 Screen Targeting Noncoding Regions Reveals a Deep Intronic Founder Variant in Belgian Stargardt Patients. *Hum. Mutat.* 36, 39–42. doi: 10.1002/humu.22716

Bauwens, M., Garanto, A., Sangermano, R., Naessens, S., Weisschuh, N., De Zaeytijd, J., et al. (2019). ABCA4-associated disease as a model for missing heritability in autosomal recessive disorders: novel noncoding splice, cis-regulatory, structural, and recurrent hypomorphic variants. *Genet. Med.* 21, 1761–1771. doi: 10.1038/s41436-018-0420-y

Baux, D., Blanchet, C., Hamel, C., Meunier, I., Larrieu, L., Faugère, V., et al. (2014). Enrichment of LOVD-USHbases with 152 USH2A Genotypes Defines an Extensive Mutational Spectrum and Highlights Missense Hotspots. *Hum. Mutat.* 35, 1179–1186. doi: 10.1002/humu.22608

Baux, D., Larrieu, L., Blanchet, C., Hamel, C., Ben Salah, S., Vielle, A., et al. (2007). Molecular and in silico analyses of the full-length isoform of usherin identify new pathogenic alleles in Usher type II patients. *Hum. Mutat.* 28, 781–789. doi: 10.1002/humu.20513

Baux, D., Vaché, C., Blanchet, C., Willems, M., Baudoin, C., Moclyn, M., et al. (2017). Combined genetic approaches yield a 48% diagnostic rate in a large cohort of French hearing-impaired patients. *Sci. Rep.* 7, 16783. doi: 10.1038/s41598-017-16846-9

Bax, N. M., Sangermano, R., Roosing, S., Thiadens, A. A. H. J., Hoefsloot, L. H., van den Born, L. I., et al. (2015). Heterozygous Deep-Intronic Variants and

Deletions in ABCA4 in Persons with Retinal Dystrophies and One Exonic ABCA4 Variant. *Hum. Mutat.* 36, 43–47. doi: 10.1002/humu.22717

Baxter, M. F., and Borchert, G. A. (2024). Gene Therapy for Achromatopsia. *Int. J. Mol. Sci.* 25, 9739. doi: 10.3390/ijms25179739

Ben-Yosef, T., and Friedman, T. B. (2003). The genetic bases for syndromic and nonsyndromic deafness among Jews. *Trends Mol. Med.* 9, 496–502. doi: 10.1016/j.molmed.2003.09.004

Benlloch-Navarro, S., Trachsel-Moncho, L., Fernández-Carbonell, Á., Olivar, T., Soria, J. M., Almansa, I., et al. (2019). Progesterone anti-inflammatory properties in hereditary retinal degeneration. *J. Steroid Biochem. Mol. Biol.* 189, 291–301. doi: 10.1016/j.jsbmb.2019.01.007

Bennett, C. F. (2019). Therapeutic Antisense Oligonucleotides Are Coming of Age. *Annu. Rev. Med.* 70, 307–321. doi: 10.1146/annurev-med-041217-010829

Bennett, C. F., and Swayze, E. E. (2010). RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform. *Annu. Rev. Pharmacol. Toxicol.* 50, 259–293. doi: 10.1146/annurev.pharmtox.010909.105654

Bennett, J. (2017). Taking Stock of Retinal Gene Therapy: Looking Back and Moving Forward. *Mol. Ther.* 25, 1076–1094. doi: 10.1016/j.ymthe.2017.03.008

Bernal, S. (2003). Mutations in USH2A in Spanish patients with autosomal recessive retinitis pigmentosa: high prevalence and phenotypic variation. *J. Med. Genet.* 40, 8e – 8. doi: 10.1136/jmg.40.1.e8

Bernal, S., Medà, C., Solans, T., Ayuso, C., Garcia-Sandoval, B., Valverde, D., et al. (2005). Clinical and genetic studies in Spanish patients with Usher syndrome type II: description of new mutations and evidence for a lack of genotype-phenotype correlation. *Clin. Genet.* 68, 204–214. doi: 10.1111/j.1399-0004.2005.00481.x

Bernardis, I., Chiesi, L., Tenedini, E., Artuso, L., Percesepe, A., Artusi, V., et al. (2016). Unravelling the Complexity of Inherited Retinal Dystrophies Molecular Testing : Added Value of Targeted Next-Generation Sequencing. 2016. doi: 10.1155/2016/6341870

Besnard, T., García-García, G., Baux, D., Vaché, C., Faugère, V., Larrieu, L., et al. (2014). Experience of targeted Usher exome sequencing as a clinical test. *Mol. Genet. Genomic Med.* 2, 30–43. doi: 10.1002/mgg3.25

Beyter, D., Ingimundardottir, H., Oddsson, A., Eggertsson, H. P., Bjornsson, E., Jonsson, H., et al. (2021). Long-read sequencing of 3,622 Icelanders provides insight into the role of structural variants in human diseases and other traits. *Nat. Genet.* 53, 779–786. doi: 10.1038/s41588-021-00865-4

Bhardwaj, A., Yadav, A., Yadav, M., and Tanwar, M. (2022). Genetic dissection of non-syndromic retinitis pigmentosa. *Indian J. Ophthalmol.* 70, 2355. doi: 10.4103/ijo.IJO_46_22

Biasutto, P., Adamson, P., Dulla, K., Murray, S., Monia, B., and McCaleb, M. (2019). Allele specific knock-down of human P23H rhodopsin mRNA and prevention of retinal degeneration in humanized P23H rhodopsin knock-in mouse, following treatment with an intravitreal GAPmer antisense oligonucleotide (QR-1123). *Invest Ophthalmol Vis Sci*, 5719.

Binder, S., Stolba, U., Krebs, I., Kellner, L., Jahn, C., Feichtinger, H., et al. (2002). Transplantation of autologous retinal pigment epithelium in eyes with foveal neovascularization resulting from age-related macular degeneration: a pilot study. *Am. J. Ophthalmol.* 133, 215–225. doi: 10.1016/S0002-9394(01)01373-3

Biswas, P., Villanueva, A. L., Soto-Hermida, A., Duncan, J. L., Matsui, H., Borooah, S., et al. (2021). Deciphering the genetic architecture and ethnographic distribution of IRD in three ethnic populations by whole genome sequence analysis. *PLOS Genet.* 17, e1009848. doi: 10.1371/journal.pgen.1009848

Black, D. L. (2003). Mechanisms of Alternative Pre-Messenger RNA Splicing. *Annu. Rev. Biochem.* 72, 291–336. doi: 10.1146/annurev.biochem.72.121801.161720

Bocquet, B., Borday, C., Erkilic, N., Mamaeva, D., Donval, A., Masson, C., et al. (2023). TBC1D32 variants disrupt retinal ciliogenesis and cause retinitis pigmentosa. *JCI Insight* 8. doi: 10.1172/jci.insight.169426

Bohlen, J., Fenzl, K., Kramer, G., Bukau, B., and Teleman, A. A. (2020). Selective 40S Footprinting Reveals Cap-Tethered Ribosome Scanning in Human Cells. *Mol. Cell* 79, 561-574.e5. doi: 10.1016/j.molcel.2020.06.005

Bonilha, V. L., Trzupek, K. M., Li, Y., Francis, P. J., Hollyfield, J. G., Rayborn, M. E., et al. (2008). Choroideremia: Analysis of the Retina from a Female Symptomatic Carrier. *Ophthalmic Genet.* 29, 99–110. doi: 10.1080/13816810802206499

Bonnet, C., and El-Amraoui, A. (2012). Usher syndrome (sensorineural deafness and retinitis pigmentosa). *Curr. Opin. Neurol.* 25, 42–49. doi: 10.1097/WCO.0b013e32834ef8b2

Bonnet, C., Grati, M., Marlin, S., Levilliers, J., Hardelin,

J. P., Parodi, M., et al. (2011). Complete exon sequencing of all known Usher syndrome genes greatly improves molecular diagnosis. *Orphanet J. Rare Dis.* 6, 21. doi: 10.1186/1750-1172-6-21

Bonnet, C., Riahi, Z., Chantot-bastaraud, S., Smagghe, L., Letexier, M., Marcaillou, C., et al. (2016). An innovative strategy for the molecular diagnosis of Usher syndrome identi fi es causal biallelic mutations in 93 % of European patients. 1730–1738. doi: 10.1038/ejhg.2016.99

Boon, C. J. F., den Hollander, A. I., Hoyng, C. B., Cremers, F. P. M., Klevering, B. J., and Keunen, J. E. E. (2008). The spectrum of retinal dystrophies caused by mutations in the peripherin/RDS gene. *Prog. Retin. Eye Res.* 27, 213–235. doi: 10.1016/j.preteyeres.2008.01.002

Booth, K. T., Kahrizi, K., Babanejad, M., Daghagh, H., Bademci, G., Arzhangi, S., et al. (2018). Variants in CIB2 cause DFNB48 and not USH1J. *Clin. Genet.* 93, 812–821. doi: 10.1111/cge.13170

Borella, Y., Dhaenens, C.-M., Grunewald, O., and Caputo, G. (2024). Wagner syndrome: Novel VCAN variant and prophylactic management with encircling band and retinopexy. *Am. J. Ophthalmol. Case Reports* 34, 102061. doi: 10.1016/j.ajoc.2024.102061

Bork, J. M., Peters, L. M., Riazuddin, S., Bernstein, S. L., Ahmed, Z. M., Ness, S. L., et al. (2001). Usher Syndrome 1D and Nonsyndromic Autosomal Recessive Deafness DFNB12 Are Caused by Allelic Mutations of the Novel Cadherin-Like Gene CDH23. *Am. J. Hum. Genet.* 68, 26–37. doi: 10.1086/316954

Bottillo, I., De Luca, A., Schirinzi, A., Guida, V., Torrente, I., Calvieri, S., et al. (2007). Functional analysis of splicing mutations in exon 7 of NF1gene. *BMC Med. Genet.* 8, 4. doi: 10.1186/1471-2350-8-4

Brar, A. S., Parameswarappa, D. C., Takkar, B., Narayanan, R., Jalali, S., Mandal, S., et al. (2024). Gene Therapy for Inherited Retinal Diseases: From Laboratory Bench to Patient Bedside and Beyond. *Ophthalmol. Ther.* 13, 21–50. doi: 10.1007/s40123-023-00862-2

Braun, T. A., Mullins, R. F., Wagner, A. H., Andorf, J. L., Johnston, R. M., Bakall, B. B., et al. (2013). Non-exomic and synonymous variants in ABCA4 are an important cause of Stargardt disease. *Hum. Mol. Genet.* 22, 5136–45. doi: 10.1093/hmg/ddt367

Bravo-Gil, N., Méndez-Vidal, C., Romero-Pérez, L., González-Del Pozo, M., Rodríguez-De La Ruá, E., Dopazo, J., et al. (2016). Improving the management of Inherited Retinal Dystrophies by targeted sequencing of a population-specific gene panel. *Sci. Rep.* 6, 1–10. doi: 10.1038/srep23910

Brewster, B. L., Rossiello, F., French, J. D., Edwards, S. L., Wong, M., Wronski, A., et al. (2012). Identification of fifteen novel germline variants in the BRCA1 3'UTR reveals a variant in a breast cancer case that introduces a functional miR-103 target site. *Hum. Mutat.* 33, 1665–1675. doi: 10.1002/humu.22159

Britten-Jones, A. C., Gocuk, S. A., Goh, K. L., Huq, A., Edwards, T. L., and Ayton, L. N. (2023a). The Diagnostic Yield of Next Generation Sequencing in Inherited Retinal Diseases: A Systematic Review and Meta-analysis. *Am. J. Ophthalmol.* 249, 57–73. doi: 10.1016/j.ajo.2022.12.027

Britten-Jones, A. C., Gocuk, S. A., Goh, K. L., Huq, A., Edwards, T. L., and Ayton, L. N. (2023b). The Diagnostic Yield of Next Generation Sequencing in Inherited Retinal Diseases: A Systematic Review and Meta-analysis. *Am. J. Ophthalmol.* 249, 57–73. doi: 10.1016/j.ajo.2022.12.027

Bruel, A.-L., Franco, B., Duffourd, Y., Thevenon, J., Jego, L., Lopez, E., et al. (2017). Fifteen years of research on oral–facial–digital syndromes: from 1 to 16 causal genes. *J. Med. Genet.* 54, 371–380. doi: 10.1136/jmedgenet-2016-104436

Bruninx, R., and Lepièce, G. (2020). [Retinitis pigmentosa]. *Rev. Med. Liege* 75, 73–74. Available at: http://www.ncbi.nlm.nih.gov/pubmed/32030928

Bryant, L., Lozynska, O., Maguire, A., Aleman, T., and Bennett, J. (2017). Prescreening whole exome sequencing results from patients with retinal degeneration for variants in genes associated with retinal degeneration. *Clin. Ophthalmol.* Volume 12, 49–63. doi: 10.2147/OPTH.S147684

Buch, P. K., Bainbridge, J. W., and Ali, R. R. (2008). AAV-mediated gene therapy for retinal disorders: from mouse to man. *Gene Ther.* 15, 849–857. doi: 10.1038/gt.2008.66

Burke, T. R., Fishman, G. A., Zernant, J., Schubert, C., Tsang, S. H., Theodore Smith, R., et al. (2012). Retinal Phenotypes in Patients Homozygous for the G1961E Mutation in the ABCA4 Gene. *Investig. Ophthalmol. Vis. Sci.* 53, 4458–4467. doi: 10.1167/iovs.11-9166

Buskin, A., Zhu, L., Chichagova, V., Basu, B., Mozaffari-Jovin, S., Dolan, D., et al. (2018). Disrupted alternative splicing for genes implicated in splicing and ciliogenesis causes PRPF31 retinitis pigmentosa. *Nat. Commun.* 9, 4234. doi: 10.1038/s41467-018-06448-y

Cáceres, J. F., and Kornblihtt, A. R. (2002). Alternative splicing: multiple control mechanisms and involvement in human disease. *Trends Genet.* 18, 186–193. doi: 10.1016/S0168-9525(01)02626-9

Calvo, S. E., Pagliarini, D. J., and Mootha, V. K. (2009).

Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc. Natl. Acad. Sci.* 106, 7507–7512. doi: 10.1073/pnas.0810916106

Carss, K., Arno, G., Erwood, M., Stephens, J., Sanchis-Juan, A., Hull, S., et al. (2017). Comprehensive Rare Variant Analysis via Whole-Genome Sequencing to Determine the Molecular Pathology of Inherited Retinal Disease. *Am. J. Hum. Genet.* 100, 75–90. doi: 10.1016/j.ajhg.2016.12.003

Cartegni, L., Chew, S. L., and Krainer, A. R. (2002). Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.* 3, 285–298. doi: 10.1038/nrg775

Castiglione, A., and Möller, C. (2022). Usher Syndrome. *Audiol. Res.* 12, 42–65. doi: 10.3390/audiolres12010005

Cavalieri, V., Baiamonte, E., and Lo Iacono, M. (2018). Non-Primate Lentiviral Vectors and Their Applications in Gene Therapy for Ocular Disorders. *Viruses* 10, 316. doi: 10.3390/v10060316

Charng, J., Cideciyan, A. V, Jacobson, S. G., Sumaroka, A., Schwartz, S. B., Swider, M., et al. (2019). Variegated yet non-random rod and cone photoreceptor disease patterns in RPGR-ORF15-associated retinal degeneration. *Hum. Mol. Genet.* 28, 175–175. doi: 10.1093/hmg/ddy342

Chen, C., Sun, L., Li, S., Huang, L., Zhang, T., Wang, Z., et al. (2020). The spectrum of genetic mutations in patients with asymptomatic mild familial exudative vitreoretinopathy. *Exp. Eye Res.* 192, 107941. doi: 10.1016/j.exer.2020.107941

Chen, M., and Manley, J. L. (2009). Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nat. Rev. Mol. Cell Biol.* 10, 741–754. doi: 10.1038/nrm2777

Chen, T.-C., Huang, D.-S., Lin, C.-W., Yang, C.-H., Yang, C.-M., Wang, V. Y., et al. (2021). Genetic characteristics and epidemiology of inherited retinal degeneration in Taiwan. *npj Genomic Med.* 6, 16. doi: 10.1038/s41525-021-00180-1

Chen, X., Liu, Y., Sheng, X., Tam, P. O. S., Zhao, K., Chen, X., et al. (2014). PRPF4 mutations cause autosomal dominant retinitis pigmentosa. *Hum. Mol. Genet.* 23, 2926–2939. doi: 10.1093/hmg/ddu005

Cherry, T. J., Yang, M. G., Harmin, D. A., Tao, P., Timms, A. E., Bauwens, M., et al. (2020). Mapping the cis -regulatory architecture of the human retina reveals noncoding genetic variation in disease. *Proc. Natl. Acad. Sci.* 117, 9001–9012. doi: 10.1073/pnas.1922501117

Chi, X., Gatti, P., and Papoian, T. (2017). Safety of antisense oligonucleotide and siRNA-based therapeutics. *Drug Discov. Today* 22, 823–833. doi: 10.1016/j.drudis.2017.01.013

Chiang, J. P. W., Lamey, T. M., Wang, N. K., Duan, J., Zhou, W., McLaren, T. L., et al. (2018). Development of High-Throughput Clinical Testing of RPGR ORF15 Using a Large Inherited Retinal Dystrophy Cohort. *Investig. Opthalmology Vis. Sci.* 59, 4434. doi: 10.1167/iovs.18-24555

Chiang, T.-K., and Yu, M. (2023). Electrophysiological Evaluation of Macular Dystrophies. *J. Clin. Med.* 12, 1430. doi: 10.3390/jcm12041430

Choi, B. Y., Park, G., Gim, J., Kim, A. R., Kim, B.-J., Kim, H.-S., et al. (2013). Diagnostic Application of Targeted Resequencing for Familial Nonsyndromic Hearing Loss. *PLoS One* 8, e68692. doi: 10.1371/journal.pone.0068692

Cideciyan, A. V., Jacobson, S. G., Drack, A. V., Ho, A. C., Charng, J., Garafalo, A. V., et al. (2019). Effect of an intravitreal antisense oligonucleotide on vision in Leber congenital amaurosis due to a photoreceptor cilium defect. *Nat. Med.* 25, 225–228. doi: 10.1038/s41591-018-0295-0

Cideciyan, A. V., Jacobson, S. G., Ho, A. C., Swider, M., Sumaroka, A., Roman, A. J., et al. (2023). Durable vision improvement after a single intravitreal treatment with antisense oligonucleotide in CEP290-LCA: Replication in two eyes. *Am. J. Ophthalmol. Case Reports* 32, 101873. doi: 10.1016/j.ajoc.2023.101873

Coco-Martin, R. M., Sanchez-Tocino, H. T., Desco, C., Usategui-Martín, R., and Tellería, J. J. (2020). PRPH2-Related Retinal Diseases: Broadening the Clinical Spectrum and Describing a New Mutation. *Genes (Basel).* 11, 773. doi: 10.3390/genes11070773

Collin, R. W. J., and Garanto, A. (2017). Applications of antisense oligonucleotides for the treatment of inherited retinal diseases. *Curr. Opin. Ophthalmol.* 28, 260–266. doi: 10.1097/ICU.0000000000000363

Collin, R. W. J., Littink, K. W., Klevering, B. J., van den Born, L. I., Koenekoop, R. K., Zonneveld, M. N., et al. (2008). Identification of a 2 Mb Human Ortholog of Drosophila eyes shut/spacemaker that Is Mutated in Patients with Retinitis Pigmentosa. *Am. J. Hum. Genet.* 83, 594–603. doi: 10.1016/j.ajhg.2008.10.014

Collison, F. T., Lee, W., Fishman, G. A., Park, J. C., Zernant, J., McAnany, J. J., et al. (2019). CLINICAL CHARACTERIZATION OF STARGARDT DISEASE PATIENTS WITH THE p.N1868I ABCA4 MUTATION. *Retina* 39, 2311–2325. doi: 10.1097/IAE.0000000000002316

Coppieters, F., Casteels, I., Meire, F., De Jaegere, S., Hooghe, S., van Regemorter, N., et al. (2010). Genetic screening of LCA in Belgium: Predominance of CEP290 and identification of potential modifier alleles in AHI1 of CEP290-related phenotypes. *Hum. Mutat.* 31. doi: 10.1002/humu.21336

Coppieters, F., Todeschini, A. L., Fujimaki, T., Baert, A., Bruyne, M., Cauwenbergh, C., et al. (2015). Hidden Genetic Variation in LCA9-Associated Congenital Blindness Explained by 5'UTR Mutations and Copy-Number Variations of NMNAT1. *Hum. Mutat.* 36, 1188–1196. doi: 10.1002/humu.22899

Cornelis, S. S., Bax, N. M., Zernant, J., Allikmets, R., Fritsche, L. G., den Dunnen, J. T., et al. (2017). In Silico Functional Meta-Analysis of 5,962 ABCA4 Variants in 3,928 Retinal Dystrophy Cases. *Hum. Mutat.* 38, 400–408. doi: 10.1002/humu.23165

Corradi, Z., Khan, M., Hitti-Malin, R., Mishra, K., Whelan, L., Cornelis, S. S., et al. (2023). Targeted sequencing and in vitro splice assays shed light on ABCA4-associated retinopathies missing heritability. *Hum. Genet. Genomics Adv.* 4, 100237. doi: 10.1016/j.xhgg.2023.100237

Corton, M., Tatu, S. D., Avila-Fernandez, A., Vallespín, E., Tapias, I., Cantalapiedra, D., et al. (2013). High frequency of CRB1 mutations as cause of Early-Onset Retinal Dystrophies in the Spanish population. *Orphanet J. Rare Dis.* 8. doi: 10.1186/1750-1172-8-20

Costa, K. A., Salles, M. V., Whitebirch, C., Chiang, J., and Sallum, J. M. F. (2017). Gene panel sequencing in Brazilian patients with retinitis pigmentosa. *Int. J. Retin. Vitr.* 3, 33. doi: 10.1186/s40942-017-0087-6

Cremers, F. (1998). Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene ABCR. *Hum. Mol. Genet.* 7, 355–362. doi: 10.1093/hmg/7.3.355

Cremers, F. P. M., Cornelis, S. S., Runhart, E. H., and Astuti, G. D. N. (2018). Author Response: Penetrance of the ABCA4 p.Asn1868Ile Allele in Stargardt Disease. *Investig. Opthalmology Vis. Sci.* 59, 5566. doi: 10.1167/iovs.18-25944

Cremers, F. P. M., Kimberling, W. J., Kulm, M., de Brouwer, A. P., van Wijk, E., te Brinke, H., et al. (2006). Development of a genotyping microarray for Usher syndrome. *J. Med. Genet.* 44, 153–160. doi: 10.1136/jmg.2006.044784

Cremers, F. P. M., Lee, W., Collin, R. W. J., and Allikmets, R. (2020). Clinical spectrum, genetic complexity and therapeutic approaches for retinal disease caused by ABCA4 mutations. *Prog. Retin. Eye Res.* 79, 100861. doi: 10.1016/j.preteyeres.2020.100861

Crooke, S. T., Wang, S., Vickers, T. A., Shen, W., and Liang, X. (2017). Cellular uptake and trafficking of antisense oligonucleotides. *Nat. Biotechnol.* 35, 230–237. doi: 10.1038/nbt.3779

da Cruz, L., Dorn, J. D., Humayun, M. S., Dagnelie, G., Handa, J., Barale, P.-O., et al. (2016). Five-Year Safety and Performance Results from the Argus II Retinal Prosthesis System Clinical Trial. *Ophthalmology* 123, 2248–2254. doi: 10.1016/j.ophtha.2016.06.049

Dabrowski, M., Bukowy-Bieryllo, Z., and Zietkiewicz, E. (2018). Advances in therapeutic use of a drug-stimulated translational readthrough of premature termination codons. *Mol. Med.* 24, 25. doi: 10.1186/s10020-018-0024-7

Daich Varela, M., Bellingham, J., Motta, F., Jurkute, N., Ellingford, J. M., Quinodoz, M., et al. (2023). Multidisciplinary team directed analysis of whole genome sequencing reveals pathogenic non-coding variants in molecularly undiagnosed inherited retinal dystrophies. *Hum. Mol. Genet.* 32, 595–607. doi: 10.1093/hmg/ddac227

Daiger, S. P., Bowne, S. J., and Sullivan, L. S. (2015). Genes and Mutations Causing Autosomal Dominant Retinitis Pigmentosa. *Cold Spring Harb. Perspect. Med.* 5, a017129. doi: 10.1101/cshperspect.a017129

Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., et al. (2021). Twelve years of SAMtools and BCFtools. *Gigascience* 10. doi: 10.1093/gigascience/giab008

Dass, S., Scoles, D., Trese, M. G. J., and Drenser, K. A. (2023). Retinal detachment in a pediatric patient with enhanced S-cone syndrome. *J. Am. Assoc. Pediatr. Ophthalmol. Strabismus* 27, 229–231. doi: 10.1016/j.jaapos.2023.04.014

Davis, R. L., Homer, V. M., George, P. M., and Brennan, S. O. (2009). A deep intronic mutation in FGB creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment. *Hum. Mutat.* 30, 221–227. doi: 10.1002/humu.20839

De Angeli, P., Flores-Tufiño, A., Stingl, K., Kühlewein, L., Roschi, E., Wissinger, B., et al. (2024). Splicing defects and CRISPR-Cas9 correction in isogenic homozygous photoreceptor precursors harboring clustered deep-intronic ABCA4 variants. *Mol. Ther. - Nucleic Acids* 35, 102113. doi: 10.1016/j.omtn.2023.102113

de Bruijn, S. E., Fadaie, Z., Cremers, F. P. M., Kremer, H., and Roosing, S. (2021). The Impact of Modern Technologies on Molecular Diagnostic Success Rates, with a Focus on Inherited Retinal

Dystrophy and Hearing Loss. *Int. J. Mol. Sci.* 22, 2943. doi: 10.3390/ijms22062943

de Bruijn, S. E., Fiorentino, A., Ottaviani, D., Fanucchi, S., Melo, U. S., Corral-Serrano, J. C., et al. (2020). Structural Variants Create New Topological-Associated Domains and Ectopic Retinal Enhancer-Gene Contact in Dominant Retinitis Pigmentosa. *Am. J. Hum. Genet.* 107, 802–814. doi: 10.1016/j.ajhg.2020.09.002

de Bruijn, S. E., Rodenburg, K., Corominas, J., Ben-Yosef, T., Reurink, J., Kremer, H., et al. (2023). Optical genome mapping and revisiting short-read genome sequencing data reveal previously overlooked structural variants disrupting retinal disease-associated genes. *Genet. Med.* 25, 100345. doi: 10.1016/j.gim.2022.11.013

de Sousa Dias, M., Hernan, I., Delás, B., Pascual, B., Borràs, E., Gamundi, M. J., et al. (2015). New COL6A6 variant detected by whole-exome sequencing is linked to break points in intron 4 and 3'-UTR, deleting exon 5 of RHO, and causing adRP. *Mol. Vis.* 21, 857–870.

Del Pozo-Valero, M., Riveiro-Alvarez, R., Blanco-Kelly, F., Aguirre-Lamban, J., Martin-Merida, I., Iancu, I.-F., et al. (2020). Genotype–Phenotype Correlations in a Spanish Cohort of 506 Families With Biallelic ABCA4 Pathogenic Variants. *Am. J. Ophthalmol.* 219, 195–204. doi: 10.1016/j.ajo.2020.06.027

Del Pozo-Valero, M., Riveiro-Alvarez, R., Martin-Merida, I., Blanco-Kelly, F., Swafiri, S., Lorda-Sanchez, I., et al. (2022). Impact of Next Generation Sequencing in Unraveling the Genetics of 1036 Spanish Families With Inherited Macular Dystrophies. *Investig. Opthalmology Vis. Sci.* 63, 11. doi: 10.1167/iovs.63.2.11

Delmaghani, S., and El-Amraoui, A. (2022). The genetic and phenotypic landscapes of Usher syndrome: from disease mechanisms to a new classification. *Hum. Genet.* 141, 709–735. doi: 10.1007/s00439-022-02448-7

Den Hollander, A. I., Black, A., Bennett, J., and Cremers, F. P. M. (2010). Lighting a candle in the dark: Advances in genetics and gene therapy of recessive retinal dystrophies. *J. Clin. Invest.* 120, 3042–3053. doi: 10.1172/JCI42258

Den Hollander, A. I., Koenekoop, R. K., Yzer, S., Lopez, I., Arends, M. L., Voesenek, K. E. J., et al. (2006). Mutations in the CEP290 (NPHP6) gene are a frequent cause of leber congenital amaurosis. *Am. J. Hum. Genet.* 79, 556–561. doi: 10.1086/507318

den Hollander, A. I., McGee, T. L., Ziviello, C., Banfi, S., Dryja, T. P., Gonzalez-Fernandez, F., et al. (2009). A homozygous missense mutation in the IRBP gene (RBP3) associated with autosomal recessive retinitis pigmentosa. *Investig. Ophthalmol. Vis. Sci.* 50, 1864–1872. doi: 10.1167/iovs.08-2497

den Hollander, A. I., ten Brink, J. B., de Kok, Y. J. M., van Soest, S., van den Born, L. I., van Driel, M. A., et al. (1999). Mutations in a human homologue of Drosophila crumbs cause retinitis pigmentosa (RP12). *Nat. Genet.* 23, 217–221. doi: 10.1038/13848

Deng, C., Zhao, P. Y., Branham, K., Schlegel, D., Fahim, A. T., Jayasundera, T. K., et al. (2022). Real-world outcomes of voretigene neparvovec treatment in pediatric patients with RPE65-associated Leber congenital amaurosis. *Graefe's Arch. Clin. Exp. Ophthalmol.* 260, 1543–1550. doi: 10.1007/s00417-021-05508-2

Devanna, P., van de Vorst, M., Pfundt, R., Gilissen, C., and Vernes, S. C. (2018). Genome-wide investigation of an ID cohort reveals de novo 3'UTR variants affecting gene expression. *Hum. Genet.* 137, 717–721. doi: 10.1007/s00439-018-1925-9

Dhir, A., and Buratti, E. (2010). Alternative splicing: role of pseudoexons in human disease and potential therapeutic strategies. *FEBS J.* 277, 841–855. doi: 10.1111/j.1742-4658.2009.07520.x

Di Resta, C., Spiga, I., Presi, S., Merella, S., Pipitone, G. B., Manitto, M. P., et al. (2018). Integration of multigene panels for the diagnosis of hereditary retinal disorders using Next Generation Sequencing and bioinformatics approaches. *Electron. J. Int. Fed. Clin. Chem. Lab. Med.* 29, 15–25.

Di Scipio, M., Tavares, E., Deshmukh, S., Audo, I., Green-Sanderson, K., Zubak, Y., et al. (2020). Phenotype Driven Analysis of Whole Genome Sequencing Identifies Deep Intronic Variants that Cause Retinal Dystrophies by Aberrant Exonization. *Investig. Opthalmology Vis. Sci.* 61, 36. doi: 10.1167/iovs.61.10.36

Diakatou, M., Manes, G., Bocquet, B., Meunier, I., and Kalatzis, V. (2019). Genome Editing as a Treatment for the Most Prevalent Causative Genes of Autosomal Dominant Retinitis Pigmentosa. *Int. J. Mol. Sci.* 20, 2542. doi: 10.3390/ijms20102542

Dietrich, K. (2002). A novel mutation of the RP1 gene (Lys778ter) associated with autosomal dominant retinitis pigmentosa. *Br. J. Ophthalmol.* 86, 328–332. doi: 10.1136/bjo.86.3.328

Diñeiro, M., Capín, R., Cifuentes, G. Á., Fernández-Vega, B., Villota, E., Otero, A., et al. (2020). Comprehensive genomic diagnosis of inherited retinal and optical nerve disorders reveals hidden syndromes and personalized therapeutic options. *Acta Ophthalmol.*, aos.14479. doi: 10.1111/aos.14479

Dockery, A., Stephenson, K., Keegan, D., Wynne, N., Silvestri, G., Humphries, P., et al. (2017). Target 5000: Target Capture Sequencing for Inherited Retinal Degenerations. *Genes (Basel).* 8, 304. doi: 10.3390/genes8110304

Dockery, A., Whelan, L., Humphries, P., and Farrar, G. J. (2021). Next-Generation Sequencing Applications for Inherited Retinal Diseases. *Int. J. Mol. Sci.* 22, 5684. doi: 10.3390/ijms22115684

Doudna, J. A., and Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science (80-.).* 346. doi: 10.1126/science.1258096

Dowling, J. E. (1987). The Retina: An Approachable Part of the Brain. *Belkn. Press Harvard Univ. Press*.

Dreyer, B., Tranebjærg, L., Brox, V., Rosenberg, T., Möller, C., Beneyto, M., et al. (2001). A Common Ancestral Origin of the Frequent and Widespread 2299delG USH2A Mutation. *Am. J. Hum. Genet.* 69, 228–234. doi: 10.1086/321269

Dreyer, B., Tranebjærg, L., Rosenberg, T., Weston, M. D., Kimberling, W. J., and Nilssen, Ø. (2000). Identification of novel USH2A mutations: implications for the structure of USH2A protein. *Eur. J. Hum. Genet.* 8, 500–506. doi: 10.1038/sj.ejhg.5200491

Dryja, T. P., Hahn, L. B., Kajiwara, K., and Berson, E. L. (1997). Dominant and digenic mutations in the peripherin/RDS and ROM1 genes in retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 38, 1972–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9331261

Dueñas Rey, A., del Pozo Valero, M., Bouckaert, M., Wood, K. A., Van den Broeck, F., Daich Varela, M., et al. (2024). Combining a prioritization strategy and functional studies nominates 5'UTR variants underlying inherited retinal disease. *Genome Med.* 16, 7. doi: 10.1186/s13073-023-01277-1

Duijkers, L., van den Born, L., Neidhardt, J., Bax, N., Pierrache, L., Klevering, B., et al. (2018). Antisense Oligonucleotide-Based Splicing Correction in Individuals with Leber Congenital Amaurosis due to Compound Heterozygosity for the c.2991+1655A>G Mutation in CEP290. *Int. J. Mol. Sci.* 19, 753. doi: 10.3390/ijms19030753

Dulla, K., Aguila, M., Lane, A., Jovanovic, K., Parfitt, D. A., Schulkens, I., et al. (2018). Splice-Modulating Oligonucleotide QR-110 Restores CEP290 mRNA and Function in Human c.2991+1655A>G LCA10 Models. *Mol. Ther. - Nucleic Acids* 12, 730–740. doi: 10.1016/j.omtn.2018.07.010

Dulla, K., Slijkerman, R., van Diepen, H. C., Albert, S., Dona, M., Beumer, W., et al. (2021). Antisense oligonucleotide-based treatment of retinitis pigmentosa caused by USH2A exon 13 mutations. *Mol. Ther.* doi: 10.1016/j.ymthe.2021.04.024

DuPont, M., Jones, E. M., Xu, M., and Chen, R. (2018). Investigating the disease association of USH2A p.C759F variant by leveraging large retinitis pigmentosa cohort data. *Ophthalmic Genet.* 39, 291–292. doi: 10.1080/13816810.2017.1418388

Dyka, F. M., Molday, L. L., Chiodo, V. A., Molday, R. S., and Hauswirth, W. W. (2019). Dual ABCA4 -AAV Vector Treatment Reduces Pathogenic Retinal A2E Accumulation in a Mouse Model of Autosomal Recessive Stargardt Disease. *Hum. Gene Ther.* 30, 1361–1370. doi: 10.1089/hum.2019.132

Ebermann, I., Phillips, J. B., Liebau, M. C., Koenekoop, R. K., Schermer, B., Lopez, I., et al. (2010). PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. *J. Clin. Invest.* 120, 1812–1823. doi: 10.1172/JCI39715

Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., et al. (2009). Real-Time DNA Sequencing from Single Polymerase Molecules. *Science (80-.).* 323, 133–138. doi: 10.1126/science.1162986

El-Amraoui, A., and Petit, C. (2005). Usher I syndrome: unravelling the mechanisms that underlie the cohesion of the growing hair bundle in inner ear sensory cells. *J. Cell Sci.* 118, 4593–4603. doi: 10.1242/jcs.02636

El-Amraoui, A., and Petit, C. (2014). The retinal phenotype of Usher syndrome: Pathophysiological insights from animal models. *C. R. Biol.* 337, 167–177. doi: 10.1016/j.crvi.2013.12.004

El-Kadiry, A. E.-H., Rafei, M., and Shammaa, R. (2021). Cell Therapy: Types, Regulation, and Clinical Benefits. *Front. Med.* 8. doi: 10.3389/fmed.2021.756029

Ellingford, J. M., Barton, S., Bhaskar, S., O'Sullivan, J., Williams, S. G., Lamb, J. A., et al. (2016). Molecular findings from 537 individuals with inherited retinal disease. *J. Med. Genet.* 53, 761–767. doi: 10.1136/jmedgenet-2016-103837

Elnahry, A. G., Hassan, F. K., and Abdel-Kader, A. A. (2018). Bevacizumab for the treatment of intraretinal cystic spaces in a patient with gyrate atrophy of the choroid and retina. *Ophthalmic Genet.* 39, 759–762. doi: 10.1080/13816810.2018.1536220

Espinós, C., Millán, J. M., Beneyto, M., and Nájera, C. (1998). Epidemiology of Usher Syndrome in Valencia and Spain. *Public Health Genomics* 1, 223–228. doi: 10.1159/000016167

Esteve-Garcia, A., Cobos, E., Sau, C., Padró-Miquel, A., Català-Mora, J., Barberán-Martínez, P., et al. (2024). Deciphering complexity: TULP1 variants linked to an atypical retinal dystrophy

phenotype. *Front. Genet.* 15. doi: 10.3389/fgene.2024.1352063

Eudy, J. D., Weston, M. D., Yao, S., Hoover, D. M., Rehm, H. L., Ma-Edmonds, M., et al. (1998). Mutation of a gene encoding a protein with extracellular matrix motifs in Usher syndrome type IIa. *Science* 280, 1753–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9624053

Ezquerra-Inchausti, M., Anasagasti, A., Barandika, O., Garai-Aramburu, G., Galdós, M., López de Munain, A., et al. (2018). A new approach based on targeted pooled DNA sequencing identifies novel mutations in patients with Inherited Retinal Dystrophies. *Sci. Rep.* 8, 1–12. doi: 10.1038/s41598-018-33810-3

Fadaie, Z., Khan, M., Del Pozo-Valero, M., Cornelis, S. S., Ayuso, C., Cremers, F. P. M., et al. (2019). Identification of splice defects due to noncanonical splice site or deep-intronic variants in ABCA4. *Hum. Mutat.* 40, 2365–2376. doi: 10.1002/humu.23890

Fadaie, Z., Neveling, K., Mantere, T., Derks, R., Haer-Wigman, L., den Ouden, A., et al. (2021a). Long-read technologies identify a hidden inverted duplication in a family with choroideremia. *HGG Adv.* 2, 100046. doi: 10.1016/j.xhgg.2021.100046

Fadaie, Z., Whelan, L., Ben-Yosef, T., Dockery, A., Corradi, Z., Gilissen, C., et al. (2021b). Whole genome sequencing and in vitro splice assays reveal genetic causes for inherited retinal diseases. *npj Genomic Med.* 6, 97. doi: 10.1038/s41525-021-00261-1

Falese, L., Sandza, K., Yates, B., Triffault, S., Gangar, S., Long, B., et al. (2017). Strategy to detect pre-existing immunity to AAV gene therapy. *Gene Ther.* 24, 768–778. doi: 10.1038/gt.2017.95

Farag, S., Yusuf, I. H., Kaukonen, M., Taylor, L. J., Charbel Issa, P., and MacLaren, R. E. (2024). Hypomorphic CDHR1 variants may result in retinitis pigmentosa with relative preservation of cone function. *Ophthalmic Genet.* 45, 201–206. doi: 10.1080/13816810.2023.2255265

Farrar, G. J., Carrigan, M., Dockery, A., Millington-Ward, S., Palfi, A., Chadderton, N., et al. (2017). Toward an elucidation of the molecular genetics of inherited retinal degenerations. *Hum. Mol. Genet.* 26, R2–R11. doi: 10.1093/hmg/ddx185

Farrar, G. J., Millington-Ward, S., Chadderton, N., Humphries, P., and Kenna, P. F. (2012). Gene-based therapies for dominantly inherited retinopathies. *Gene Ther.* 19, 137–144. doi: 10.1038/gt.2011.172

Fear, V. S., Forbes, C. A., Anderson, D., Rauschert, S., Syn, G., Shaw, N., et al. (2022). Functional validation of variants of unknown significance using CRISPR gene editing and transcriptomics: A Kleefstra syndrome case study. *Gene* 821, 146287. doi: 10.1016/j.gene.2022.146287

Fenner, B. J., Tan, T.-E., Barathi, A. V., Tun, S. B. B., Yeo, S. W., Tsai, A. S. H., et al. (2022). Gene-Based Therapeutics for Inherited Retinal Diseases. *Front. Genet.* 12. doi: 10.3389/fgene.2021.794805

Fernández, A., Josa, S., and Montoliu, L. (2017). A history of genome editing in mammals. *Mamm. Genome* 28, 237–246. doi: 10.1007/s00335-017-9699-2

Filatova, A., Reveguk, I., Piatkova, M., Bessonova, D., Kuziakova, O., Demakova, V., et al. (2023). Annotation of uORFs in the OMIM genes allows to reveal pathogenic variants in 5'UTRs. *Nucleic Acids Res.* 51, 1229–1244. doi: 10.1093/nar/gkac1247

Finkel, R. S., Mercuri, E., Darras, B. T., Connolly, A. M., Kuntz, N. L., Kirschner, J., et al. (2017). Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* 377, 1723–1732. doi: 10.1056/NEJMoa1702752

Fishilevich, S., Nudel, R., Rappaport, N., Hadar, R., Plaschkes, I., Iny Stein, T., et al. (2017). GeneHancer: genome-wide integration of enhancers and target genes in GeneCards. *Database* 2017. doi: 10.1093/database/bax028

Fishman, G. A. (2003). ABCA4 Gene Sequence Variations in Patients With Autosomal Recessive Cone-Rod Dystrophy. *Arch. Ophthalmol.* 121, 851. doi: 10.1001/archopht.121.6.851

Fowler, A., Mahamdallie, S., Ruark, E., Seal, S., Ramsay, E., Clarke, M., et al. (2016). Accurate clinical detection of exon copy number variants in a targeted NGS panel using DECoN. *Wellcome Open Res.* 1, 20. doi: 10.12688/wellcomeopenres.10069.1

Fowler, N. H., El-Rashedy, M. I., Chishti, E. A., Vander Kooi, C. W., and Maldonado, R. S. (2021). Multimodal imaging and genetic findings in a case of ARSG-related atypical Usher syndrome. *Ophthalmic Genet.* 42, 338–343. doi: 10.1080/13816810.2021.1891552

Fraile-Bethencourt, E., Valenzuela-Palomo, A., Díez-Gómez, B., Caloca, M. J., Gómez-Barrero, S., and Velasco, E. A. (2019). Minigene Splicing Assays Identify 12 Spliceogenic Variants of BRCA2 Exons 14 and 15. *Front. Genet.* 10. doi: 10.3389/fgene.2019.00503

Frio, T. R., McGee, T. L., Wade, N. M., Iseli, C., Beckmann, J. S., Berson, E. L., et al. (2009). A single-base substitution within an intronic repetitive element causes dominant retinitis pigmentosa with reduced penetrance. *Hum. Mutat.* 30, 1340–1347. doi: 10.1002/humu.21071

Fu, Q., Xu, M., Chen, X., Sheng, X., Yuan, Z., Liu, Y., et al. (2017). CEP78 is mutated in a distinct type of Usher syndrome. *J. Med. Genet.* 54, 190–195. doi: 10.1136/jmedgenet-2016-104166

Fuster-García, C., García-Bohórquez, B., Rodríguez-Muñoz, A., Aller, E., Jaijo, T., Millán, J. M., et al. (2021). Usher Syndrome: Genetics of a Human Ciliopathy. *Int. J. Mol. Sci.* 22, 6723. doi: 10.3390/ijms22136723

Fuster-García, C., García-García, G., González-Romero, E., Jaijo, T., Sequedo, M. D., Ayuso, C., et al. (2017). USH2A Gene Editing Using the CRISPR System. *Mol. Ther. Nucleic Acids* 8, 529–541. doi: 10.1016/j.omtn.2017.08.003

Fuster-García, C., García-García, G., Jaijo, T., Blanco-Kelly, F., Tian, L., Hakonarson, H., et al. (2019). Expanding the Genetic Landscape of Usher-Like Phenotypes. *Investig. Opthalmology Vis. Sci.* 60, 4701. doi: 10.1167/iovs.19-27470

Fuster-García, C., García-García, G., Jaijo, T., Fornés, N., Ayuso, C., Fernández-Burriel, M., et al. (2018). High-throughput sequencing for the molecular diagnosis of Usher syndrome reveals 42 novel mutations and consolidates CEP250 as Usher-like disease causative. *Sci. Rep.* 8, 17113. doi: 10.1038/s41598-018-35085-0

Gaj, T., Gersbach, C. A., and Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31, 397–405. doi: 10.1016/j.tibtech.2013.04.004

Ganapathi, M., Thomas-Wilson, A., Buchovecky, C., Dharmadhikari, A., Barua, S., Lee, W., et al. (2022). Clinical exome sequencing for inherited retinal degenerations at a tertiary care center. *Sci. Rep.* 12, 9358. doi: 10.1038/s41598-022-13026-2

Gao, F.-J., Wang, D.-D., Chen, F., Sun, H.-X., Hu, F.-Y., Xu, P., et al. (2021). Prevalence and genetic-phenotypic characteristics of patients with USH2A mutations in a large cohort of Chinese patients with inherited retinal disease. *Br. J. Ophthalmol.* 105, 87–92. doi: 10.1136/bjophthalmol-2020-315878

Garanto, A., Chung, D. C., Duijkers, L., Corral-Serrano, J. C., Messchaert, M., Xiao, R., et al. (2016). In vitro and in vivo rescue of aberrant splicing in CEP290 -associated LCA by antisense oligonucleotide delivery. *Hum. Mol. Genet.*, ddw118. doi: 10.1093/hmg/ddw118

Garanto, A., and Collin, R. W. J. (2018). Design and In Vitro Use of Antisense Oligonucleotides to Correct Pre-mRNA Splicing Defects in Inherited Retinal Dystrophies. *Methods Mol. Biol.* 1715, 61–78. doi: 10.1007/978-1-4939-7522-8_5

Garanto, A., Duijkers, L., Tomkiewicz, T. Z., and Collin, R. W. J. (2019). Antisense Oligonucleotide Screening to Optimize the Rescue of the Splicing Defect Caused by the Recurrent Deep-Intronic ABCA4 Variant c.4539+2001G>A in Stargardt Disease. *Genes (Basel).* 10, 452. doi: 10.3390/genes10060452

García-Bohórquez, B., Barberán-Martínez, P., Aller, E., Jaijo, T., Mínguez, P., Rodilla, C., et al. (2024). Exploring non-coding variants and evaluation of antisense oligonucleotides for splicing redirection in Usher syndrome. *Mol. Ther. - Nucleic Acids* 35, 102374. doi: 10.1016/j.omtn.2024.102374

García-garcía, G., Aller, E., Jaijo, T., Aparisi, M. J., Larrieu, L., Faugère, V., et al. (2014). Novel deletions involving the USH2A gene in patients with Usher syndrome and retinitis pigmentosa. 106, 1398–1410.

Garcia-Garcia, G., Aparisi, M. J., Jaijo, T., Rodrigo, R., Leon, A. M., Avila-fernandez, A., et al. (2011). Mutational screening of the USH2A gene in Spanish USH patients reveals 23 novel pathogenic mutations. 1–13.

García-García, G., Besnard, T., Baux, D., Vaché, C., Aller, E., Malcolm, S., et al. (2013). The contribution of GPR98 and DFNB31 genes to a Spanish Usher syndrome type 2 cohort. *Mol. Vis.* 19, 367–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23441107

García Bohórquez, B., Aller, E., Rodríguez Muñoz, A., Jaijo, T., García García, G., and Millán, J. M. (2021). Updating the Genetic Landscape of Inherited Retinal Dystrophies. *Front. Cell Dev. Biol.* 9. doi: 10.3389/fcell.2021.645600

Gasparini, S. J., Llonch, S., Borsch, O., and Ader, M. (2019). Transplantation of photoreceptors into the degenerative retina: Current state and future perspectives. *Prog. Retin. Eye Res.* 69, 1–37. doi: 10.1016/j.preteyeres.2018.11.001

Gass, J. D. M. (1997). Stereoscopic atlas of macular diseases. *Diagnosis Treat.*, 158–65.

Gebauer, F., Schwarzl, T., Valcárcel, J., and Hentze, M. W. (2021). RNA-binding proteins in human genetic disease. *Nat. Rev. Genet.* 22, 185–198. doi: 10.1038/s41576-020-00302-y

Géléoc, G. G. S., and El-Amraoui, A. (2020). Disease mechanisms and gene therapy for Usher syndrome. *Hear. Res.* 394, 107932. doi: 10.1016/j.heares.2020.107932

Geoffroy, V., Lamouche, J.-B., Guignard, T., Nicaise, S., Kress, A., Scheidecker, S., et al. (2023). The AnnotSV webserver in 2023: updated visualization and ranking. *Nucleic Acids Res.* 51, W39–W45. doi: 10.1093/nar/gkad426

Georgiou, M., Fujinami, K., and Michaelides, M. (2021).

Inherited retinal diseases: Therapeutics, clinical trials and end points—A review. *Clin. Experiment. Ophthalmol.* 49, 270–288. doi: 10.1111/ceo.13917

Gerber, S., Rozet, J. M., van de Pol, T. J. R., Hoyng, C. B., Munnich, A., Blankenagel, A., et al. (1998). Complete Exon–Intron Structure of the Retina-Specific ATP Binding Transporter Gene (ABCR) Allows the Identification of Novel Mutations Underlying Stargardt Disease. *Genomics* 48, 139–142. doi: 10.1006/geno.1997.5164

Ghanbari, M., Erkeland, S. J., Xu, L., Colijn, J. M., Franco, O. H., Dehghan, A., et al. (2017). Genetic variants in microRNAs and their binding sites within gene 3'UTRs associate with susceptibility to age-related macular degeneration. *Hum. Mutat.* 38, 827–838. doi: 10.1002/humu.23226

Ghosh, S., Brown, A. M., Jenkins, C., and Campbell, K. (2020). Viral Vector Systems for Gene Therapy: A Comprehensive Literature Review of Progress and Biosafety Challenges. *Appl. Biosaf.* 25, 7–18. doi: 10.1177/1535676019899502

Giese, A. P. J., Tang, Y.-Q., Sinha, G. P., Bowl, M. R., Goldring, A. C., Parker, A., et al. (2017). CIB2 interacts with TMC1 and TMC2 and is essential for mechanotransduction in auditory hair cells. *Nat. Commun.* 8, 43. doi: 10.1038/s41467-017-00061-1

Gilhooley, M. J., Hickey, D. G., Lindner, M., Palumaa, T., Hughes, S., Peirson, S. N., et al. (2021). ON-bipolar cell gene expression during retinal degeneration: Implications for optogenetic visual restoration. *Exp. Eye Res.* 207, 108553. doi: 10.1016/j.exer.2021.108553

Gilhooley, M. J., Lindner, M., Palumaa, T., Hughes, S., Peirson, S. N., and Hankins, M. W. (2022). A systematic comparison of optogenetic approaches to visual restoration. *Mol. Ther. - Methods Clin. Dev.* 25, 111–123. doi: 10.1016/j.omtm.2022.03.003

Gill, J. S., Georgiou, M., Kalitzeos, A., Moore, A. T., and Michaelides, M. (2019). Progressive cone and cone-rod dystrophies: clinical features, molecular genetics and prospects for therapy. *Br. J. Ophthalmol.* 103, 711–720. doi: 10.1136/bjophthalmol-2018-313278

Girach, A., Audo, I., Birch, D. G., Huckfeldt, R. M., Lam, B. L., Leroy, B. P., et al. (2022). RNA-based therapies in inherited retinal diseases. *Ther. Adv. Ophthalmol.* 14, 251584142211346. doi: 10.1177/25158414221134602

Glisovic, T., Bachorik, J. L., Yong, J., and Dreyfuss, G. (2008). RNA-binding proteins and post-transcriptional gene regulation. *FEBS Lett.* 582, 1977–1986. doi: 10.1016/j.febslet.2008.03.004

Glockle, N., Kohl, S., Mohr, J., Scheurenbrand, T., Sprecher, A., Weisschuh, N., et al. (2014). Panel-based next generation sequencing as a reliable and efficient technique to detect mutations in unselected patients with retinal dystrophies. *Eur. J. Hum. Genet.* 22, 99–104. doi: 10.1038/ejhg.2013.72

Goldmann, T., Overlack, N., Möller, F., Belakhov, V., van Wyk, M., Baasov, T., et al. (2012). A comparative evaluation of NB30, NB54 and PTC124 in translational read-through efficacy for treatment of an USH1C nonsense mutation. *EMBO Mol. Med.* 4, 1186–99. doi: 10.1002/emmm.201201438

Goldmann, T., Overlack, N., Wolfrum, U., and Nagel-Wolfrum, K. (2011). PTC124-Mediated Translational Readthrough of a Nonsense Mutation Causing Usher Syndrome Type 1C. *Hum. Gene Ther.* 22, 537–547. doi: 10.1089/hum.2010.067

González-del Pozo, M., Borrego, S., Barragán, I., Pieras, J. I., Santoyo, J., Matamala, N., et al. (2011). Mutation screening of multiple genes in Spanish patients with autosomal recessive retinitis pigmentosa by targeted resequencing. *PLoS One* 6. doi: 10.1371/journal.pone.0027894

González-del Pozo, M., Fernández-Suárez, E., Bravo-Gil, N., Méndez-Vidal, C., Martín-Sánchez, M., Rodríguez-de la Rúa, E., et al. (2022). A comprehensive WGS-based pipeline for the identification of new candidate genes in inherited retinal dystrophies. *npj Genomic Med.* 7, 17. doi: 10.1038/s41525-022-00286-0

González-del Pozo, M., Martín-Sánchez, M., Bravo-Gil, N., Méndez-Vidal, C., Chimenea, Á., Rodríguez-de la Rúa, E., et al. (2018). Searching the second hit in patients with inherited retinal dystrophies and monoallelic variants in ABCA4, USH2A and CEP290 by whole-gene targeted sequencing. *Sci. Rep.* 8, 13312. doi: 10.1038/s41598-018-31511-5

Gonzalez-Fernandez, F., Kurz, D., Bao, Y., Newman, S., Conway, B. P., Young, J. E., et al. (1999). 11-cis retinol dehydrogenase mutations as a major cause of the congenital night-blindness disorder known as fundus albipunctatus. *Mol. Vis.* 5, 41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10617778

Goutaki, M., Lam, Y. T., Alexandru, M., Anagiotos, A., Armengot, M., Boon, M., et al. (2023). Characteristics of Otologic Disease Among Patients With Primary Ciliary Dyskinesia. *JAMA Otolaryngol. Neck Surg.* 149, 587. doi: 10.1001/jamaoto.2023.0841

Haer-Wigman, L., van Zelst-Stams, W. A., Pfundt, R., van den Born, L. I., Klaver, C. C., Verheij, J. B., et al. (2017). Diagnostic exome sequencing in 266 Dutch patients with visual impairment. *Eur. J.*

Hum. Genet. 25, 591–599. doi: 10.1038/ejhg.2017.9

Hamel, C. (2006). Retinitis pigmentosa. *Orphanet J. Rare Dis.* 1, 40. doi: 10.1186/1750-1172-1-40

Hamel, C. P. (2007). Cone rod dystrophies. *Orphanet J. Rare Dis.* 2, 7. doi: 10.1186/1750-1172-2-7

Hamel, C. P. (2014). Gene discovery and prevalence in inherited retinal dystrophies. *Comptes Rendus - Biol.* 337, 160–166. doi: 10.1016/j.crvi.2013.12.001

Han, J. H., Cancellieri, F., Perea-Romero, I., Ayuso, C., Quinodoz, M., and Rivolta, C. (2024). The p.C759F Variant in USH2A Is a Pathogenic Mutation: Systematic Literature Review and Meta-Analysis of 667 Genotypes. *Ophthalmic Res.* 67, 107–114. doi: 10.1159/000535545

Hanany, M., Rivolta, C., and Sharon, D. (2020). Worldwide carrier frequency and genetic prevalence of autosomal recessive inherited retinal diseases. *Proc. Natl. Acad. Sci.* 117, 2710–2716. doi: 10.1073/pnas.1913179117

Hanein, S., Perrault, I., Gerber, S., Tanguy, G., Barbet, F., Ducroq, D., et al. (2004). Leber congenital amaurosis: Comprehensive survey of the genetic heterogeneity, refinement of the clinical definition, and genotype-phenotype correlations as a strategy for molecular diagnosis. *Hum. Mutat.* 23, 306–317. doi: 10.1002/humu.20010

Haq, W., Arango-Gonzalez, B., Zrenner, E., Euler, T., and Schubert, T. (2014). Synaptic remodeling generates synchronous oscillations in the degenerated outer mouse retina. *Front. Neural Circuits* 8. doi: 10.3389/fncir.2014.00108

Haque, U. S., and Yokota, T. (2024). Recent Progress in Gene-Targeting Therapies for Spinal Muscular Atrophy: Promises and Challenges. *Genes (Basel).* 15, 999. doi: 10.3390/genes15080999

Harris, S. C., Chong, K., Chitayat, D., Gilmore, K. L., Jorge, A. A. L., Freire, B. L., et al. (2023). Diagnosis of <scp> *TBC1D32* </scp> -associated conditions: Expanding the phenotypic spectrum of a complex ciliopathy. *Am. J. Med. Genet. Part A* 191, 1282–1292. doi: 10.1002/ajmg.a.63150

Hartong, D. T., Berson, E. L., and Dryja, T. P. (2006). Retinitis pigmentosa. *Lancet (London, England)* 368, 1795–809. doi: 10.1016/S0140-6736(06)69740-7

Hauswirth, W. W. (2014). Retinal Gene Therapy Using Adeno-Associated Viral Vectors: Multiple Applications for a Small Virus. *Hum. Gene Ther.* 25, 671–678. doi: 10.1089/hum.2014.2530

Havens, M. A., and Hastings, M. L. (2016). Splice-switching antisense oligonucleotides as therapeutic drugs. *Nucleic Acids Res.* 44, 6549–6563. doi: 10.1093/nar/gkw533

Hayasaka, S., Shiono, T., Mizuno, K., Sasayama, C., Akiya, S., Tanaka, Y., et al. (1986). Gyrate atrophy of the choroid and retina: 15 Japanese patients. *Br. J. Ophthalmol.* 70, 612–614. doi: 10.1136/bjo.70.8.612

Heath Jeffery, R. C., Mukhtar, S. A., McAllister, I. L., Morgan, W. H., Mackey, D. A., and Chen, F. K. (2021). Inherited retinal diseases are the most common cause of blindness in the working-age population in Australia. *Ophthalmic Genet.* 42, 431–439. doi: 10.1080/13816810.2021.1913610

Hernández-Juárez, J., Rodríguez-Uribe, G., and Borooah, S. (2021). Toward the Treatment of Inherited Diseases of the Retina Using CRISPR-Based Gene Editing. *Front. Med.* 8. doi: 10.3389/fmed.2021.698521

Herrera, W., Aleman, T. S., Cideciyan, A. V., Roman, A. J., Banin, E., Ben-Yosef, T., et al. (2008). Retinal Disease in Usher Syndrome III Caused by Mutations in the Clarin-1 Gene. *Investig. Opthalmology Vis. Sci.* 49, 2651. doi: 10.1167/iovs.07-1505

Hietamäki, J., Gregory, L. C., Ayoub, S., Iivonen, A.-P., Vaaralahti, K., Liu, X., et al. (2020). Loss-of-Function Variants in TBC1D32 Underlie Syndromic Hypopituitarism. *J. Clin. Endocrinol. Metab.* 105, 1748–1758. doi: 10.1210/clinem/dgaa078

Hinnebusch, A. G., Ivanov, I. P., and Sonenberg, N. (2016). Translational control by 5′-untranslated regions of eukaryotic mRNAs. *Science (80-.).* 352, 1413–1416. doi: 10.1126/science.aad9868

Hirakawa, M. P., Krishnakumar, R., Timlin, J. A., Carney, J. P., and Butler, K. S. (2020). Gene editing and CRISPR in the clinic: current and future perspectives. *Biosci. Rep.* 40. doi: 10.1042/BSR20200127

Hitti-Malin, R. J., Panneman, D. M., Corradi, Z., Boonen, E. G. M., Astuti, G., Dhaenens, C.-M., et al. (2024). Towards Uncovering the Role of Incomplete Penetrance in Maculopathies through Sequencing of 105 Disease-Associated Genes. *Biomolecules* 14, 367. doi: 10.3390/biom14030367

Hope, C. I., Bundey, S., Proops, D., and Fielder, A. R. (1997). Usher syndrome in the city of Birmingham---prevalence and clinical classification. *Br. J. Ophthalmol.* 81, 46–53. doi: 10.1136/bjo.81.1.46

Hu, F., Gao, F., Li, J., Xu, P., Wang, D., Chen, F., et al. (2020). Novel variants associated with Stargardt disease in Chinese patients. *Gene* 754, 144890. doi: 10.1016/j.gene.2020.144890

Hu, M. L., Edwards, T. L., O'Hare, F., Hickey, D. G.,

Wang, J.-H., Liu, Z., et al. (2021). Gene therapy for inherited retinal diseases: progress and possibilities. *Clin. Exp. Optom.* 104, 444–454. doi: 10.1080/08164622.2021.1880863

Hu, X., Zhang, B., Li, X., Li, M., Wang, Y., Dan, H., et al. (2023). The application and progression of CRISPR/Cas9 technology in ophthalmological diseases. *Eye* 37, 607–617. doi: 10.1038/s41433-022-02169-1

Huang, C.-H., Yang, C.-M., Yang, C.-H., Hou, Y.-C., and Chen, T.-C. (2021). Leber's Congenital Amaurosis: Current Concepts of Genotype-Phenotype Correlations. *Genes (Basel).* 12, 1261. doi: 10.3390/genes12081261

Hussain, H. M. J., Wang, M., Huang, A., Schmidt, R., Qian, X., Yang, P., et al. (2023). Novel Pathogenic Mutations Identified from Whole-Genome Sequencing in Unsolved Cases of Patients Affected with Inherited Retinal Diseases. *Genes (Basel).* 14, 447. doi: 10.3390/genes14020447

Huynh, A., Gray, P. E., Sullivan, A., Mackie, J., Guerin, A., Rao, G., et al. (2024). A Novel Case of IFNAR1 Deficiency Identified a Common Canonical Splice Site Variant in DOCK8 in Western Polynesia: The Importance of Validating Variants of Unknown Significance in Under-Represented Ancestries. *J. Clin. Immunol.* 44, 170. doi: 10.1007/s10875-024-01774-x

Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. S., and Weissman, J. S. (2009). Genome-Wide Analysis in Vivo of Translation with Nucleotide Resolution Using Ribosome Profiling. *Science (80-.).* 324, 218–223. doi: 10.1126/science.1168978

Ingolia, N. T., Lareau, L. F., and Weissman, J. S. (2011). Ribosome Profiling of Mouse Embryonic Stem Cells Reveals the Complexity and Dynamics of Mammalian Proteomes. *Cell* 147, 789–802. doi: 10.1016/j.cell.2011.10.002

Irigoyen, C., Amenabar Alonso, A., Sanchez-Molina, J., Rodríguez-Hidalgo, M., Lara-López, A., and Ruiz-Ederra, J. (2022). Subretinal Injection Techniques for Retinal Disease: A Review. *J. Clin. Med.* 11, 4717. doi: 10.3390/jcm11164717

Ishikawa, H., Thompson, J., Yates, J. R., and Marshall, W. F. (2012). Proteomic Analysis of Mammalian Primary Cilia. *Curr. Biol.* 22, 414–419. doi: 10.1016/j.cub.2012.01.031

Jacobson, S. G., and Cideciyan, A. V. (2010). Treatment Possibilities for Retinitis Pigmentosa. *N. Engl. J. Med.* 363, 1669–1671. doi: 10.1056/NEJMcibr1007685

Jacobson, S. G., Cideciyan, A. V, Iannaccone, A., Weleber, R. G., Fishman, G. A., Maguire, A. M., et al. (2000). Disease expression of RP1 mutations causing autosomal dominant retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.* 41, 1898–908. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10845615

Jacoszek, A., Pollak, A., Płoski, R., and Ołdak, M. (2017). Advances in genetic hearing loss: CIB2 gene. *Eur. Arch. Oto-Rhino-Laryngology* 274, 1791–1795. doi: 10.1007/s00405-016-4330-9

Jaganathan, K., Kyriazopoulou Panagiotopoulou, S., McRae, J. F., Darbandi, S. F., Knowles, D., Li, Y. I., et al. (2019). Predicting Splicing from Primary Sequence with Deep Learning. *Cell* 176, 535-548.e24. doi: 10.1016/j.cell.2018.12.015

Jain, R., and Daigavane, S. (2024). Advances and Challenges in Gene Therapy for Inherited Retinal Dystrophies: A Comprehensive Review. *Cureus*. doi: 10.7759/cureus.69895

Jamshidi, F., Place, E. M., Mehrotra, S., Navarro-Gomez, D., Maher, M., Branham, K. E., et al. (2019). Contribution of noncoding pathogenic variants to RPGRIP1-mediated inherited retinal degeneration. *Genet. Med.* 21, 694–704. doi: 10.1038/s41436-018-0104-7

Jauregui, R., Park, K. S., Tanaka, A. J., Cho, A., Paavo, M., Zernant, J., et al. (2019). Spectrum of Disease Severity and Phenotype in Choroideremia Carriers. *Am. J. Ophthalmol.* 207, 77–86. doi: 10.1016/j.ajo.2019.06.002

Jayakody, S. A., Gonzalez-Cordero, A., Ali, R. R., and Pearson, R. A. (2015). Cellular strategies for retinal repair by photoreceptor replacement. *Prog. Retin. Eye Res.* 46, 31–66. doi: 10.1016/j.preteyeres.2015.01.003

Jespersgaard, C., Bertelsen, M., Arif, F., Gellert-Kristensen, H. G., Fang, M., Jensen, H., et al. (2020). Bi-Allelic Pathogenic Variations in MERTK Including Deletions Are Associated with an Early Onset Progressive Form of Retinitis Pigmentosa. *Genes (Basel).* 11, 1517. doi: 10.3390/genes11121517

Jespersgaard, C., Fang, M., Bertelsen, M., Dang, X., Jensen, H., Chen, Y., et al. (2019). Molecular genetic analysis using targeted NGS analysis of 677 individuals with retinal dystrophy. *Sci. Rep.* 9, 1–7. doi: 10.1038/s41598-018-38007-2

Jo, W. G., Lee, C. S., and Han, J. (2023). Clinical and Genetic Findings in Korean Patients with Choroideremia. *Korean J. Ophthalmol.* doi: 10.3341/kjo.2023.0020

Johansson, L. F., van Dijk, F., de Boer, E. N., van Dijk-Bos, K. K., Jongbloed, J. D. H., van der Hout, A. H., et al. (2016). CoNVaDING: Single Exon Variation Detection in Targeted NGS Data. *Hum. Mutat.* 37, 457–464. doi: 10.1002/humu.22969

Jouret, G., Poirsier, C., Spodenkiewicz, M., Jaquin, C., Gouy, E., Arndt, C., et al. (2019). Genetics of

Usher Syndrome: New Insights From a Meta-analysis. *Otol. Neurotol.* 40, 121–129. doi: 10.1097/MAO.0000000000002054

Jurkute, N., Cancellieri, F., Pohl, L., Li, C. H. Z., Heaton, R. A., Reurink, J., et al. (2022). Biallelic variants in coenzyme Q10 biosynthesis pathway genes cause a retinitis pigmentosa phenotype. *npj Genomic Med.* 7, 60. doi: 10.1038/s41525-022-00330-z

Kajiwara, K., Berson, E., and Dryja, T. (1994). Digenic retinitis pigmentosa due to mutations at the unlinked peripherin/RDS and ROM1 loci. *Science (80-.).* 264, 1604–1608. doi: 10.1126/science.8202715

Karali, M., and Banfi, S. (2015). Inherited Retinal Dystrophies: The role of gene expression regulators. *Int. J. Biochem. Cell Biol.* 61, 115–119. doi: 10.1016/j.biocel.2015.02.007

Karali, M., Testa, F., Di Iorio, V., Torella, A., Zeuli, R., Scarpato, M., et al. (2022). Genetic epidemiology of inherited retinal diseases in a large patient cohort followed at a single center in Italy. *Sci. Rep.* 12, 20815. doi: 10.1038/s41598-022-24636-1

Karam, A., Delvallée, C., Estrada-Cuzcano, A., Geoffroy, V., Lamouche, J.-B., Leuvrey, A.-S., et al. (2023). WGS Revealed Novel BBS5 Pathogenic Variants, Missed by WES, Causing Ciliary Structure and Function Defects. *Int. J. Mol. Sci.* 24, 8729. doi: 10.3390/ijms24108729

Kaway, C. S., Adams, M. K. M., Jenkins, K. S., and Layton, C. J. (2017). A Novel ABCA4 Mutation Associated with a Late-Onset Stargardt Disease Phenotype: A Hypomorphic Allele. *Case Rep. Ophthalmol.* 8, 180–184. doi: 10.1159/000464129

Kefalov, V. J. (2012). Rod and Cone Visual Pigments and Phototransduction through Pharmacological, Genetic, and Physiological Approaches. *J. Biol. Chem.* 287, 1635–1641. doi: 10.1074/jbc.R111.303008

Khan, A. O., Becirovic, E., Betz, C., Neuhaus, C., Altmüller, J., Maria Riedmayr, L., et al. (2017). A deep intronic CLRN1 (USH3A) founder mutation generates an aberrant exon and underlies severe Usher syndrome on the Arabian Peninsula. *Sci. Rep.* 7, 1–10. doi: 10.1038/s41598-017-01577-8

Khan, M., Arno, G., Fakin, A., Parfitt, D. A., Dhooge, P. P. A., Albert, S., et al. (2020a). Detailed Phenotyping and Therapeutic Strategies for Intronic ABCA4 Variants in Stargardt Disease. *Mol. Ther. - Nucleic Acids* 21, 412–427. doi: 10.1016/j.omtn.2020.06.007

Khan, M., Cornelis, S. S., De Pozo-Valero, M. l, Whelan, L., Runhart, E. H., Mishra, K., et al. (2020b). Resolving the dark matter of ABCA4 for 1054 Stargardt disease probands through integrated genomics and transcriptomics. *Genet. Med.* 22, 1235–1246. doi: 10.1038/s41436-020-0787-4

Khan, M., Cornelis, S. S., Khan, M. I., Elmelik, D., Manders, E., Bakker, S., et al. (2019). Cost-effective molecular inversion probe-based ABCA4 sequencing reveals deep-intronic variants in Stargardt disease. *Hum. Mutat.* 40, 1749–1759. doi: 10.1002/humu.23787

Khan, M., and Cremers, F. P. M. (2020). ABCA4-Associated Stargardt Disease. *Klin. Monbl. Augenheilkd.* 237, 267–274. doi: 10.1055/a-1057-9939

Khan, M. I., Kersten, F. F. J., Azam, M., Collin, R. W. J., Hussain, A., Shah, S. T.-A., et al. (2011). CLRN1 Mutations Cause Nonsyndromic Retinitis Pigmentosa. *Ophthalmology* 118, 1444–1448. doi: 10.1016/j.ophtha.2010.10.047

Khan, M. Y., Ibraheim, A. S., and Firoozmand, S. (1994). Gyrate atrophy of the choroid and retina with hyperornithinaemia, cystinuria and lysinuria. *Eye* 8, 284–287. doi: 10.1038/eye.1994.58

Khateb, S., Kowalewski, B., Bedoni, N., Damme, M., Pollack, N., Saada, A., et al. (2018). A homozygous founder missense variant in arylsulfatase G abolishes its enzymatic activity causing atypical Usher syndrome in humans. *Genet. Med.* 20, 1004–1012. doi: 10.1038/gim.2017.227

Khateb, S., Zelinger, L., Mizrahi-meissonnier, L., Ayuso, C., Koenekoop, R. K., Laxer, U., et al. (2014a). A homozygous nonsense CEP250 mutation combined with a heterozygous nonsense C2orf71 mutation is associated with atypical Usher syndrome. 460–469. doi: 10.1136/jmedgenet-2014-102287

Khateb, S., Zelinger, L., Mizrahi-Meissonnier, L., Ayuso, C., Koenekoop, R. K., Laxer, U., et al. (2014b). A homozygous nonsense CEP250 mutation combined with a heterozygous nonsense C2orf71 mutation is associated with atypical Usher syndrome. *J. Med. Genet.* 51, 460–469. doi: 10.1136/jmedgenet-2014-102287

Khvorova, A., and Watts, J. K. (2017). The chemical evolution of oligonucleotide therapies of clinical utility. *Nat. Biotechnol.* 35, 238–248. doi: 10.1038/nbt.3765

Kim, B. M., Song, H. S., Kim, J.-Y., Kwon, E. Y., Ha, S. Y., Kim, M., et al. (2022). Functional characterization of ABCA4 genetic variants related to Stargardt disease. *Sci. Rep.* 12, 22282. doi: 10.1038/s41598-022-26912-6

Kim, S., Scheffler, K., Halpern, A. L., Bekritsky, M. A., Noh, E., Källberg, M., et al. (2018). Strelka2: fast and accurate calling of germline and somatic variants. *Nat. Methods* 15, 591–594. doi: 10.1038/s41592-018-0051-x

Kimberling, W. J., Hildebrand, M. S., Shearer, A. E., Jensen, M. L., Halder, J. A., Trzupek, K., et al. (2010). Frequency of Usher syndrome in two pediatric populations: Implications for genetic screening of deaf and hard of hearing children. *Genet. Med.* 12, 512–516. doi: 10.1097/GIM.0b013e3181e5afb8

Kinnick, T. R., Mullins, R. F., Dev, S., Leys, M., Mackey, D. A., Kay, C. N., et al. (2011). AUTOSOMAL RECESSIVE VITELLIFORM MACULAR DYSTROPHY IN A LARGE COHORT OF VITELLIFORM MACULAR DYSTROPHY PATIENTS. *Retina* 31, 581–595. doi: 10.1097/IAE.0b013e318203ee60

Kiser, P. D., Zhang, J., Sharma, A., Angueyra, J. M., Kolesnikov, A. V., Badiee, M., et al. (2018). Retinoid isomerase inhibitors impair but do not block mammalian cone photoreceptor function. *J. Gen. Physiol.* 150, 571–590. doi: 10.1085/jgp.201711815

Ko, H. W., Norman, R. X., Tran, J., Fuller, K. P., Fukuda, M., and Eggenschwiler, J. T. (2010). Broad-Minded Links Cell Cycle-Related Kinase to Cilia Assembly and Hedgehog Signal Transduction. *Dev. Cell* 18, 237–247. doi: 10.1016/j.devcel.2009.12.014

Kocaaga, A., Aköz, İ. Ö., Demir, N. U., and Paksoy, B. (2023). Identification of novel variants in retinitis pigmentosa genes by whole-exome sequencing. *Rev. Assoc. Med. Bras.* 69. doi: 10.1590/1806-9282.20221073

Koenekoop, R. K. (2004). An overview of leber congenital amaurosis: a model to understand human retinal development. *Surv. Ophthalmol.* 49, 379–398. doi: 10.1016/j.survophthal.2004.04.003

Koenekoop, R. K., Lopez, I., den Hollander, A. I., Allikmets, R., and Cremers, F. P. (2007). Genetic testing for retinal dystrophies and dysfunctions: benefits, dilemmas and solutions. *Clin. Experiment. Ophthalmol.* 35, 473–485. doi: 10.1111/j.1442-9071.2007.01534.x

Kohl, S., Christ-Adler, M., Apfelstedt-Sylla, E., Kellner, U., Eckstein, A., Zrenner, E., et al. (1997). RDS/peripherin gene mutations are frequent causes of central retinal dystrophies. *J. Med. Genet.* 34, 620–626. doi: 10.1136/jmg.34.8.620

Komro, J., Skender, S., Ross, B. X., and Lin, X. (2022). Autosomal Dominant Vitreoretinochoroidopathy With a Novel BEST1 Mutation and a Review of Reported Mutations. *Cureus*. doi: 10.7759/cureus.32990

Kono, N., and Arakawa, K. (2019). Nanopore sequencing: Review of potential applications in functional genomics. *Dev. Growth Differ.* 61, 316–326. doi: 10.1111/dgd.12608

Koolen, L., Gagliardi, G., ten Brink, S. C. A., de Breuk, A., Heesterbeek, T. J., Hoyng, C. B., et al. (2022). Generation and characterization of human induced pluripotent stem cells (iPSCs) from three patients with age-related macular degeneration carrying rare variants in the CFH gene. *Stem Cell Res.* 60, 102669. doi: 10.1016/j.scr.2022.102669

Koyanagi, Y., Akiyama, M., Nishiguchi, K. M., Momozawa, Y., Kamatani, Y., Takata, S., et al. (2019). Genetic characteristics of retinitis pigmentosa in 1204 Japanese patients. *J. Med. Genet.* 56, 662–670. doi: 10.1136/jmedgenet-2018-105691

Krawitz, P. M., Schiska, D., Krüger, U., Appelt, S., Heinrich, V., Parkhomchuk, D., et al. (2014). Screening for single nucleotide variants, small indels and exon deletions with a next-generation sequencing based gene panel approach for <scp>U</scp> sher syndrome. *Mol. Genet. Genomic Med.* 2, 393–401. doi: 10.1002/mgg3.92

Kremer, H., van Wijk, E., Märker, T., Wolfrum, U., and Roepman, R. (2006). Usher syndrome: molecular links of pathogenesis, proteins and pathways. *Hum. Mol. Genet.* 15, R262–R270. doi: 10.1093/hmg/ddl205

Kuijper, E. C., Bergsma, A. J., Pijnappel, W. W. M. P., and Aartsma-Rus, A. (2021). Opportunities and challenges for antisense oligonucleotide therapies. *J. Inherit. Metab. Dis.* 44, 72–87. doi: 10.1002/jimd.12251

Kumaran, N., Moore, A. T., Weleber, R. G., and Michaelides, M. (2017). Leber congenital amaurosis/early-onset severe retinal dystrophy: clinical features, molecular genetics and therapeutic interventions. *Br. J. Ophthalmol.* 101, 1147–1154. doi: 10.1136/bjophthalmol-2016-309975

Kurzawa-Akanbi, M., Tzoumas, N., Corral-Serrano, J. C., Guarascio, R., Steel, D. H., Cheetham, M. E., et al. (2024). Pluripotent stem cell-derived models of retinal disease: Elucidating pathogenesis, evaluating novel treatments, and estimating toxicity. *Prog. Retin. Eye Res.* 100, 101248. doi: 10.1016/j.preteyeres.2024.101248

Kute, P. M., Soukarieh, O., Tjeldnes, H., Trégouët, D.-A., and Valen, E. (2022). Small Open Reading Frames, How to Find Them and Determine Their Function. *Front. Genet.* 12. doi: 10.3389/fgene.2021.796060

LaDuca, H., Farwell, K. D., Vuong, H., Lu, H.-M., Mu, W., Shahmirzadi, L., et al. (2017). Exome sequencing covers >98% of mutations identified on targeted next generation sequencing panels. *PLoS One* 12, e0170843. doi: 10.1371/journal.pone.0170843

Le Quesne Stabej, P., Saihan, Z., Rangesh, N., Steele-

Stallard, H. B., Ambrose, J., Coffey, A., et al. (2012). Comprehensive sequence analysis of nine Usher syndrome genes in the UK National Collaborative Usher Study. *J. Med. Genet.* 49, 27–36. doi: 10.1136/jmedgenet-2011-100468

Lee, K., Berg, J. S., Milko, L., Crooks, K., Lu, M., Bizon, C., et al. (2015). High Diagnostic Yield of Whole Exome Sequencing in Participants With Retinal Dystrophies in a Clinical Ophthalmology Setting. *Am. J. Ophthalmol.* 160, 354-363.e9. doi: 10.1016/j.ajo.2015.04.026

Lee, S., Liu, B., Lee, S., Huang, S.-X., Shen, B., and Qian, S.-B. (2012). Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution. *Proc. Natl. Acad. Sci.* 109. doi: 10.1073/pnas.1207846109

Lee, Y., and Rio, D. C. (2015). Mechanisms and Regulation of Alternative Pre-mRNA Splicing. *Annu. Rev. Biochem.* 84, 291–323. doi: 10.1146/annurev-biochem-060614-034316

Lenassi, E., Vincent, A., Li, Z., Saihan, Z., Coffey, A. J., Steele-Stallard, H. B., et al. (2015). A detailed clinical and molecular survey of subjects with nonsyndromic USH2A retinopathy reveals an allelic hierarchy of disease-causing variants. *Eur. J. Hum. Genet.* 23, 1318–1327. doi: 10.1038/ejhg.2014.283

Lentz, J. J., Jodelka, F. M., Hinrich, A. J., McCaffrey, K. E., Farris, H. E., Spalitta, M. J., et al. (2013). Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. *Nat. Med.* 19, 345–350. doi: 10.1038/nm.3106

Leppek, K., Das, R., and Barna, M. (2018). Functional 5′ UTR mRNA structures in eukaryotic translation regulation and how to find them. *Nat. Rev. Mol. Cell Biol.* 19, 158–174. doi: 10.1038/nrm.2017.103

Leventea, E., Zhu, Z., Fang, X., Nikolaeva, Y., Markham, E., Hirst, R. A., et al. (2021). Ciliopathy genes are required for apical secretion of Cochlin, an otolith crystallization factor. *Proc. Natl. Acad. Sci.* 118. doi: 10.1073/pnas.2102562118

Lewis, R. A., Shroyer, N. F., Singh, N., Allikmets, R., Hutchinson, A., Li, Y., et al. (1999). Genotype/Phenotype Analysis of a Photoreceptor-Specific ATP-Binding Cassette Transporter Gene, ABCR, in Stargardt Disease. *Am. J. Hum. Genet.* 64, 422–434. doi: 10.1086/302251

Li, D., Mastaglia, F. L., Fletcher, S., and Wilton, S. D. (2018). Precision Medicine through Antisense Oligonucleotide-Mediated Exon Skipping. *Trends Pharmacol. Sci.* 39, 982–994. doi: 10.1016/j.tips.2018.09.001

Li, G., Wu, J., and Wang, X. (2024). Predicting functional UTR variants by integrating region-specific features. *Brief. Bioinform.* 25. doi: 10.1093/bib/bbae248

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Liew, G., Michaelides, M., and Bunce, C. (2014). A comparison of the causes of blindness certifications in England and Wales in working age adults (16–64 years), 1999–2000 with 2009–2010. *BMJ Open* 4, e004015. doi: 10.1136/bmjopen-2013-004015

Lindner, M., Gilhooley, M. J., Hughes, S., and Hankins, M. W. (2022). Optogenetics for visual restoration: From proof of principle to translational challenges. *Prog. Retin. Eye Res.* 91, 101089. doi: 10.1016/j.preteyeres.2022.101089

Liquori, A., Vaché, C., Baux, D., Blanchet, C., Hamel, C., Malcolm, S., et al. (2016). Whole USH2A Gene Sequencing Identifies Several New Deep Intronic Mutations. *Hum. Mutat.* 37, 184–193. doi: 10.1002/humu.22926

Littink, K. W., Pott, J.-W. R., Collin, R. W. J., Kroes, H. Y., Verheij, J. B. G. M., Blokland, E. A. W., et al. (2010). A Novel Nonsense Mutation in CEP290 Induces Exon Skipping and Leads to a Relatively Mild Retinal Phenotype. *Investig. Opthalmology Vis. Sci.* 51, 3646. doi: 10.1167/iovs.09-5074

Liu, H., Lu, S., Chen, M., Gao, N., Yang, Y., Hu, H., et al. (2024a). Towards Stem/Progenitor Cell-Based Therapies for Retinal Degeneration. *Stem Cell Rev. Reports* 20, 1459–1479. doi: 10.1007/s12015-024-10740-4

Liu, H., Xu, J., Lan, Y., Lim, H.-W., and Jiang, R. (2021). The Scleraxis Transcription Factor Directly Regulates Multiple Distinct Molecular and Cellular Processes During Early Tendon Cell Differentiation. *Front. Cell Dev. Biol.* 9. doi: 10.3389/fcell.2021.654397

Liu, X., Hu, F., Zhang, D., Li, Z., He, J., Zhang, S., et al. (2024b). Whole genome sequencing enables new genetic diagnosis for inherited retinal diseases by identifying pathogenic variants. *npj Genomic Med.* 9, 6. doi: 10.1038/s41525-024-00391-2

Liu, X. Z., Li, Y. Y., and Yang, L. P. (2020). [Comparison study of whole exome sequencing and targeted panel sequencing in molecular diagnosis of inherited retinal dystrophies]. *Beijing Da Xue Xue Bao.* 52, 836–844. Available at: http://www.ncbi.nlm.nih.gov/pubmed/33047716

Liu, Y., Zong, X., Cao, W., Zhang, W., Zhang, N., and Yang, N. (2024c). Gene Therapy for Retinitis Pigmentosa: Current Challenges and New Progress. *Biomolecules* 14, 903. doi:

10.3390/biom14080903

Lizardi, P. M., Forloni, M., and Wajapeyee, N. (2011). Genome-wide approaches for cancer gene discovery. *Trends Biotechnol.* 29, 558–568. doi: 10.1016/j.tibtech.2011.06.003

Loose, M., Malla, S., and Stout, M. (2016). Real-time selective sequencing using nanopore technology. *Nat. Methods* 13, 751–754. doi: 10.1038/nmeth.3930

Lord, J., and Baralle, D. (2021). Splicing in the Diagnosis of Rare Disease: Advances and Challenges. *Front. Genet.* 12. doi: 10.3389/fgene.2021.689892

Lotery, A. J. (2001). Mutations in the CRB1 Gene Cause Leber Congenital Amaurosis. *Arch. Ophthalmol.* 119, 415. doi: 10.1001/archopht.119.3.415

MacDonald, I. M., Russell, L., and Chan, C.-C. (2009). Choroideremia: New Findings from Ocular Pathology and Review of Recent Literature. *Surv. Ophthalmol.* 54, 401–407. doi: 10.1016/j.survophthal.2009.02.008

Maerker, T., van Wijk, E., Overlack, N., Kersten, F. F. J., McGee, J., Goldmann, T., et al. (2008). A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. *Hum. Mol. Genet.* 17, 71–86. doi: 10.1093/hmg/ddm285

Magi, A., Semeraro, R., Mingrino, A., Giusti, B., and D'Aurizio, R. (2017). Nanopore sequencing data analysis: state of the art, applications and challenges. *Brief. Bioinform.* doi: 10.1093/bib/bbx062

Malka, S., Biswas, P., Berry, A.-M., Sangermano, R., Ullah, M., Lin, S., et al. (2024). Substitution of a single non-coding nucleotide upstream of TMEM216 causes non-syndromic retinitis pigmentosa and is associated with reduced TMEM216 expression. *Am. J. Hum. Genet.* 111, 2012–2030. doi: 10.1016/j.ajhg.2024.07.020

Malm, E., Ponjavic, V., Möller, C., Kimberling, W. J., and Andréasson, S. (2011). Phenotypes in Defined Genotypes Including Siblings with Usher Syndrome. *Ophthalmic Genet.* 32, 65–74. doi: 10.3109/13816810.2010.536064

Malvasi, M., Casillo, L., Avogaro, F., Abbouda, A., and Vingolo, E. M. (2023). Gene Therapy in Hereditary Retinal Dystrophies: The Usefulness of Diagnostic Tools in Candidate Patient Selections. *Int. J. Mol. Sci.* 24, 13756. doi: 10.3390/ijms241813756

Manes, G., Guillaumie, T., Vos, W. L., Devos, A., Zeitz, C., Marquette, V., et al. (2015). US.

Manley, A., Meshkat, B. I., Jablonski, M. M., and Hollingsworth, T. J. (2023). Cellular and Molecular Mechanisms of Pathogenesis Underlying Inherited Retinal Dystrophies. *Biomolecules* 13, 271. doi: 10.3390/biom13020271

Mansard, L., Baux, D., Vaché, C., Blanchet, C., Meunier, I., Willems, M., et al. (2021). The Study of a 231 French Patient Cohort Significantly Extends the Mutational Spectrum of the Two Major Usher Genes MYO7A and USH2A. *Int. J. Mol. Sci.* 22, 13294. doi: 10.3390/ijms222413294

Mantere, T., Kersten, S., and Hoischen, A. (2019). Long-Read Sequencing Emerging in Medical Genetics. *Front. Genet.* 10. doi: 10.3389/fgene.2019.00426

Mäntyjärvi, M., and Tuppurainen, K. (1998). Colour vision in gyrate atrophy. *Vision Res.* 38, 3409–3412. doi: 10.1016/S0042-6989(98)00073-X

Maria Oonk, A. M., van Huet, R. A. C., Leijendeckers, J. M., Oostrik, J., Venselaar, H., van Wijk, E., et al. (2015). Nonsyndromic hearing loss caused by USH1G mutations: widening the USH1G disease spectrum. *Ear Hear.* 36, 205–11. doi: 10.1097/AUD.0000000000000095

Marouf, A., Johnson, B., and Alagramam, K. N. (2022). Usher syndrome IIIA: a review of the disorder and preclinical research advances in therapeutic approaches. *Hum. Genet.* 141, 759–783. doi: 10.1007/s00439-022-02446-9

Mastrorosa, F. K., Miller, D. E., and Eichler, E. E. (2023). Applications of long-read sequencing to Mendelian genetics. *Genome Med.* 15, 42. doi: 10.1186/s13073-023-01194-3

Mata, N. L., Radu, R. A., Clemmons, R. S., and Travis, G. H. (2002). Isomerization and Oxidation of Vitamin A in Cone-Dominant Retinas. *Neuron* 36, 69–80. doi: 10.1016/S0896-6273(02)00912-1

Matalkah, F., Jeong, B., Sheridan, M., Horstick, E., Ramamurthy, V., and Stoilov, P. (2022). The Musashi proteins direct post-transcriptional control of protein expression and alternate exon splicing in vertebrate photoreceptors. *Commun. Biol.* 5, 1011. doi: 10.1038/s42003-022-03990-w

Mathur, P., and Yang, J. (2015). Usher syndrome: Hearing loss, retinal degeneration and associated abnormalities. *Biochim. Biophys. Acta* 1852, 406–20. doi: 10.1016/j.bbadis.2014.11.020

Maw, M., Kumaramanickavel, G., Kar, B., John, S., Bridges, R., and Denton, M. (1998). Two Indian siblings with Oguchi disease are homozygous for an arrestin mutation encoding premature termination. *Hum. Mutat.* 11, S317–S319. doi: 10.1002/humu.1380110199

May-Simera, H. L., Wan, Q., Jha, B. S., Hartford, J., Khristov, V., Dejene, R., et al. (2018). Primary Cilium-Mediated Retinal Pigment Epithelium Maturation Is Disrupted in Ciliopathy Patient

Cells. *Cell Rep.* 22, 189–205. doi: 10.1016/j.celrep.2017.12.038

Mayya, V. K., and Duchaine, T. F. (2019). Ciphers and Executioners: How 3'-Untranslated Regions Determine the Fate of Messenger RNAs. *Front. Genet.* 10. doi: 10.3389/fgene.2019.00006

Mazzarotto, F., Olivotto, I., and Walsh, R. (2020). Advantages and Perils of Clinical Whole-Exome and Whole-Genome Sequencing in Cardiomyopathy. *Cardiovasc. Drugs Ther.* 34, 241–253. doi: 10.1007/s10557-020-06948-4

Mburu, P., Mustapha, M., Varela, A., Weil, D., El-Amraoui, A., Holme, R. H., et al. (2003). Defects in whirlin, a PDZ domain molecule involved in stereocilia elongation, cause deafness in the whirler mouse and families with DFNB31. *Nat. Genet.* 34, 421–428. doi: 10.1038/ng1208

McClements, M. E., Barnard, A. R., Singh, M. S., Charbel Issa, P., Jiang, Z., Radu, R. A., et al. (2019). An AAV Dual Vector Strategy Ameliorates the Stargardt Phenotype in Adult Abca4 −/− Mice. *Hum. Gene Ther.* 30, 590–600. doi: 10.1089/hum.2018.156

McClements, M. E., Staurenghi, F., MacLaren, R. E., and Cehajic-Kapetanovic, J. (2020). Optogenetic Gene Therapy for the Degenerate Retina: Recent Advances. *Front. Neurosci.* 14. doi: 10.3389/fnins.2020.570909

McGee, T. L., Seyedahmadi, B. J., Sweeney, M. O., Dryja, T. P., and Berson, E. L. (2010). Novel mutations in the long isoform of the USH2A gene in patients with Usher syndrome type II or non-syndromic retinitis pigmentosa. *J. Med. Genet.* 47, 499–506. doi: 10.1136/jmg.2009.075143

McGillivray, P., Ault, R., Pawashe, M., Kitchen, R., Balasubramanian, S., and Gerstein, M. (2018). A comprehensive catalog of predicted functional upstream open reading frames in humans. *Nucleic Acids Res.* 46, 3326–3338. doi: 10.1093/nar/gky188

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R. S., Thormann, A., et al. (2016). The Ensembl Variant Effect Predictor. *Genome Biol.* 17, 122. doi: 10.1186/s13059-016-0974-4

Megaw, R. D., Soares, D. C., and Wright, A. F. (2015). RPGR: Its role in photoreceptor physiology, human disease, and future therapies. *Exp. Eye Res.* 138, 32–41. doi: 10.1016/j.exer.2015.06.007

Méjécase, C., Hummel, A., Mohand-Saïd, S., Andrieu, C., El Shamieh, S., Antonio, A., et al. (2019). Whole exome sequencing resolves complex phenotype and identifies CC2D2A mutations underlying non-syndromic rod-cone dystrophy. *Clin. Genet.* 95, 329–333. doi: 10.1111/cge.13453

Mendell, J. R., Connolly, A. M., Lehman, K. J., Griffin, D. A., Khan, S. Z., Dharia, S. D., et al. (2022). Testing preexisting antibodies prior to AAV gene transfer therapy: rationale, lessons and future considerations. *Mol. Ther. - Methods Clin. Dev.* 25, 74–83. doi: 10.1016/j.omtm.2022.02.011

Merkle, T., Merz, S., Reautschnig, P., Blaha, A., Li, Q., Vogel, P., et al. (2019). Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides. *Nat. Biotechnol.* 37, 133–138. doi: 10.1038/s41587-019-0013-6

Michaelides, M. (2004). The cone dysfunction syndromes. *Br. J. Ophthalmol.* 88, 291–297. doi: 10.1136/bjo.2003.027102

Millán, J. M., Aller, E., Jaijo, T., Blanco-Kelly, F., Gimenez-Pardo, A., and Ayuso, C. (2011). An Update on the Genetics of Usher Syndrome. *J. Ophthalmol.* 2011, 1–8. doi: 10.1155/2011/417217

Millo, T., Rivera, A., Obolensky, A., Marks-Ohana, D., Xu, M., Li, Y., et al. (2022). Identification of autosomal recessive novel genes and retinal phenotypes in members of the solute carrier (SLC) superfamily. *Genet. Med.* 24, 1523–1535. doi: 10.1016/j.gim.2022.03.020

Monies, D., Abouelhoda, M., Assoum, M., Moghrabi, N., Rafiullah, R., Almontashiri, N., et al. (2019). Lessons Learned from Large-Scale, First-Tier Clinical Exome Sequencing in a Highly Consanguineous Population. *Am. J. Hum. Genet.* 104, 1182–1201. doi: 10.1016/j.ajhg.2019.04.011

Moon, K.-H., Ma, J.-H., Min, H., Koo, H., Kim, H., Ko, H. W., et al. (2020). Dysregulation of sonic hedgehog signaling causes hearing loss in ciliopathy mouse models. *Elife* 9. doi: 10.7554/eLife.56551

Moran, A. L., Fehilly, J. D., Blacque, O., and Kennedy, B. N. (2023). Gene therapy for RAB28: What can we learn from zebrafish? *Vision Res.* 210, 108270. doi: 10.1016/j.visres.2023.108270

Moseley, J., Leest, T., Larsson, K., Magrelli, A., and Stoyanova-Beninska, V. (2024). Inherited retinal dystrophies and orphan designations in the European Union. *Eur. J. Ophthalmol.* doi: 10.1177/11206721241236214

Mustapha, M., Chouery, E., Chardenoux, S., Naboulsi, M., Paronnaud, J., Lemainque, A., et al. (2002). DFNB31, a recessive form of sensorineural hearing loss, maps to chromosome 9q32-34. *Eur. J. Hum. Genet.* 10, 210–212. doi: 10.1038/sj.ejhg.5200780

Nájera, C., Beneyto, M., Blanca, J., Aller, E., Fontcuberta, A., Millán, J. M., et al. (2002). Mutations in Myosin VIIA (MYO7A) and Usherin (USH2A) in Spanish patients with usher syndrome types I and II, respectively. *Hum. Mutat.* 20, 76–77. doi: 10.1002/humu.9042

Nakamichi, K., Van Gelder, R. N., Chao, J. R., and Mustafi, D. (2023). Targeted adaptive long-read sequencing for discovery of complex phased variants in inherited retinal disease patients. *Sci. Rep.* 13, 8535. doi: 10.1038/s41598-023-35791-4

Nash, B. M., Ma, A., Ho, G., Farnsworth, E., Minoche, A. E., Cowley, M. J., et al. (2022). Whole Genome Sequencing, Focused Assays and Functional Studies Increasing Understanding in Cryptic Inherited Retinal Dystrophies. *Int. J. Mol. Sci.* 23, 3905. doi: 10.3390/ijms23073905

Nasonkin, I., Illing, M., Koehler, M. R., Schmid, M., Molday, R. S., and Weber, B. H. F. (1998). Mapping of the rod photoreceptor ABC transporter (ABCR) to 1p21-p22.1 and identification of novel mutations in Stargardt's disease. *Hum. Genet.* 102, 21–26. doi: 10.1007/s004390050649

Ness, S. L. (2003). Genetic homogeneity and phenotypic variability among Ashkenazi Jews with Usher syndrome type III. *J. Med. Genet.* 40, 767–772. doi: 10.1136/jmg.40.10.767

Neuhaus, C., Eisenberger, T., Decker, C., Nagl, S., Blank, C., Issa, P. C., et al. (2017). Next-generation sequencing reveals the mutational landscape of clinically diagnosed Usher syndrome : copy number variations , phenocopies , a predominant target for translational read-through , and PEX26 mutated in Heimler syndrome. 531–552. doi: 10.1002/mgg3.312

Neveling, K., den Hollander, A. I., Cremers, F. P. M., and Collin, R. W. J. (2012). "Identification and Analysis of Inherited Retinal Disease Genes," 3–23. doi: 10.1007/978-1-62703-080-9_1

Nikopoulos, K., Cisarova, K., Quinodoz, M., Koskiniemi-Kuendig, H., Miyake, N., Farinelli, P., et al. (2019). A frequent variant in the Japanese population determines quasi-Mendelian inheritance of rare retinal ciliopathy. *Nat. Commun.* 10, 2884. doi: 10.1038/s41467-019-10746-4

Nishi, T., Kaneko, I., Iwanaga, S., and Yuda, M. (2024). PbARID-associated chromatin remodeling events are essential for gametocyte development in Plasmodium. *Nucleic Acids Res.* 52, 5624–5642. doi: 10.1093/nar/gkae207

Nishiguchi, K. M., Fujita, K., Miya, F., Katayama, S., and Nakazawa, T. (2020). Single AAV-mediated mutation replacement genome editing in limited number of photoreceptors restores vision in mice. *Nat. Commun.* 11, 482. doi: 10.1038/s41467-019-14181-3

Nishiguchi, K. M., and Rivolta, C. (2012). Genes Associated with Retinitis Pigmentosa and Allied Diseases Are Frequently Mutated in the General Population. *PLoS One* 7, e41902. doi: 10.1371/journal.pone.0041902

Nishimura, D. Y., Baye, L. M., Perveen, R., Searby, C. C., Avila-Fernandez, A., Pereiro, I., et al. (2010). Discovery and Functional Analysis of a Retinitis Pigmentosa Gene, C2ORF71. *Am. J. Hum. Genet.* 86, 686–695. doi: 10.1016/j.ajhg.2010.03.005

Nixon, T., Meredith, S., and Snead, M. (2024). "Hereditary Vitreoretinopathies," in *Reference Module in Neuroscience and Biobehavioral Psychology*, (Elsevier). doi: 10.1016/B978-0-443-13820-1.00010-4

Nolen, R. M., Hufnagel, R. B., Friedman, T. B., Turriff, A. E., Brewer, C. C., Zalewski, C. K., et al. (2020). Atypical and ultra-rare Usher syndrome: a review. *Ophthalmic Genet.* 41, 401–412. doi: 10.1080/13816810.2020.1747090

Nuzbrokh, Y., Ragi, S. D., and Tsang, S. H. (2021). Gene therapy for inherited retinal diseases. *Ann. Transl. Med.* 9, 1278–1278. doi: 10.21037/atm-20-4726

Olivares-González, L., Velasco, S., Campillo, I., and Rodrigo, R. (2021). Retinal Inflammation, Cell Death and Inherited Retinal Dystrophies. *Int. J. Mol. Sci.* 22, 2096. doi: 10.3390/ijms22042096

Ordoñez-Labastida, V., Chacon-Camacho, O. F., Lopez-Rodriguez, V. R., and Zenteno, J. C. (2023). USH2A mutational spectrum causing syndromic and non-syndromic retinal dystrophies in a large cohort of Mexican patients. *Mol. Vis.* 29, 31–38. Available at: http://www.ncbi.nlm.nih.gov/pubmed/37287646

Oshima, A., Jaijo, T., Aller, E., Millan, J. M., Carney, C., Usami, S., et al. (2008). Mutation profile of theCDH23 gene in 56 probands with Usher syndrome type I. *Hum. Mutat.* 29, E37–E46. doi: 10.1002/humu.20761

Ouyang, X., Xia, X., Verpy, E., Du, L., Pandya, A., Petit, C., et al. (2002). Mutations in the alternatively spliced exons of USH1C cause non-syndromic recessive deafness. *Hum. Genet.* 111, 26–30. doi: 10.1007/s00439-002-0736-0

Parfitt, D. A., Lane, A., Ramsden, C. M., Carr, A.-J. F., Munro, P. M., Jovanovic, K., et al. (2016). Identification and Correction of Mechanisms Underlying Inherited Blindness in Human iPSC-Derived Optic Cups. *Cell Stem Cell* 18, 769–781. doi: 10.1016/j.stem.2016.03.021

Parmeggiani, F., S. Sorrentino, F., Ponzin, D., Barbaro, V., Ferrari, S., and Di Iorio, E. (2011). Retinitis Pigmentosa: Genes and Disease Mechanisms. *Curr. Genomics* 12, 238–249. doi: 10.2174/138920211795860107

Parnami, K., and Bhattacharyya, A. (2023). Current approaches to vision restoration using optogenetic therapy. *Front. Cell. Neurosci.* 17. doi: 10.3389/fncel.2023.1236826

Patel, N., Khan, A. O., Alsahli, S., Abdel-Salam, G., Nowilaty, S. R., Mansour, A. M., et al. (2018). Genetic investigation of 93 families with microphthalmia or posterior microphthalmos. *Clin. Genet.* 93, 1210–1222. doi: 10.1111/cge.13239

Payne, A. M., Downes, S. M., Bessant, D. A. R., Bird, A. C., and Bhattacharya, S. S. (1998). Founder Effect, Seen in the British Population, of the 172 Peripherin/RDS Mutation—and Further Refinement of Genetic Positioning of the Peripherin/RDS Gene. *Am. J. Hum. Genet.* 62, 192–195. doi: 10.1086/301679

Peng, Y.-W., Hao, Y., Petters, R. M., and Wong, F. (2000). Ectopic synaptogenesis in the mammalian retina caused by rod photoreceptor-specific mutations. *Nat. Neurosci.* 3, 1121–1127. doi: 10.1038/80639

Perea-Romero, I., Gordo, G., Iancu, I. F., Del Pozo-Valero, M., Almoguera, B., Blanco-Kelly, F., et al. (2021). Genetic landscape of 6089 inherited retinal dystrophies affected cases in Spain and their therapeutic and extended epidemiological implications. *Sci. Rep.* 11, 1526. doi: 10.1038/s41598-021-81093-y

Peter, V. G., Quinodoz, M., Sadio, S., Held, S., Rodrigues, M., Soares, M., et al. (2021). New clinical and molecular evidence linking mutations in ARSG to Usher syndrome type IV. *Hum. Mutat.* 42, 261–271. doi: 10.1002/humu.24150

Peter, V. G., Quinodoz, M., Sadio, S., Held, S., Rodrigues, M., Soares, M., et al. (2022). New clinical and molecular evidence linking mutations in ARSG to Usher syndrome type IV. *Hum. Mutat.* 43, 2326–2327. doi: 10.1002/humu.24496

Pham, Q., Glicksman, J., and Chatterjee, A. (2024). Chemical approaches to probe and engineer AAV vectors. *Nanoscale* 16, 13820–13833. doi: 10.1039/D4NR01300J

Pierce, E. A., Aleman, T. S., Jayasundera, K. T., Ashimatey, B. S., Kim, K., Rashid, A., et al. (2024). Gene Editing for CEP290 -Associated Retinal Degeneration. *N. Engl. J. Med.* 390, 1972–1984. doi: 10.1056/NEJMoa2309915

Pierrache, L. H. M., Hartel, B. P., van Wijk, E., Meester-Smoor, M. A., Cremers, F. P. M., de Baere, E., et al. (2016). Visual Prognosis in USH2A-Associated Retinitis Pigmentosa Is Worse for Patients with Usher Syndrome Type IIa Than for Those with Nonsyndromic Retinitis Pigmentosa. *Ophthalmology* 123, 1151–1160. doi: 10.1016/j.ophtha.2016.01.021

Pinilla, I., Maneu, V., Campello, L., Fernández-Sánchez, L., Martínez-Gil, N., Kutsyr, O., et al. (2022). Inherited Retinal Dystrophies: Role of Oxidative Stress and Inflammation in Their Physiopathology and Therapeutic Implications. *Antioxidants* 11, 1086. doi: 10.3390/antiox11061086

Plagnol, V., Curtis, J., Epstein, M., Mok, K. Y., Stebbings, E., Grigoriadou, S., et al. (2012). A robust model for read count data in exome sequencing experiments and implications for copy number variant calling. *Bioinformatics* 28, 2747–2754. doi: 10.1093/bioinformatics/bts526

Popp, M. W.-L., and Maquat, L. E. (2013). Organizing Principles of Mammalian Nonsense-Mediated mRNA Decay. *Annu. Rev. Genet.* 47, 139–165. doi: 10.1146/annurev-genet-111212-133424

Povysil, G., Tzika, A., Vogt, J., Haunschmid, V., Messiaen, L., Zschocke, J., et al. (2017). panelcn.MOPS: Copy-number detection in targeted NGS panel data for clinical diagnostics. *Hum. Mutat.* 38, 889–897. doi: 10.1002/humu.23237

Prosseda, P. P., Tran, M., Kowal, T., Wang, B., and Sun, Y. (2022). Advances in Ophthalmic Optogenetics: Approaches and Applications. *Biomolecules* 12, 269. doi: 10.3390/biom12020269

Pulman, J., Sahel, J.-A., and Dalkara, D. (2022). New Editing Tools for Gene Therapy in Inherited Retinal Dystrophies. *Cris. J.* 5, 377–388. doi: 10.1089/crispr.2021.0141

Radu, R. A., Yuan, Q., Hu, J., Peng, J. H., Lloyd, M., Nusinowitz, S., et al. (2008). Accelerated Accumulation of Lipofuscin Pigments in the RPE of a Mouse Model for ABCA4 -Mediated Retinal Dystrophies following Vitamin A Supplementation. *Investig. Opthalmology Vis. Sci.* 49, 3821. doi: 10.1167/iovs.07-1470

Radziwon, A., Arno, G., K. Wheaton, D., McDonagh, E. M., Baple, E. L., Webb-Jones, K., et al. (2017). Single-base substitutions in the CHM promoter as a cause of choroideremia. *Hum. Mutat.* 38, 704–715. doi: 10.1002/humu.23212

Rahman, M. T., Chari, D. A., Ishiyama, G., Lopez, I., Quesnel, A. M., Ishiyama, A., et al. (2022). Cochlear implants: Causes, effects and mitigation strategies for the foreign body response and inflammation. *Hear. Res.* 422, 108536. doi: 10.1016/j.heares.2022.108536

Rahman, N., Georgiou, M., Khan, K. N., and Michaelides, M. (2020). Macular dystrophies: clinical and imaging features, molecular genetics and therapeutic options. *Br. J. Ophthalmol.* 104, 451–460. doi: 10.1136/bjophthalmol-2019-315086

Rehman, A. U., Santos-Cortez, R. L. P., Morell, R. J., Drummond, M. C., Ito, T., Lee, K., et al. (2014). Mutations in TBC1D24, a Gene Associated With Epilepsy, Also Cause Nonsyndromic Deafness DFNB86. *Am. J. Hum. Genet.* 94, 144–152. doi:

10.1016/j.ajhg.2013.12.004

Reiter, J. F., and Leroux, M. R. (2017). Genes and molecular pathways underpinning ciliopathies. *Nat. Rev. Mol. Cell Biol.* 18, 533–547. doi: 10.1038/nrm.2017.60

Renz, P. F., Valdivia-Francia, F., and Sendoel, A. (2020). Some like it translated: small ORFs in the 5'UTR. *Exp. Cell Res.* 396, 112229. doi: 10.1016/j.yexcr.2020.112229

Reurink, J., Weisschuh, N., Garanto, A., Dockery, A., van den Born, L. I., Fajardy, I., et al. (2023). Whole genome sequencing for USH2A-associated disease reveals several pathogenic deep-intronic variants that are amenable to splice correction. *Hum. Genet. Genomics Adv.* 4, 100181. doi: 10.1016/j.xhgg.2023.100181

Riazuddin, S., Belyantseva, I. A., Giese, A. P. J., Lee, K., Indzhykulian, A. A., Nandamuri, S. P., et al. (2012). Alterations of the CIB2 calcium- and integrin-binding protein cause Usher syndrome type 1J and nonsyndromic deafness DFNB48. *Nat. Genet.* 44, 1265–1271. doi: 10.1038/ng.2426

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., et al. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* 17, 405–423. doi: 10.1038/gim.2015.30

Riepe, T. V., Khan, M., Roosing, S., Cremers, F. P. M., and 't Hoen, P. A. C. (2021). Benchmarking deep learning splice prediction tools using functional splice assays. *Hum. Mutat.* 42, 799–810. doi: 10.1002/humu.24212

Riera, M., Navarro, R., Ruiz-Nogales, S., Méndez, P., Burés-Jelstrup, A., Corcóstegui, B., et al. (2017). Whole exome sequencing using Ion Proton system enables reliable genetic diagnosis of inherited retinal dystrophies. *Sci. Rep.* 7, 42078. doi: 10.1038/srep42078

Rivolta, C., Berson, E. L., and Dryja, T. P. (2002). Paternal uniparental heterodisomy with partial isodisomy of chromosome 1 in a patient with retinitis pigmentosa without hearing loss and a missense mutation in the Usher syndrome type II gene USH2A. *Arch. Ophthalmol. (Chicago, Ill. 1960)* 120, 1566–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12427073

Rivolta, C., Sweklo, E. A., Berson, E. L., and Dryja, T. P. (2000). Report Missense Mutation in the USH2A Gene: Association with Recessive Retinitis Pigmentosa without Hearing Loss. *Am. J. Hum. Genet* 66, 1975–1978. doi: 10.1086/302926

Roche, S. L., Ruiz-Lopez, A. M., Moloney, J. N., Byrne, A. M., and Cotter, T. G. (2018). Microglial-induced Müller cell gliosis is attenuated by progesterone in a mouse model of retinitis pigmentosa. *Glia* 66, 295–310. doi: 10.1002/glia.23243

Rodríguez-Muñoz, A., Aller, E., Jaijo, T., González-García, E., Cabrera-Peset, A., Gallego-Pinazo, R., et al. (2020). Expanding the Clinical and Molecular Heterogeneity of Nonsyndromic Inherited Retinal Dystrophies. *J. Mol. Diagnostics* 22, 532–543. doi: 10.1016/j.jmoldx.2020.01.003

Rodríguez-Muñoz, A., García-Bohórquez, B., Udaondo, P., Hervás-Ontiveros, A., Salom, D., Aller, E., et al. (2021). Concomitant mutations in inherited retinal dystrophies. *Retina* Publish Ah. doi: 10.1097/IAE.0000000000003103

Rodriguez-Muñoz, A., Liquori, A., García-Bohorquez, B., Jaijo, T., Aller, E., Millán, J. M., et al. (2022). Functional assays of non-canonical splice-site variants in inherited retinal dystrophies genes. *Sci. Rep.* 12, 68. doi: 10.1038/s41598-021-03925-1

Romano, M., Buratti, E., and Baralle, D. (2013). Role of Pseudoexons and Pseudointrons in Human Cancer. *Int. J. Cell Biol.* 2013, 1–16. doi: 10.1155/2013/810572

Romo, L., Findlay, S. D., and Burge, C. B. (2024). Regulatory features aid interpretation of 3'UTR variants. *Am. J. Hum. Genet.* 111, 350–363. doi: 10.1016/j.ajhg.2023.12.017

Rowlands, C. F., Baralle, D., and Ellingford, J. M. (2019). Machine Learning Approaches for the Prioritization of Genomic Variants Impacting Pre-mRNA Splicing. *Cells* 8, 1513. doi: 10.3390/cells8121513

Rowlands, C., Thomas, H. B., Lord, J., Wai, H. A., Arno, G., Beaman, G., et al. (2021). Comparison of in silico strategies to prioritize rare genomic variants impacting RNA splicing for the diagnosis of genomic disorders. *Sci. Rep.* 11, 20607. doi: 10.1038/s41598-021-99747-2

Rozet, J. M., Gerber, S., Souied, E., Perrault, I., Châtelin, S., Ghazi, I., et al. (1998). Spectrum of ABCR gene mutations in autosomal recessive macular dystrophies. *Eur. J. Hum. Genet.* 6, 291–295. doi: 10.1038/sj.ejhg.5200221

Ruberto, F. P., Balzano, S., Namburi, P., Kimchi, A., Pescini-Gobert, R., Obolensky, A., et al. (2021). Heterozygous deletions of noncoding parts of the PRPF31 gene cause retinitis pigmentosa via reduced gene expression. *Mol. Vis.* 27, 107–116. Available at: http://www.ncbi.nlm.nih.gov/pubmed/33907366

Runhart, E. H., Sangermano, R., Cornelis, S. S., Verheij, J. B. G. M., Plomp, A. S., Boon, C. J. F., et al.

(2018). The Common ABCA4 Variant p.Asn1868Ile Shows Nonpenetrance and Variable Expression of Stargardt Disease When Present in trans With Severe Variants. *Investig. Opthalmology Vis. Sci.* 59, 3220. doi: 10.1167/iovs.18-23881

Russell, S., Bennett, J., Wellman, J. A., Chung, D. C., Yu, Z.-F., Tillman, A., et al. (2017). Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65 -mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet* 390, 849–860. doi: 10.1016/S0140-6736(17)31868-8

Sahel, J. A., Marazova, K., and Audo, I. (2015). Clinical characteristics and current therapies for inherited retinal degenerations. *Cold Spring Harb. Perspect. Med.* 5, 1–25. doi: 10.1101/cshperspect.a017111

Sakharkar, M. K., Chow, V. T. K., and Kangueane, P. (2004). Distributions of exons and introns in the human genome. *In Silico Biol.* 4, 387–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15217358

Salles, M. V., Motta, F. L., Martin, R., Filippelli-Silva, R., Dias da Silva, E., Varela, P., et al. (2018). Variants in the ABCA4 gene in a Brazilian population with Stargardt disease. *Mol. Vis.* 24, 546–559. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30093795

Salmaninejad, A., Motaee, J., Farjami, M., Alimardani, M., Esmaeilie, A., and Pasdar, A. (2019). Next-generation sequencing and its application in diagnosis of retinitis pigmentosa. *Ophthalmic Genet.* 40, 393–402. doi: 10.1080/13816810.2019.1675178

Sanchez-Navarro, I., R. J. da Silva, L., Blanco-Kelly, F., Zurita, O., Sanchez-Bolivar, N., Villaverde, C., et al. (2018). Combining targeted panel-based resequencing and copy-number variation analysis for the diagnosis of inherited syndromic retinopathies and associated ciliopathies. *Sci. Rep.* 8, 5285. doi: 10.1038/s41598-018-23520-1

Sanchis-Juan, A., Stephens, J., French, C., Gleadall, N., Mégy, K., Penkett, C., et al. (2018). Complex Structural Variants Resolved by Short-Read and Long-Read Whole Genome Sequencing in Mendelian Disorders. *bioRxiv*, 281683. doi: 10.1101/281683

Sandberg, M. A., Rosner, B., Weigel-DiFranco, C., McGee, T. L., Dryja, T. P., and Berson, E. L. (2008). Disease Course in Patients with Autosomal Recessive Retinitis Pigmentosa due to the USH2A Gene. *Investig. Opthalmology Vis. Sci.* 49, 5532. doi: 10.1167/iovs.08-2009

Sangermano, R., Bax, N. M., Bauwens, M., van den Born, L. I., De Baere, E., Garanto, A., et al. (2016). Photoreceptor Progenitor mRNA Analysis Reveals Exon Skipping Resulting from the ABCA4 c.5461-10T→C Mutation in Stargardt Disease. *Ophthalmology* 123, 1375–1385. doi: 10.1016/j.ophtha.2016.01.053

Sangermano, R., Garanto, A., Khan, M., Runhart, E. H., Bauwens, M., Bax, N. M., et al. (2019). Deep-intronic ABCA4 variants explain missing heritability in Stargardt disease and allow correction of splice defects by antisense oligonucleotides. *Genet. Med.* 21, 1751–1760. doi: 10.1038/s41436-018-0414-9

Sangermano, R., Gupta, P., Price, C., Han, J., Navarro, J., Condroyer, C., et al. (2024). Coding and non-coding variants in the ciliopathy gene CFAP410 cause early-onset non-syndromic retinal degeneration. *Res. Sq.* doi: 10.21203/rs.3.rs-3871956/v1

Sangermano, R., Khan, M., Cornelis, S. S., Richelle, V., Albert, S., Garanto, A., et al. (2018). ABCA4 midigenes reveal the full splice spectrum of all reported noncanonical splice site variants in Stargardt disease. *Genome Res.* 28, 100–110. doi: 10.1101/gr.226621.117

Santana, L. S., Reyes, A., Hoersch, S., Ferrero, E., Kolter, C., Gaulis, S., et al. (2024). Benchmarking tools for transcription factor prioritization. *Comput. Struct. Biotechnol. J.* 23, 2190–2199. doi: 10.1016/j.csbj.2024.05.016

Schadt, E. E., Turner, S., and Kasarskis, A. (2010). A window into third-generation sequencing. *Hum. Mol. Genet.* 19, R227–R240. doi: 10.1093/hmg/ddq416

Schellens, R., de Vrieze, E., Slijkerman, R., Kremer, H., and van Wijk, E. (2022). Generation of Humanized Zebrafish Models for the In Vivo Assessment of Antisense Oligonucleotide-Based Splice Modulation Therapies. *Methods Mol. Biol.* 2434, 281–299. doi: 10.1007/978-1-0716-2010-6_19

Schellens, R. T. W., Broekman, S., Peters, T., Graave, P., Malinar, L., Venselaar, H., et al. (2023). A protein domain-oriented approach to expand the opportunities of therapeutic exon skipping for USH2A-associated retinitis pigmentosa. *Mol. Ther. - Nucleic Acids* 32, 980–994. doi: 10.1016/j.omtn.2023.05.020

Schulz, H. L., Grassmann, F., Kellner, U., Spital, G., Rüther, K., Jägle, H., et al. (2017). Mutation Spectrum of the ABCA4 Gene in 335 Stargardt Disease Patients From a Multicenter German Cohort—Impact of Selected Deep Intronic Variants and Common SNPs. *Investig. Opthalmology Vis. Sci.* 58, 394. doi: 10.1167/iovs.16-19936

Schuster, S. L., and Hsieh, A. C. (2019). The Untranslated Regions of mRNAs in Cancer. *Trends in Cancer* 5, 245–262. doi: 10.1016/j.trecan.2019.02.011

Schwartz, S. B., Aleman, T. S., Cideciyan, A. V., Windsor, E. A. M., Sumaroka, A., Roman, A. J., et al. (2005). Disease Expression in Usher Syndrome Caused by VLGR1 Gene Mutation (USH2C) and Comparison with USH2A Phenotype. *Investig. Opthalmology Vis. Sci.* 46, 734. doi: 10.1167/iovs.04-1136

Schwartz, S. D., Hubschman, J.-P., Heilwell, G., Franco-Cardenas, V., Pan, C. K., Ostrick, R. M., et al. (2012). Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* 379, 713–720. doi: 10.1016/S0140-6736(12)60028-2

Schwartz, S. D., Regillo, C. D., Lam, B. L., Eliott, D., Rosenfeld, P. J., Gregori, N. Z., et al. (2015). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet* 385, 509–516. doi: 10.1016/S0140-6736(14)61376-3

Schwerk, J., and Savan, R. (2015). Translating the Untranslated Region. *J. Immunol.* 195, 2963–2971. doi: 10.4049/jimmunol.1500756

Seth, P. P., Tanowitz, M., and Bennett, C. F. (2019). Selective tissue targeting of synthetic nucleic acid drugs. *J. Clin. Invest.* 129, 915–925. doi: 10.1172/JCI125228

Sfeir, A., and Symington, L. S. (2015). Microhomology-Mediated End Joining: A Back-up Survival Mechanism or Dedicated Pathway? *Trends Biochem. Sci.* 40, 701–714. doi: 10.1016/j.tibs.2015.08.006

Shahryari, A., Saghaeian Jazi, M., Mohammadi, S., Razavi Nikoo, H., Nazari, Z., Hosseini, E. S., et al. (2019). Development and Clinical Translation of Approved Gene Therapy Products for Genetic Disorders. *Front. Genet.* 10. doi: 10.3389/fgene.2019.00868

Shchaslyvyi, A. Y., Antonenko, S. V., Tesliuk, M. G., and Telegeev, G. D. (2023). Current State of Human Gene Therapy: Approved Products and Vectors. *Pharmaceuticals* 16, 1416. doi: 10.3390/ph16101416

Shearer, A. E., and Smith, R. J. H. (2012). Genetics: advances in genetic testing for deafness. *Curr. Opin. Pediatr.* 24, 679–686. doi: 10.1097/MOP.0b013e3283588f5e

Sheck, L., Davies, W. I. L., Moradi, P., Robson, A. G., Kumaran, N., Liasis, A. C., et al. (2018). Leber Congenital Amaurosis Associated with Mutations in CEP290, Clinical Phenotype, and Natural History in Preparation for Trials of Novel Therapies. *Ophthalmology* 125, 894–903. doi: 10.1016/j.ophtha.2017.12.013

Shen, C., You, B., Chen, Y.-N., Li, Y., Li, W., and Wei, W.-B. (2022). Whole-exome sequencing identified genes known to be responsible for retinitis pigmentosa in 28 Chinese families. *Mol. Vis.* 28, 96–113. Available at: http://www.ncbi.nlm.nih.gov/pubmed/35814500

Shi, H., Wang, H., Zhang, C., Lu, Y., Yao, J., Chen, Z., et al. (2022). Mutations in OSBPL2 cause hearing loss associated with primary cilia defects via sonic hedgehog signaling. *JCI Insight* 7. doi: 10.1172/jci.insight.149626

Shroyer, N. F., Lewis, R. A., Yatsenko, A. N., and Lupski, J. R. (2001). Null missense ABCR (ABCA4) mutations in a family with stargardt disease and retinitis pigmentosa. *Investig. Ophthalmol. Vis. Sci.* 42, 2757–2761.

Simunovic, M. P., Shen, W., Lin, J. Y., Protti, D. A., Lisowski, L., and Gillies, M. C. (2019). Optogenetic approaches to vision restoration. *Exp. Eye Res.* 178, 15–26. doi: 10.1016/j.exer.2018.09.003

Singh, G., and Cooper, T. A. (2006). Minigene reporter for identification and analysis of cis elements and trans factors affecting pre-mRNA splicing. *Biotechniques* 41, 177–181. doi: 10.2144/000112208

Slavotinek, A. M., Garcia, S. T., Chandratillake, G., Bardakjian, T., Ullah, E., Wu, D., et al. (2015). Exome sequencing in 32 patients with anophthalmia/microphthalmia and developmental eye defects. *Clin. Genet.* 88, 468–473. doi: 10.1111/cge.12543

Slijkerman, R., Goloborodko, A., Broekman, S., de Vrieze, E., Hetterschijt, L., Peters, T., et al. (2018a). Poor Splice-Site Recognition in a Humanized Zebrafish Knockin Model for the Recurrent Deep-Intronic c.7595-2144A>G Mutation in USH2A. *Zebrafish* 15, 597–609. doi: 10.1089/zeb.2018.1613

Slijkerman, R., Kremer, H., and van Wijk, E. (2018b). "Antisense Oligonucleotide Design and Evaluation of Splice-Modulating Properties Using Cell-Based Assays," 519–530. doi: 10.1007/978-1-4939-8651-4_34

Slijkerman, R. W., Vaché, C., Dona, M., García-García, G., Claustres, M., Hetterschijt, L., et al. (2016). Antisense Oligonucleotide-based Splice Correction for USH2A-associated Retinal Degeneration Caused by a Frequent Deep-intronic Mutation. *Mol. Ther. - Nucleic Acids* 5, e381. doi: 10.1038/mtna.2016.89

Smith, C. I. E., and Zain, R. (2019). Therapeutic Oligonucleotides: State of the Art. *Annu. Rev. Pharmacol. Toxicol.* 59, 605–630. doi:

10.1146/annurev-pharmtox-010818-021050

Smith, R. J. H., Berlin, C. I., Hejtmancik, J. F., Keats, B. J. B., Kimberling, W. J., Lewis, R. A., et al. (1994). Clinical diagnosis of the Usher syndromes. *Am. J. Med. Genet.* 50, 32–38. doi: 10.1002/ajmg.1320500107

Soens, Z. T., Branch, J., Wu, S., Yuan, Z., Li, Y., Li, H., et al. (2017). Leveraging splice-affecting variant predictors and a minigene validation system to identify Mendelian disease-causing variants among exon-captured variants of uncertain significance. *Hum. Mutat.* 38, 1521–1533. doi: 10.1002/humu.23294

Soucy, M., Kolesnikova, M., Kim, A. H., and Tsang, S. H. (2023). Phenotypic variability in PRPH2 as demonstrated by a family with incomplete penetrance of autosomal dominant cone-rod dystrophy. *Doc. Ophthalmol.* 146, 267–272. doi: 10.1007/s10633-022-09916-5

Srivastava, S., Ramsbottom, S. A., Molinari, E., Alkanderi, S., Filby, A., White, K., et al. (2017). A human patient-derived cellular model of Joubert syndrome reveals ciliary defects which can be rescued with targeted therapies. *Hum. Mol. Genet.* 26, 4657–4667. doi: 10.1093/hmg/ddx347

Stallworth, J. Y., Blair, D. R., Slavotinek, A., Moore, A. T., Duncan, J. L., and de Alba Campomanes, A. G. (2023). Retinopathy and optic atrophy in a case of COQ2 -related primary coenzyme Q 10 deficiency. *Ophthalmic Genet.* 44, 486–490. doi: 10.1080/13816810.2022.2141792

Stark, Z., Tan, T. Y., Chong, B., Brett, G. R., Yap, P., Walsh, M., et al. (2016). A prospective evaluation of whole-exome sequencing as a first-tier molecular test in infants with suspected monogenic disorders. *Genet. Med.* 18, 1090–1096. doi: 10.1038/gim.2016.1

Steele-Stallard, H. B., Le Quesne Stabej, P., Lenassi, E., Luxon, L. M., Claustres, M., Roux, A.-F., et al. (2013). Screening for duplications, deletions and a common intronic mutation detects 35% of second mutations in patients with USH2A monoallelic mutations on Sanger sequencing. *Orphanet J. Rare Dis.* 8, 122. doi: 10.1186/1750-1172-8-122

Stellacci, E., Niceta, M., Bruselles, A., Straface, E., Tatti, M., Carvetta, M., et al. (2024). Whole Genome Sequencing Solves an Atypical Form of Bardet–Biedl Syndrome: Identification of Novel Pathogenic Variants of BBS9. *Int. J. Mol. Sci.* 25, 8313. doi: 10.3390/ijms25158313

Stenirri, S., Fermo, I., Battistella, S., Galbiati, S., Soriani, N., Paroni, R., et al. (2004). Denaturing HPLC Profiling of the ABCA4 Gene for Reliable Detection of Allelic Variations. *Clin. Chem.* 50, 1336–1343. doi: 10.1373/clinchem.2004.033241

Stephenson, M. L., and Zamecnik, P. C. (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl. Acad. Sci.* 75, 285–288. doi: 10.1073/pnas.75.1.285

Steri, M., Idda, M. L., Whalen, M. B., and Orrù, V. (2018). Genetic variants in mRNA untranslated regions. *WIREs RNA* 9. doi: 10.1002/wrna.1474

Sterne-Weiler, T., Howard, J., Mort, M., Cooper, D. N., and Sanford, J. R. (2011). Loss of exon identity is a common mechanism of human inherited disease. *Genome Res.* 21, 1563–1571. doi: 10.1101/gr.118638.110

Stingl, K., Schippert, R., Bartz-Schmidt, K. U., Besch, D., Cottriall, C. L., Edwards, T. L., et al. (2017). Interim Results of a Multicenter Trial with the New Electronic Subretinal Implant Alpha AMS in 15 Patients Blind from Inherited Retinal Degenerations. *Front. Neurosci.* 11. doi: 10.3389/fnins.2017.00445

Stockman, A., and Sharpe, L. T. (2000). The spectral sensitivities of the middle- and long-wavelength-sensitive cones derived from measurements in observers of known genotype. *Vision Res.* 40, 1711–1737. doi: 10.1016/S0042-6989(00)00021-3

Stone, E. M., Andorf, J. L., Whitmore, S. S., DeLuca, A. P., Giacalone, J. C., Streb, L. M., et al. (2017). Clinically Focused Molecular Investigation of 1000 Consecutive Families with Inherited Retinal Disease. *Ophthalmology* 124, 1314–1331. doi: 10.1016/j.ophtha.2017.04.008

Strubbe, I., Van Cauwenbergh, C., De Zaeytijd, J., De Jaegere, S., De Bruyne, M., Rosseel, T., et al. (2021). Phenocopy of a heterozygous carrier of X-linked retinitis pigmentosa due to mosaicism for a RHO variant. *Sci. Rep.* 11, 117. doi: 10.1038/s41598-020-80400-3

Sun, D., Schur, R. M., Sears, A. E., Gao, S.-Q., Vaidya, A., Sun, W., et al. (2020). Non-viral Gene Therapy for Stargardt Disease with ECO/pRHO-ABCA4 Self-Assembled Nanoparticles. *Mol. Ther.* 28, 293–303. doi: 10.1016/j.ymthe.2019.09.010

Sundaram, V., Moore, A. T., Ali, R. R., and Bainbridge, J. W. (2012). Retinal dystrophies and gene therapy. *Eur. J. Pediatr.* 171, 757–65. doi: 10.1007/s00431-011-1615-2

Surendran, H., Nandakumar, S., Reddy K, V. B., Stoddard, J., Mohan K, V., Upadhyay, P. K., et al. (2021). Transplantation of retinal pigment epithelium and photoreceptors generated concomitantly via small molecule-mediated differentiation rescues visual function in rodent models of retinal degeneration. *Stem Cell Res. Ther.* 12, 70. doi: 10.1186/s13287-021-02134-x

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E. J., et al. (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* 540, 144–149. doi: 10.1038/nature20565

Sweeney, M. O., McGee, T. L., Berson, E. L., and Dryja, T. P. (2007). Low prevalence of lecithin retinol acyltransferase mutations in patients with Leber congenital amaurosis and autosomal recessive retinitis pigmentosa. *Mol. Vis.* 13, 588–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17438524

Takki, K. K., and Milton, R. C. (1981). The Natural History of Gyrate Atrophy of the Choroid and Retina. *Ophthalmology* 88, 292–301. doi: 10.1016/S0161-6420(81)35031-3

Tan, T.-E., Tang, R. W. C., Chan, C. M., Mathur, R. S., and Fenner, B. J. (2023). Diagnostic Challenges in ABCA4-Associated Retinal Degeneration: One Gene, Many Phenotypes. *Diagnostics* 13, 3530. doi: 10.3390/diagnostics13233530

Tao, T., Xu, N., Li, J., Li, H., Qu, J., Yin, H., et al. (2021). Ocular Features and Mutation Spectrum of Patients With Familial Exudative Vitreoretinopathy. *Investig. Opthalmology Vis. Sci.* 62, 4. doi: 10.1167/iovs.62.15.4

Tatour, Y., and Ben-Yosef, T. (2020). Syndromic Inherited Retinal Diseases: Genetic, Clinical and Diagnostic Aspects. *Diagnostics* 10, 779. doi: 10.3390/diagnostics10100779

Tavakoli, S., Peynshaert, K., Lajunen, T., Devoldere, J., del Amo, E. M., Ruponen, M., et al. (2020). Ocular barriers to retinal delivery of intravitreal liposomes: Impact of vitreoretinal interface. *J. Control. Release* 328, 952–961. doi: 10.1016/j.jconrel.2020.10.028

Teo, K. Y. C., Lee, S. Y., Barathi, A. V., Tun, S. B. B., Tan, L., and Constable, I. J. (2018). Surgical Removal of Internal Limiting Membrane and Layering of AAV Vector on the Retina Under Air Enhances Gene Transfection in a Nonhuman Primate. *Investig. Opthalmology Vis. Sci.* 59, 3574. doi: 10.1167/iovs.18-24333

Thiadens, A. A. H. J., Phan, T. M. L., Zekveld-Vroon, R. C., Leroy, B. P., Van Den Born, L. I., Hoyng, C. B., et al. (2012). Clinical course, genetic etiology, and visual outcome in cone and cone-rod dystrophy. *Ophthalmology* 119, 819–826. doi: 10.1016/j.ophtha.2011.10.011

Thuma, T. B. T., Procopio, R. A., Jimenez, H. J., Gunton, K. B., and Pulido, J. S. (2024). Hypomorphic variants in inherited retinal and ocular diseases: A review of the literature with clinical cases. *Surv. Ophthalmol.* 69, 337–348. doi: 10.1016/j.survophthal.2023.11.006

Tian, T., Chen, C., Zhang, X., Zhang, Q., and Zhao, P. (2019). Clinical and Genetic Features of Familial Exudative Vitreoretinopathy With Only-Unilateral Abnormalities in a Chinese Cohort. *JAMA Ophthalmol.* 137, 1054. doi: 10.1001/jamaophthalmol.2019.1493

Tiwari, A., Bahr, A., Bähr, L., Fleischhauer, J., Zinkernagel, M. S., Winkler, N., et al. (2016). Next generation sequencing based identification of disease-associated mutations in Swiss patients with retinal dystrophies. *Sci. Rep.* 6, 28755. doi: 10.1038/srep28755

Tlili, A., Charfedine, I., Lahmar, I., Benzina, Z., Amor Mohamed, B., Weil, D., et al. (2005). Identification of a novel frameshift mutation in the DFNB31/WHRN gene in a Tunisian consanguineous family with hereditary non-syndromic recessive hearing loss. *Hum. Mutat.* 25, 503–503. doi: 10.1002/humu.9333

To, K., Adamian, M., and Berson, E. L. (2004). Histologic study of retinitis pigmentosa due to a mutation in the RP13 gene (PRPC8): comparison with rhodopsin Pro23His, Cys110Arg, and Glu181Lys. *Am. J. Ophthalmol.* 137, 946–948. doi: 10.1016/j.ajo.2003.10.047

Toms, M., Dubis, A. M., de Vrieze, E., Tracey-White, D., Mitsios, A., Hayes, M., et al. (2020a). Clinical and preclinical therapeutic outcome metrics for USH2A-related disease. *Hum. Mol. Genet.* 29, 1882–1899. doi: 10.1093/hmg/ddaa004

Toms, M., Pagarkar, W., and Moosajee, M. (2020b). Usher syndrome: clinical features, molecular genetics and advancing therapeutics. *Ther. Adv. Ophthalmol.* 12, 251584142095219. doi: 10.1177/2515841420952194

Toualbi, L., Toms, M., and Moosajee, M. (2020). USH2A-retinopathy: From genetics to therapeutics. *Exp. Eye Res.* 201, 108330. doi: 10.1016/j.exer.2020.108330

Trizuljak, J., Likavcová, P., Staňo Kozubík, K., Vrzalová, Z., Hynšt, J., Deissová, T., et al. (2024). Impact of thrombocytopenia-associated c.-118C>T and c.-140C>G ANKRD26 5'UTR variants in three-generational pedigree. *Platelets* 35. doi: 10.1080/09537104.2024.2388103

Trujillo, M. J., Martinez-Gimeno, M., Gim&#65533;nez, A., Lorda, I., Bueno, J., Garc&#65533;a-Sandoval, B., et al. (2001). Two novel mutations (Y141H; C214Y) and previously published mutation (R142W) in the RDS-peripherin gene in autosomal dominant macular dystrophies in Spanish families. *Hum. Mutat.* 17, 80–80. doi: 10.1002/1098-1004(2001)17:1<80::AID-HUMU27>3.0.CO;2-G

Tsang, S. H., and Sharma, T. (2018). "Stargardt Disease," 139–151. doi: 10.1007/978-3-319-95046-4_27

Tsui, J. C., Aleman, T. S., Tapino, P. J., and Kim, B. J. (2024). Detailed Phenotype Supports Pathogenicity of Hypomorphic Variant in ABCC6-Associated Pattern Dystrophy. *Case Rep. Ophthalmol.* 15, 497–506. doi: 10.1159/000538045

Vaché, C., Besnard, T., le Berre, P., García-García, G., Baux, D., Larrieu, L., et al. (2012). Usher syndrome type 2 caused by activation of an USH2A pseudoexon: Implications for diagnosis and therapy. *Hum. Mutat.* 33, 104–108. doi: 10.1002/humu.21634

Valkenburg, D., van Cauwenbergh, C., Lorenz, B., van Genderen, M. M., Bertelsen, M., Pott, J.-W. R., et al. (2018). Clinical Characterization of 66 Patients With Congenital Retinal Disease Due to the Deep-Intronic c.2991+1655A>G Mutation in CEP290. *Invest. Ophthalmol. Vis. Sci.* 59, 4384–4391. doi: 10.1167/iovs.18-24817

van Dijk, E. L., Jaszczyszyn, Y., Naquin, D., and Thermes, C. (2018). The Third Revolution in Sequencing Technology. *Trends Genet.* 34, 666–681. doi: 10.1016/j.tig.2018.05.008

Van Schil, K., Naessens, S., Van de Sompele, S., Carron, M., Aslanidis, A., Van Cauwenbergh, C., et al. (2018). Mapping the genomic landscape of inherited retinal disease genes prioritizes genes prone to coding and noncoding copy-number variations. *Genet. Med.* 20, 202–213. doi: 10.1038/gim.2017.97

Varela, M. D., Bellingham, J., Motta, F., Jurkute, N., Ellingford, J. M., Quinodoz, M., et al. (2022). Multi-disciplinary team directed analysis of whole genome sequencing reveals pathogenic non-coding variants in molecularly undiagnosed inherited retinal dystrophies. *Hum. Mol. Genet.* doi: 10.1093/hmg/ddac227

Vázquez-Domínguez, I., Anido, A. A., Duijkers, L., Hoppenbrouwers, T., Hoogendoorn, A. D. M., Koster, C., et al. (2024). Efficacy, biodistribution and safety comparison of chemically modified antisense oligonucleotides in the retina. *Nucleic Acids Res.* 52, 10447–10463. doi: 10.1093/nar/gkae686

Velde, H. M., Reurink, J., Held, S., Li, C. H. Z., Yzer, S., Oostrik, J., et al. (2022). Usher syndrome type IV: clinically and molecularly confirmed by novel ARSG variants. *Hum. Genet.* 141, 1723–1738. doi: 10.1007/s00439-022-02441-0

Verbakel, S. K., van Huet, R. A. C., Boon, C. J. F., den Hollander, A. I., Collin, R. W. J., Klaver, C. C. W., et al. (2018). Non-syndromic retinitis pigmentosa. *Prog. Retin. Eye Res.* 66, 157–186. doi: 10.1016/j.preteyeres.2018.03.005

Vervoort, R., Lennon, A., Bird, A. C., Tulloch, B., Axton, R., Miano, M. G., et al. (2000). Mutational hot spot within a new RPGR exon in X-linked retinitis pigmentosa. *Nat. Genet.* 25, 462–6. doi: 10.1038/78182

Vincent, A., Forster, N., Maynes, J. T., Paton, T. A., Billingsley, G., Roslin, N. M., et al. (2014). OTX2 mutations cause autosomal dominant pattern dystrophy of the retinal pigment epithelium. *J. Med. Genet.* 51, 797–805. doi: 10.1136/jmedgenet-2014-102620

Vingolo, E. M., Mascolo, S., Miccichè, F., and Manco, G. (2024). Retinitis Pigmentosa: From Pathomolecular Mechanisms to Therapeutic Strategies. *Medicina (B. Aires).* 60, 189. doi: 10.3390/medicina60010189

Wade, E. M., Daniel, P. B., Jenkins, Z. A., McInerney-Leo, A., Leo, P., Morgan, T., et al. (2016). Mutations in MAP3K7 that Alter the Activity of the TAK1 Signaling Complex Cause Frontometaphyseal Dysplasia. *Am. J. Hum. Genet.* 99, 392–406. doi: 10.1016/j.ajhg.2016.05.024

Wafa, T. T., Faridi, R., King, K. A., Zalewski, C., Yousaf, R., Schultz, J. M., et al. (2021). Vestibular <scp>phenotype-genotype</scp> correlation in a cohort of 90 patients with Usher syndrome. *Clin. Genet.* 99, 226–235. doi: 10.1111/cge.13868

Wagner, E. J., and Garcia-Blanco, M. A. (2001). Polypyrimidine Tract Binding Protein Antagonizes Exon Definition. *Mol. Cell. Biol.* 21, 3281–3288. doi: 10.1128/MCB.21.10.3281-3288.2001

Wagner, S., Herrmannová, A., Hronová, V., Gunišová, S., Sen, N. D., Hannan, R. D., et al. (2020). Selective Translation Complex Profiling Reveals Staged Initiation and Co-translational Assembly of Initiation Factor Complexes. *Mol. Cell* 79, 546-560.e7. doi: 10.1016/j.molcel.2020.06.004

Wai, H. A., Lord, J., Lyon, M., Gunning, A., Kelly, H., Cibin, P., et al. (2020). Blood RNA analysis can increase clinical diagnostic rate and resolve variants of uncertain significance. *Genet. Med.* 22, 1005–1014. doi: 10.1038/s41436-020-0766-9

Waldock, W. J., Taylor, L. J., Sperring, S., Staurenghi, F., Martinez-Fernandez de la Camara, C., Whitfield, J., et al. (2024). A hypomorphic variant of choroideremia is associated with a novel intronic mutation that leads to exon skipping. *Ophthalmic Genet.* 45, 210–217. doi: 10.1080/13816810.2023.2270554

Walker, L. C., Hoya, M. de la, Wiggins, G. A. R., Lindy, A., Vincent, L. M., Parsons, M. T., et al. (2023). Using the ACMG/AMP framework to capture evidence related to predicted and observed impact on splicing: Recommendations from the ClinGen SVI Splicing Subgroup. *Am. J. Hum. Genet.* 110, 1046–1067. doi:

10.1016/j.ajhg.2023.06.002

Wang, C., Li, J., Takemaru, K.-I., Jiang, X., Xu, G., and Wang, B. (2018a). Centrosomal protein Dzip1l binds Cby, promotes ciliary bud formation, and acts redundantly with Bromi to regulate ciliogenesis in the mouse. *Development* 145. doi: 10.1242/dev.164236

Wang, G.-S., and Cooper, T. A. (2007). Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat. Rev. Genet.* 8, 749–761. doi: 10.1038/nrg2164

Wang, J.-S., Estevez, M. E., Cornwall, M. C., and Kefalov, V. J. (2009). Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat. Neurosci.* 12, 295–302. doi: 10.1038/nn.2258

Wang, J.-S., and Kefalov, V. J. (2011). The Cone-specific visual cycle. *Prog. Retin. Eye Res.* 30, 115–128. doi: 10.1016/j.preteyeres.2010.11.001

Wang, J., Zhang, V. W., Feng, Y., Tian, X., Li, F. Y., Truong, C., et al. (2014). Dependable and efficient clinical utility of target capture-based deep sequencing in molecular diagnosis of retinitis pigmentosa. *Investig. Ophthalmol. Vis. Sci.* 55, 6213–6223. doi: 10.1167/iovs.14-14936

Wang, L., Zhang, J., Chen, N., Wang, L., Zhang, F., Ma, Z., et al. (2018b). Application of whole exome and targeted panel sequencing in the clinical molecular diagnosis of 319 Chinese families with inherited retinal dystrophy and comparison study. *Genes (Basel).* 9, 1–11. doi: 10.3390/genes9070360

Wang, S., Zhang, Q., Zhang, X., Wang, Z., and Zhao, P. (2016). Clinical and genetic characteristics of Leber congenital amaurosis with novel mutations in known genes based on a Chinese eastern coast Han population. *Graefe's Arch. Clin. Exp. Ophthalmol.* 254, 2227–2238. doi: 10.1007/s00417-016-3428-5

Wang, Y., Li, J., Yao, X., Li, W., Du, H., Tang, M., et al. (2017). Loss of CIB2 Causes Profound Hearing Loss and Abolishes Mechanoelectrical Transduction in Mice. *Front. Mol. Neurosci.* 10. doi: 10.3389/fnmol.2017.00401

WANG, Y., LIU, J., HUANG, B., XU, Y.-M., LI, J., HUANG, L.-F., et al. (2015). Mechanism of alternative splicing and its regulation. *Biomed. Reports* 3, 152–158. doi: 10.3892/br.2014.407

Wang, Y., Wang, Q., Li, S., and Ding, X. (2023). Identification of Five Novel Variants in the TSPAN12 Gene in Chinese Families With Familial Exudative Vitreoretinopathy. *Transl. Vis. Sci. Technol.* 12, 29. doi: 10.1167/tvst.12.5.29

Wawrocka, A., Walczak-Sztulpa, J., Kuszel, L., Niedziela-Schwartz, Z., Skorczyk-Werner, A., Bernardczyk-Meller, J., et al. (2024). Coexistence of Retinitis Pigmentosa and Ataxia in Patients with PHARC, PCARP, and Oliver–McFarlane Syndromes. *Int. J. Mol. Sci.* 25, 5759. doi: 10.3390/ijms25115759

Webb, T. R., Parfitt, D. A., Gardner, J. C., Martinez, A., Bevilacqua, D., Davidson, A. E., et al. (2012). Deep intronic mutation in OFD1, identified by targeted genomic next-generation sequencing, causes a severe form of X-linked retinitis pigmentosa (RP23). *Hum. Mol. Genet.* 21, 3647–54. doi: 10.1093/hmg/dds194

Weil, D., Küssel, P., Blanchard, S., Lévy, G., Levi-Acobas, F., Drira, M., et al. (1997). The autosomal recessive isolated deafness, DFNB2, and the Usher 1B syndrome are allelic defects of the myosin-VIIA gene. *Nat. Genet.* 16, 191–193. doi: 10.1038/ng0697-191

Weleber, R. G., Michaelides, M., Trzupek, K. M., Stover, N. B., and Stone, E. M. (2011). The Phenotype of Severe Early Childhood Onset Retinal Dystrophy (SECORD) from Mutation of RPE65 and Differentiation from Leber Congenital Amaurosis. *Investig. Opthalmology Vis. Sci.* 52, 292. doi: 10.1167/iovs.10-6106

Wen, J., Chiba, A., and Cai, X. (2010). Computational identification of tissue-specific alternative splicing elements in mouse genes from RNA-Seq. *Nucleic Acids Res.* 38, 7895–7907. doi: 10.1093/nar/gkq679

Wen, S., Wang, M., Qian, X., Li, Y., Wang, K., Choi, J., et al. (2023). Systematic assessment of the contribution of structural variants to inherited retinal diseases. *Hum. Mol. Genet.* 32, 2005–2015. doi: 10.1093/hmg/ddad032

Whatley, M., Francis, A., Ng, Z. Y., Khoh, X. E., Atlas, M. D., Dilley, R. J., et al. (2020). Usher Syndrome: Genetics and Molecular Links of Hearing Loss and Directions for Therapy. *Front. Genet.* 11. doi: 10.3389/fgene.2020.565216

Wheway, G., Parry, D. A., and Johnson, C. A. (2014). The role of primary cilia in the development and disease of the retina. *Organogenesis* 10, 69–85. doi: 10.4161/org.26710

Whiffin, N., Karczewski, K. J., Zhang, X., Chothani, S., Smith, M. J., Evans, D. G., et al. (2020). Characterising the loss-of-function impact of 5' untranslated region variants in 15,708 individuals. *Nat. Commun.* 11, 2523. doi: 10.1038/s41467-019-10717-9

Wilton-Clark, H., and Yokota, T. (2023). Recent Trends in Antisense Therapies for Duchenne Muscular Dystrophy. *Pharmaceutics* 15, 778. doi: 10.3390/pharmaceutics15030778

Wojcik, M. H., Reuter, C. M., Marwaha, S., Mahmoud, M., Duyzend, M. H., Barseghyan, H., et al. (2023). Beyond the exome: What's next in diagnostic testing for Mendelian conditions. *Am. J. Hum.*

Genet. 110, 1229–1248. doi: 10.1016/j.ajhg.2023.06.009

Woods, K. N., and Pfeffer, J. (2020). Conformational perturbation, allosteric modulation of cellular signaling pathways, and disease in P23H rhodopsin. *Sci. Rep.* 10, 2657. doi: 10.1038/s41598-020-59583-2

Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M., and Bhattacharya, S. S. (2010). Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. *Nat. Rev. Genet.* 11, 273–284. doi: 10.1038/nrg2717

Xue, K., and MacLaren, R. E. (2020). Antisense oligonucleotide therapeutics in clinical trials for the treatment of inherited retinal diseases. *Expert Opin. Investig. Drugs* 29, 1163–1170. doi: 10.1080/13543784.2020.1804853

Yalla, G. R., and Kuriyan, A. E. (2024). Cell therapy for retinal disease. *Curr. Opin. Ophthalmol.* 35, 178–184. doi: 10.1097/ICU.0000000000001034

Yamada, M., Maeta, K., Suzuki, H., Kurosawa, R., Takenouchi, T., Awaya, T., et al. (2024). Successful skipping of abnormal pseudoexon by antisense oligonucleotides in vitro for a patient with beta-propeller protein-associated neurodegeneration. *Sci. Rep.* 14, 6506. doi: 10.1038/s41598-024-56704-z

Yan, D., Ouyang, X., Patterson, D. M., Du, L. L., Jacobson, S. G., and Liu, X.-Z. (2009). Mutation analysis in the long isoform of USH2A in American patients with Usher Syndrome type II. *J. Hum. Genet.* 54, 732–738. doi: 10.1038/jhg.2009.107

Yang, Z., Huang, M., Wei, X., Sun, J., and Zhang, F. (2023). Case report: Compound heterozygous nonsense <scp> *PCDH15* </scp> variant and a novel deep-intronic variant in a Chinese child with profound hearing loss. *Mol. Genet. Genomic Med.* 11. doi: 10.1002/mgg3.2193

Yu, R. Z., Grundy, J. S., and Geary, R. S. (2013). Clinical pharmacokinetics of second generation antisense oligonucleotides. *Expert Opin. Drug Metab. Toxicol.* 9, 169–182. doi: 10.1517/17425255.2013.737320

Zampaglione, E., Kinde, B., Place, E. M., Navarro-Gomez, D., Maher, M., Jamshidi, F., et al. (2020). Copy-number variation contributes 9% of pathogenicity in the inherited retinal degenerations. *Genet. Med.* 22, 1079–1087. doi: 10.1038/s41436-020-0759-8

Zaneveld, J., Siddiqui, S., Li, H., Wang, X., Wang, H., Wang, K., et al. (2015). Comprehensive analysis of patients with Stargardt macular dystrophy reveals new genotype–phenotype correlations and unexpected diagnostic revisions. *Genet. Med.* 17, 262–270. doi: 10.1038/gim.2014.174

Zeitz, C., Navarro, J., Azizzadeh Pormehr, L., Méjécase, C., Neves, L. M., Letellier, C., et al. (2024). Variants in UBAP1L lead to autosomal recessive rod-cone and cone-rod dystrophy. *Genet. Med.* 26, 101081. doi: 10.1016/j.gim.2024.101081

Zenteno, J. C., García-Montaño, L. A., Cruz-Aguilar, M., Ronquillo, J., Rodas-Serrano, A., Aguilar-Castul, L., et al. (2020). Extensive genic and allelic heterogeneity underlying inherited retinal dystrophies in Mexican patients molecularly analyzed by next-generation sequencing. *Mol. Genet. Genomic Med.* 8, 1–17. doi: 10.1002/mgg3.1044

Zernant, J., Lee, W., Collison, F. T., Fishman, G. A., Sergeev, Y. V, Schuerch, K., et al. (2017). Frequent hypomorphic alleles account for a significant fraction of ABCA4 disease and distinguish it from age-related macular degeneration. *J. Med. Genet.* 54, 404–412. doi: 10.1136/jmedgenet-2017-104540

Zernant, J., Lee, W., Nagasaki, T., Collison, F. T., Fishman, G. A., Bertelsen, M., et al. (2018). Extremely hypomorphic and severe deep intronic variants in the ABCA4 locus result in varying Stargardt disease phenotypes. *Mol. Case Stud.* 4, a002733. doi: 10.1101/mcs.a002733

Zernant, J., Schubert, C., Im, K. M., Burke, T., Brown, C. M., Fishman, G. A., et al. (2011). Analysis of the ABCA4 gene by next-generation sequencing. *Invest. Ophthalmol. Vis. Sci.* 52, 8479–87. doi: 10.1167/iovs.11-8182

Zernant, J., Xie, Y. A., Ayuso, C., Riveiro-Alvarez, R., Lopez-Martinez, M.-A., Simonelli, F., et al. (2014). Analysis of the ABCA4 genomic locus in Stargardt disease. *Hum. Mol. Genet.* 23, 6797–806. doi: 10.1093/hmg/ddu396

Zeuli, R., Karali, M., de Bruijn, S. E., Rodenburg, K., Scarpato, M., Capasso, D., et al. (2024). Whole genome sequencing identifies elusive variants in genetically unsolved Italian inherited retinal disease patients. *Hum. Genet. Genomics Adv.* 5, 100314. doi: 10.1016/j.xhgg.2024.100314

Zhai, Y., Xu, M., Radziwon, A., Dimopoulos, I. S., Crichton, P., Mah, R., et al. (2023). AAV2-Mediated Gene Therapy for Choroideremia: 5-Year Results and Alternate Anti-sense Oligonucleotide Therapy. *Am. J. Ophthalmol.* 248, 145–156. doi: 10.1016/j.ajo.2022.12.022

Zhang, A. Y., Mysore, N., Vali, H., Koenekoop, J., Cao, S. N., Li, S., et al. (2015a). Choroideremia Is a Systemic Disease With Lymphocyte Crystals and Plasma Lipid and RBC Membrane Abnormalities. *Investig. Opthalmology Vis. Sci.* 56, 8158. doi: 10.1167/iovs.14-15751

Zhang, J., Wang, Q., Qi, S., Duan, Y., Liu, Z., Liu, J., et al. (2024). An oncogenic enhancer promotes

melanoma progression via regulating ETV4 expression. *J. Transl. Med.* 22, 547. doi: 10.1186/s12967-024-05356-8

Zhang, L., Sun, Z., Zhao, P., Huang, L., Xu, M., Yang, Y., et al. (2018). Whole-exome sequencing revealed HKDC1 as a candidate gene associated with autosomal-recessive retinitis pigmentosa. *Hum. Mol. Genet.* doi: 10.1093/hmg/ddy281

Zhang, N., Tsybovsky, Y., Kolesnikov, A. V., Rozanowska, M., Swider, M., Schwartz, S. B., et al. (2015b). Protein misfolding and the pathogenesis of ABCA4-associated retinal degenerations. *Hum. Mol. Genet.* 24, 3220–3237. doi: 10.1093/hmg/ddv073

Zhang, X., Thompson, J. A., Zhang, D., Charng, J., Arunachalam, S., McLaren, T. L., et al. (2020). Characterization of CRB1 splicing in retinal organoids derived from a patient with adult-onset rod-cone dystrophy caused by the c.1892A>G and c.2548G>A variants. *Mol. Genet. Genomic Med.* 8. doi: 10.1002/mgg3.1489

Zhang, X., Wakeling, M., Ware, J., and Whiffin, N. (2021). Annotating high-impact 5'untranslated region variants with the UTRannotator. *Bioinformatics* 37, 1171–1173. doi: 10.1093/bioinformatics/btaa783

Zhang, Z., Bae, B., Cuddleston, W. H., and Miura, P. (2023). Coordination of alternative splicing and alternative polyadenylation revealed by targeted long read sequencing. *Nat. Commun.* 14, 5506. doi: 10.1038/s41467-023-41207-8

Zhou, G.-H., Ma, Y., Li, M.-L., Zhou, X.-Y., Mou, H., and Jin, Z.-B. (2020). ATP1A3 mutation as a candidate cause of autosomal dominant cone-rod dystrophy. *Hum. Genet.* 139, 1391–1401. doi: 10.1007/s00439-020-02182-y

Ziccardi, L., Cordeddu, V., Gaddini, L., Matteucci, A., Parravano, M., Malchiodi-Albedi, F., et al. (2019). Molecular Sciences Gene Therapy in Retinal Dystrophies. *Int. journalof Mol.* 20 (22), 5722.