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Targeting Vision: Insights into the Genetics of Inherited Retinal Diseases in Ireland

A thesis submitted to Trinity College Dublin, the University of Dublin for the Degree of Doctor of Philosophy

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I confirm that the work has been completed in full compliance with relevant university policies, including the Academic Integrity policy, the Trinity Policy on Good Research Practice, and the guidance on AI and GenAI in Teaching, Learning, Assessment and Research.

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This thesis includes unpublished and published work of collaborators which are duly acknowledged in the text and collaborations section.

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Summary

Inherited Retinal diseases (IRDs) are a clinically diverse group of disorders of the retina, with a highly complex genetic landscape. While they are individually classed as rare diseases, altogether IRDs have surpassed diabetic retinopathies in recent years as the most common cause of visual defects in adults of working age. The Target 5000 project was set up in 2009 in collaboration with clinicians, ophthalmologists, researchers and patient groups, with the goal of providing comprehensive genetic diagnoses to the IRD population of Ireland and establishing an IRD care pathway. As advancements in NGS technologies continue to arise, DNA sequencing and genetic screening are becoming routine practice in patient diagnosis. In turn, the identification and categorisation of disease-causing variants has become more sophisticated and efficient for hereditary diseases such as IRDs. Even so, a large proportion of IRD patients globally remain without a genetic diagnosis. This doctoral thesis focuses on sequencing, classification, computational and functional work with the aim of contributing to the growing knowledge of IRD pathogenesis and expanding the diagnostic capability of Target 5000. The utilisation of in-depth targeted/panel sequencing as a preliminary form of genetic screening for this study allowed the benefits of such methodologies to be highlighted. Additionally, the key features of more comprehensive forms of sequencing, such as whole exome or whole genome sequencing, which were used for some of the studies herein, are also highlighted.

The landscape study presented within the first research chapter (Chapter 3) of this doctoral thesis describes the use of single molecule Molecular Inversion Probes (smMIPs) as a form of targeted panel sequencing to analyse a cohort of 223 Irish patients presenting with an inherited macular dystrophy (iMD). The sequencing panel used was designed by collaborators in the Cremers Lab at RadboudUMC, Nijmegen and covers 105 iMD associated genes along with a select few AMD risk loci, deep intronic variants and regulatory sequences previously associated with disease. Library preparation, filtration and identification of candidate variants using a bespoke pipeline was completed during secondments for 6 weeks in their Institute. This study provides a first overview of the genetic architecture of iMD in the Irish population, enabling the identification and interpretation of 9 novel candidate disease-causing variants, among other outcomes. A diagnostic yield of ~33% was achieved for iMDs, and 72 patients were considered to be genetically resolved. A novel splice-altering variant in the *ABCA4* gene has been identified within the scope of this study, contributing to the growing spectrum of *ABCA4* variation, with *in vitro* functional assays for this variant being undertaken and described in Chapter 5. Similarly, a large novel

deletion in the *CDH3* gene was identified through CNV analysis. Notably, this patient is the second IRD patient globally to have a deletion incorporating exons 12 and 13 of the *CDH3* gene. In addition, the first *BBS7* pedigree reported in Ireland is described, where compound heterozygous null variants in the *BBS7* gene were identified through previous WES studies. Within this subchapter (Chapter 3B) the multifaceted BBS phenotypes experienced by two sisters with the same *BBS7* genotype are outlined, allowing the classification of one known and one novel *BBS7* variant.

Furthermore, family studies and cascade analyses undertaken as part of Target 5000 are outlined within the second research chapter (Chapter 4), providing interesting perspectives on the complexities of IRDs as clinically and genetically heterogeneous disorders. Instances of variable penetrance and hypomorphic alleles are discussed, along with the classification of further novel IRD variants utilising *in silico* tools and population data, as well as segregation and allelic information to predict pathogenicity. Such studies underpin the value of characterising variants of unknown significance as thoroughly as possible, in an effort to establish pathogenicity and provide a comprehensive genetic diagnosis to patients. Furthermore, the availability of a definitive genetic diagnosis may impact patient eligibility for participation in clinical trial(s) or treatment with approved therapies, highlighting the significant value of genetic studies such as that described in Chapter 4.

Lastly, the final research chapter (Chapter 5) highlights the value of both *in silico* predictions and *in vitro* functional assays in the classification of candidate disease-causing variants. Not only do such assays deepen our understanding of the mechanism of disease, they also allow variant classifications to potentially be up- or down-graded creating a clearer genetic diagnosis for the affected patient. Two variants with potential pathogenic impacts on splicing were analysed using midigene assays. As mentioned previously, a novel *ABCA4* variant was examined and found to lead to multimodal splice defects, with little residual wildtype transcript remaining. Additionally, a coding variant in *RPGR* was assayed and found to be the first variant identified in an IRD gene that leads to the creation of a branchpoint motif within an exon.

The results presented within this thesis highlight the significant value of target capture sequencing as a preliminary diagnostic strategy, in addition to functional analyses of putative pathogenic variants and cascade analysis to inform thorough classification. Having a greater understanding of IRDs is not only beneficial for patients in the Target 5000 cohort, but is also vital for both ocular researchers and clinicians in providing more comprehensive genetically informed care to patients globally.

Thesis Outputs

Publications

The following publications contain research that was performed in parallel with this thesis, however, the details are not discussed within the scope of the text.

Whelan, L., Dockery, A., Stephenson, K. A. J., Zhu, J., **Kopčić, E.**, Post, I. J. M., Khan, M., Corradi, Z., Wynne, N., O' Byrne, J. J., Duignan, E., Silvestri, G., Roosing, S., Cremers, F. P. M., Keegan, D. J., Kenna, P. F., & Farrar, G. J. (2023). **Detailed analysis of an enriched deep intronic ABCA4 variant in Irish Stargardt disease patients.** *Scientific reports*, 13(1), 9380.

Ridgeway, A. R., Shortall, C., Finnegan, L. K., Long, R., Matthews, E., Dockery, A., **Kopčić, E.**, Whelan, L., Kirk, C., Silvestri, G., Turner, J., Keegan, D. J., Millington-Ward, S., Chadderton, N., Duignan, E., Kenna, P. F., & Farrar, G. J. (2024). **Novel Splice-Altering Variants in the *CHM* and *CACNA1F* Genes Causative of X-Linked Choroideremia and Cone Dystrophy.** *Genes*, 16(1), 25

The following manuscripts are under preparation at the time of submission of this thesis.

Whelan, L., Cancellieri, F., Shortall, C., Dockery, A., Kaminska, K., Quinodoz, M., **Kopčić, E.**, Ridgeway, A. R., Janeschitz-Kriegl, L., Viet Tran, H., György, B., P. N. Scholl, H., Calzetti, G., Vaclavik, V., Lee, W., L.Allikmets, R., Kenna, P. F., Rivolta, C., Duignan, E., & Farrar, G. J. **A synonymous variant in *PROM1* causes recessive early onset retinal dystrophy through disrupted splicing.**

Finnegan, L. K., Ridgeway, A. R., Carrigan, M., Dempsey, H., Long, R., Matthews, E., Panneman, D., Turner, J., Dockery, A., Whelan, L., Shortall, C., **Kopčić, E.**, Cremers, F. P. M., Kirk, C., Silvestri, G., Keegan, D., Kenna, P. F., Duignan, E., Chadderton, N., & Farrar, G. J. **The landscape of variants in pre-mRNA-processing factor genes in an Irish cohort.**

Additionally, the results outlined in Chapters 3, 3B and 5 of this doctoral thesis are in preparation as first author manuscripts at the time of submission.

1. **Kopčić, E.**, et al. First novel cryptic branchpoint variant detected in Irish RPGR pedigrees with X-linked retinitis pigmentosa
2. **Kopčić, E.**, et al. Novel findings from a smMIPs-based sequencing study of 223 individuals from an Irish cohort with inherited macular degeneration
3. **Kopčić, E.**, et al. First reported BBS7-associated Bardet-Biedl Syndrome in Ireland: *A Clinical and Genetic case study*

Awards and talks (posters not included):

- Received the Travel Grant award from Women in Vision and Eye Research Ireland (WVER) to attend WVER conference in Belfast, in October 2023. Presented a short talk with findings from Chapter 3.
- Presented a short talk at ISHG Galway, in September 2023 with early results from Chapter 3.
- Received the Trinity Travel Trust grant to attend ARVO International in Seattle, in May 2024. Presented a poster with findings from Chapter 3.

Sources of Funding

Research undertaken throughout this doctoral programme was supported by grant awards from Fighting Blindness Ireland, The Health Research Board of Ireland (HRB) in conjunction with Health Research Charities Ireland (HRCI), and Taighde Éireann - Research Ireland.

During this PhD I spent approximately 6 weeks with our collaborators in Prof. Frans Cremers lab in RadboudUMC, Nijmegen, Netherlands.

Collaborations and Clinicians

Close interaction of genetic counsellors, ophthalmologists, clinicians and researchers is vital in the success of such research endeavours. Indeed, the work in this thesis has involved many national and international collaborations. Patients were assessed, their informed consent was obtained and relevant samples were taken at the Department of Ophthalmology, the Royal Victoria Eye and Ear Hospital, Dublin, Ireland by Mr. Paul Kenna, Ms. Emma Duignan and Dr. Niamh Wynne; the Mater Misericordiae University Hospital Clinical Ophthalmic Genetics Unit, Dublin, Ireland by Mr. David Keegan, Dr Kirk Stephenson, Dr. Julia Zhu, Dr. James O' Byrne and Ms Jacqueline Turner and the Belfast Health and Social Care Trust, Northern Ireland by Ms. Giuliana Silvestri and Ms. Claire Kirk.

As part of this PhD project, we have worked in collaboration with a laboratory team in RadboudUMC, Nijmegen lead by Prof. Frans Cremers. I have visited the Nijmegen team and spent 6 weeks working closely with Rebekkah Hitti-Malen and colleagues on using a newly designed MD-smMIPs sequencing panel as a first-tier sequencing method in the genetic diagnosis of an unresolved Irish cohort of MD patients. I have had the chance to learn from a wide variety of experts such as clinicians and consultants, clinical genomics specialists, and various other research scientists. All smMIPs-based sequencing was carried out during my secondments in RadboudUMC primarily with Dr. Rebekkah Hitti-Malen, Dr. Daan Panneman, Erica Boonen, Dr. Zelia Corradi, Dr. Susanne Roosing and Prof. Frans Cremers and there I was trained on how to utilise the analysis pipeline they developed. The bioinformatic pipeline employed to analyse sequencing data generated using the Target 5000 target-capture panel was designed by Dr. Matthew Carrigan and Dr. Adrian Dockery. Investigation and validation were carried out in collaboration with Anna Ridgeway, Dr. Laura Whelan, Dr. Laura Finnegan and Dr. Naomi Chadderton. Supervision and training on cloning, transfection and tissue culture methodologies were carried out by Dr. Naomi Chadderton, Dr. Sophia Millington-Ward and Dr. Arpad Palfi. Several undergraduate students aided in the re-transfection process of functional assays (Noah and Mark).

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List of abbreviations

AAV – Adeno-associated virus

ACGS – The Association for Clinical Genomic Science

ACH – Achromatopsia

ACMG – American College of Medical Genetics and Genomics

AF – Allele frequency

AG – acceptor gain

AL – acceptor loss

AMD – Age-related macular degeneration

AMP – Association for Molecular Pathology

AON – Antisense oligonucleotide

ATP – Adenosine triphosphate

BBS – Bardet Biedl syndrome

BP – Branchpoint

cGMP – Cyclic guanosine monophosphate

CNV – Copy number variant

CCRD – Cone/Cone-rod dystrophy

CRT – Central retinal thickness

CSNB – Congenital stationary night blindness

CSS – Canonical splice site variant

DG – Donor gain

DIV – Deep-intronic variant

DL – Donor loss

DMEM – Dulbecco's modified eagle media

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ERN – European Reference Networks

EMA – European Medicines Agency

ERG – Electroretinography

FAF – Fundus autofluorescence

FBS – Fetal bovine serum

FDA – U.S. Food and Drug Administration

GTP – Guanosine Triphosphate

IFT – Intra-flagellar transport

IRBP – Inter photoreceptor retinoid-binding protein

IRD – Inherited retinal disease

IS – Inner segment

iMD – Inherited macular dystrophy

LCA – Leber congenital amaurosis

LHON – Leber hereditary optic neuropathy

LOF – Loss of function

LOVD.nl – Leiden open variant database

LRAT – Lecithin retinol acyl transferase

MES – MaxEntScan

MD – Macular dystrophy

MD-BV – Best Vitelliform macular dystrophy

MDT – Multi-Disciplinary Team

NCMD – North Carolina macular dystrophy

NCSS – Non-canonical splice site variant

NGGO – National Genetics and Genomics Office

NGS – Next generation sequencing

NMD – Nonsense-mediated decay

NNS – NNSPLICE

N-Ret-PE – N-retinylidene-phosphatidylethanolamine

OS – Outer segment

OCT – Optical coherence tomography

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDE – Cyclic nucleotide phosphodiesterase

pDONR – pDonor221

PPC – Photoreceptor precursor cell

RNA – Ribonucleic acid

RP – Retinitis pigmentosa

RPE – Retinal pigment epithelium

RVEEH – Royal Victoria Eye and Ear Hospital

smMIPs – Single molecule molecular inversion probes

SNV – Single nucleotide variant

SSFL – SpliceSiteFinder-like

STGD1 – Stargardt disease

SV – Structural variant

SVI – Sequence Variant Interpretation Working Group

TCD – Trinity College Dublin

TCS – Target capture sequencing

USH – Usher’s syndrome

VA – visual acuity

VUS – Variant of unknown significance

WES – Whole exome sequencing

WGS – Whole genome sequencing

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1. General Introduction

1.1 *The eye and the layers of the retina*

For humans, vision is at the forefront of how we perceive the environment and surrounding information. Our eyes are of paramount importance to be able to adapt to the world around us effectively. The eye is a highly complex and specialised organ. It is made up of both neuronal and non-neuronal photosensitive cell types, allowing photons of light to be converted into electrochemical signals that our brain interprets as an image. The disruption of this signalling pathway can result in the gradual atrophy and loss of vision. Vision loss has an immense impact on the quality of life of a patient, and is often detrimental to their everyday movements, health spending and overall lifestyle, as well as limiting work capabilities.

Vision begins when light is received through the anterior segments of the eye and is focused by the lens onto the photosensitive layers of cells at the back of the eye, known as the retina. The retina makes up a part of the central nervous system, as it is made up of intricate neuronal cell layers, as well as photoreceptors containing light-sensitive opsins. The photoreceptors of the retina detect light and convert it to electrical signals that are carried by the optic nerve into the visual cortex of the brain to be further interpreted into what our brain perceives as an image. Five distinct types of neuronal cells can be found in the layers of the retina; photoreceptors, bipolar, horizontal, amacrine and ganglion cells (Mahabadi and Khalili, 2022). These neurons serve in both the vertical and horizontal connections in the retinal signalling pathway. The vertical signalling pathway is made up of connections between the photoreceptors, bipolar and ganglion cells. The bipolar cells collect signals from the photoreceptor cells, and transmit them to the ganglion cells where information from the individual photoreceptors is processed. These retinal ganglion cells have long axons that extend into the brain and make up the optic nerve. They facilitate the transmission of the processed signals through the optic nerve to the thalamus, where they are then sent to the visual cortex to form the final image of what we see before us (Dhande *et al.*, 2015). The horizontal signalling pathway is made up of horizontal and amacrine cells. These interneurons connect laterally across the retinal layers and work to regulate the excitatory vertical pathway and aid in the interpretations of the initial light signals (Diamond, 2017). Horizontal cells process and mediate signals between photoreceptors and bipolar cells, while amacrine cells carry out this same function but between bipolar and retinal ganglion cells. These interconnected pathways make up the cell layers of the retina and facilitate image processing and information transmission to the brain. It is also important to highlight the role

of Müller cells in the retina. They are the predominant glial cell in the retina and extend through the entire thickness of the neuronal retinal layers. Müller cells have both structural and metabolic roles in the retina, maintaining the homeostasis of the compartments surrounding the retina and between the retinal neurons (Bringmann *et al.*, 2006; Kobat and Turgut, 2020). It has also been found that Müller cells contribute to the guidance of light through the neuronal retina, allowing it to reach the photoreceptor cells to initiate vision (Agte *et al.*, 2011).

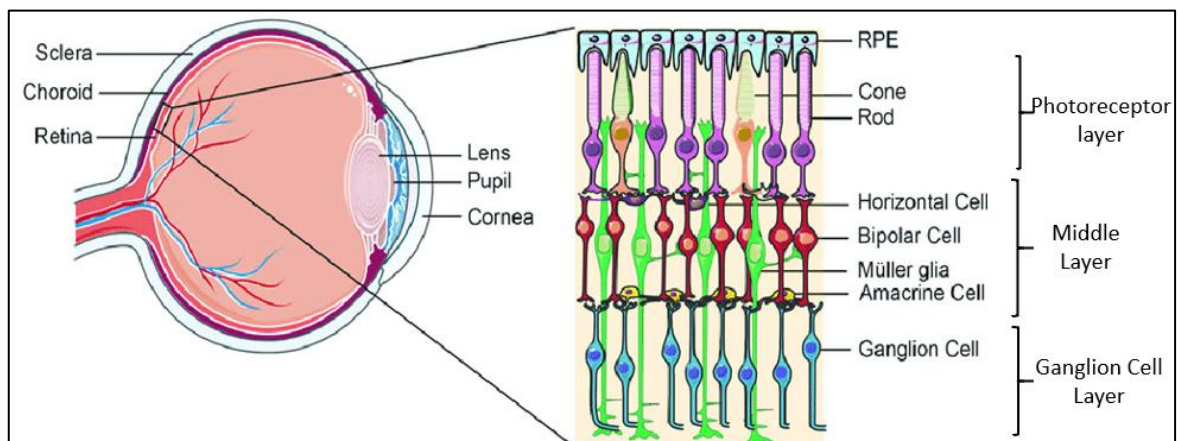


Figure 1.1 The cell layers of the retina. There are three general layers that make up the retina, each consisting of different cell types. The RPE cells border between the choroid and the photoreceptor layer. The photoreceptor layer is made up of Rods and Cones. The middle layer contains interneurons like Horizontal, Bipolar and Amacrine cells, responsible for transmitting signals from photoreceptors to Ganglion cells. (Image adapted from (Fu *et al.*, 2018)).

Photoreceptors are photosensitive cells that are specialised to detect visible light and transduce them into electrical signals to be transmitted to the visual cortex. Photoreceptor cells can be categorised as either rods or cones based on their morphological and functional characteristics, as well as their relative density in the retina. Both types of photoreceptors can be broken up into the following morphological structures; the synaptic terminal, a cell body containing the nucleus, the inner segment (IS), connecting cilium, and the outer segment (OS) (Hussey, Hadyniak and Johnston, 2022). The synaptic terminal contains glutamate neurotransmitter vesicles and allows for communication between the photoreceptors and the succeeding neuronal cells of the vertical and horizontal pathways. The IS has a predominantly metabolic and homeostatic function. Among other features, the IS contains a vast number of mitochondria to support the high energy demand of the photoreceptor cells, and serves as the production site for opsins and OS-specific proteins (Chen *et al.*, 2024). Inner and outer segments are bridged by the connecting cilium. The outer segment is a modified primary cilium, and is made up of layers of discs containing opsin photopigments that facilitate the absorption of light. OS structure differs greatly between

rods and cones. Rods have a longer outer segment than cones, with discs being stacked together to create a cylindrical structure which is fully enclosed by plasma membrane. Similarly for cones as their name suggests, the discs that build up to create their OS structure taper off at the end to create a conical structure that is shorter on average than the OS of rods, and is only partially enclosed by a membrane (Yau, 2005; Goldberg, Moritz and Williams, 2016; Hussey, Hadyniak and Johnston, 2022). The discs of both outer segments are shed terminally and replenished continually. Once the discs are shed they are phagocytosed by the retinal pigment epithelium (RPE), and new discs, in invaginations in case of the cones, are formed at the base of the OS (Anderson, Fisher and Steinberg, 1978; Hussey, Hadyniak and Johnston, 2022). The rod OS discs are shed more rapidly than that of cones, and follow the circadian rhythm for turnover.

As previously mentioned, rods and cones do not only differ in their OS shape but also in their density in the retina, light-sensitivity and native opsin. Rods have a highly light-sensitive opsin named rhodopsin, and for this reason are predominantly responsible for vision in low light, peripheral vision, and monochromatic vision. This is referred to as ‘scotopic’ vision. In humans, rods constitute approximately 95% of the photoreceptors in our retina, with no rods being present in the central fovea and the density increasing further into the periphery of the retina. Cone photoreceptors are not quite as photosensitive, therefore are involved in chromatic or colour vision, and vision in bright light. This is referred to as ‘photopic’ vision. Cones are almost 100x less sensitive to light than rods, but have a much faster kinetic response time (van Hateren and Lamb, 2006). Cones make up only 5% of the photoreceptors in our retina and are responsible for visual acuity and clarity of vision as they are concentrated in the macula and are the only photoreceptor type found in the fovea (Mannu, 2014; Lamb, 2015; Ptito, Bleau and Bouskila, 2021; Mahabadi and Khalili, 2023). Cones can be divided further into 3 different categories based on what photopigment is contained within its OS. These photopigments are made up of opsin proteins covalently bonded to a chromophore called retinal. Depending on the structure of the opsin present, cones will have different wavelength sensitivity with all three types of cones allowing for trichromatic vision. ‘Long’ L-cones have a wavelength absorption peak of around 558nm, with *OPN1LW* being the opsin gene present in these cones. ‘Medium’ M-cones have a wavelength absorption peak of around 530nm, with *OPN1MW* being the opsin gene present in these cones. And ‘Short’ S-cones have a wavelength absorption peak of around 420nm, with *OPN1SW* being the opsin gene present in these cones (Nathans, Thomas and Hogness, 1986; Neitz and Neitz, 2010). Cones map one-to-one onto bipolar cells, allowing for the more precise perception of detail. Conversely, many rod cells will synapse with one single

bipolar cell, increasing the signal amplification in dim light but with much lower precision (Ptito, Bleau and Bouskila, 2021).

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells that constitutes the outermost layer of the retina (**Figure 1.1**). The RPE supports the photoreceptors; physically and metabolically. The outer side of the RPE faces the Bruch's membrane and the choroid, while the inner side faces the photoreceptor OS. The tight junctions between the epithelial cells of the RPE allow it to strictly regulate transport between the blood of the choroid and the photoreceptor OS layer (Steinberg, 1985; Yang, Zhou and Li, 2021). In this way it makes up part of the blood-retina barrier. The RPE maintains ion, water and metabolite homeostasis in the subretinal space, as well as providing nutrients such as glucose, fatty acids and retinal to the photoreceptors from the blood (Strauss, 2005). Furthermore, a vital function of the RPE is to absorb excess light that was not captured by the photoreceptors in order to protect the retina from potential toxic photo-oxidation (Strauss, 2005; Simó *et al.*, 2010). Additionally, the RPE is responsible for the phagocytosis of the photoreceptor OS discs, allowing for retinal to be recycled and reused by the photoreceptors throughout the visual cycle and to regenerate photopigments lost (Kevany and Palczewski, 2010; Kwon and Freeman, 2020).

1.2 Phototransduction and the visual cycle

Phototransduction describes the process by which photons of light are absorbed by photoreceptors and converted to neural signals. As mentioned previously, the photopigments of photoreceptors are made up of opsins covalently bound to the chromophore 11-cis-retinal. In the dark, free cyclic guanosine monophosphate (cGMP) concentration levels are high therefore cation channels remain open. Open cation channels cause the photoreceptor to be depolarised, allowing for the release of glutamate from the synaptic terminal of the photoreceptors. When light is absorbed, the 11-cis-retinal of the photopigment is isomerised into all-trans-retinal, triggering the opsin to undergo conformational changes in a process called 'bleaching'. This bleached opsin triggers the activation of a GTP-binding protein called transducin. Transducin activates phosphodiesterase (PDE) which acts to hydrolyse cGMP. This causes the cGMP concentration to decrease and cGMP-gated channels within the plasma membrane close. The membrane is now hyperpolarised, stopping the release of the neurotransmitter glutamate from the synapse, promoting the transfer of visual information to higher-order neurons of the retina. With cation channels closed, Ca^{2+} concentration decreases in the photoreceptor OS. This decrease triggers a negative feedback loop causing the initial decrease in cGMP concentration to lessen, initiating the opening of

some cGMP-gated channels of the plasma membrane, allowing for active light adaptation of the photoreceptors to occur. This negative feedback loop is essential for cones as they function in very bright light (Lamb and Pugh, 2006; Yau, 2013; Kawamura and Tachibanaki, 2018).

The visual cycle is a process that occurs in tandem with the phototransduction pathway. It describes the recycling of all-trans-retinal and the renewal of the photoreceptor outer segments that occurs after photon absorption (Tang *et al.*, 2013; Chen *et al.*, 2024). When 11-cis-retinal is isomerised into all-trans-retinal, it detaches from the photopigment as the opsin undergoes conformational changes to initiate phototransduction. This process means that the photoreceptor cells are without light sensitive photopigments, and it is essential that they are renewed to re-establish the light-sensitivity of these cells (Lamb and Pugh, 2004). In the classic visual cycle, all-trans-retinal is reduced into all-trans-retinol by retinol dehydrogenase (RDH) enzymes. The all-trans-retinol is then removed from the OS of the photoreceptors and brought to the RPE by the retinoid-binding chaperone protein, interphotoreceptor retinoid-binding protein (IRBP). Here it is esterified by lecithin retinol acyl transferase (LRAT), and then converted to 11-cis-retinol by RPE65. 11-cis-retinol is subsequently oxidised back into 11-cis-retinal and shuttled back to the photoreceptor cells to reform photopigments, ending the classical visual cycle. While both rods and cones are thought to receive 11-cis-retinal from the RPE during the classical visual cycle, this pathway alone is not a rapid or efficient enough reaction for cone photopigment regeneration (Wang and Kefalov, 2011). It has been found that the cone visual cycle occurs in parallel to the classical visual cycle, with isomerisation of all-trans-retinol occurring in Müller cells rather than the RPE. Therefore cones can receive 11-cis-retinol from Müller cells, and are able to carry out its oxidation back into 11-cis-retinal (Miyazono *et al.*, 2008; Arshavsky, 2024).

1.3 Inherited Retinal Degenerations (IRDs)

Inherited Retinal diseases (IRDs) are a clinically diverse group of disorders of the retina, with a highly complex genetic landscape. While they are individually classed as rare diseases, altogether IRDs have in recent years surpassed diabetic retinopathies as the most common cause of visual defects in adults of working age (Liew, Michaelides and Bunce, 2014; Duncan *et al.*, 2018b; Heath Jeffery *et al.*, 2021). They can be classified into broad phenotypic subtypes based on the cell type or layers of the retina that are affected. IRDs are often associated with a gradual deterioration of vision, leading to complete blindness over time. Or they can be non-progressive and stable visual defects present since birth, such as Congenital stationary night blindness (CSNB) or colour vision phenotypes like

Achromatopsia (ACH). As well as this, IRDs have been seen to occur as part of syndromes with multisystemic features, for example, Usher syndrome (USH) and Bardet-Biedl syndrome (BBS), the latter of which will be introduced in Chapter 3.B where a *BBS7* case study will be outlined.

Significant progress has been made in elucidating the pathogenesis of IRDs. To date, just over 350 loci have been associated with various forms of IRDs as described in RetNet, with many more candidate loci under investigation (*RetNet: Summaries*, Accessed March 2025). Despite this, the genetic diagnosis for many patients is still not known. The genetic diversity observed in IRDs is not solely contributed to the vast number of genes associated with the various subtypes, but also lies within the varying types of mutations found and their locations within the causative gene. For example, *BEST1* is a gene that can cause both autosomal dominant and recessive forms of disease such as Best vitelliform macular dystrophy (MD-BV), Bestrophinopathy, Vitreoretinopathopathy, or Retinitis Pigmentosa, depending on the type of mutation found and its location within the gene (Dalvin *et al.*, 2016). Rod-cone dystrophies mainly affect rod photoreceptors and are characterized by night blindness and peripheral vision loss, such as retinitis pigmentosa (RP), where cones may begin to degenerate later over time. In contrast, cone/cone-rod dystrophies (CCRDs) present with visual acuity (VA) loss, photophobia, and colour vision abnormalities, often with no or mild rod involvement (Rodilla *et al.*, 2023).

As the clinical features of IRDs have a large amount of overlap, it is often the case that a differential diagnosis is reached and must then be refined with additional evidences. For example, as a cone dystrophy progresses and the patient begins to lose vision peripherally due to rod degeneration, this could look similar to early onset cone-rod dystrophy due to the lesser involvement of rods in both cases. Similarly, Stargardt disease (STGD1) later in life appears similar to a cone-rod dystrophy due to the initial loss centrally, and the gradual impact peripherally. Typically, the most prevalent IRDs tend to have the highest levels of heterogeneity, with variants in many genes causing the same or similar disease phenotypes. RP is a great example of this, with over 120 loci implicated in disease to date, where RP affects approximately 1 in 4000 individuals, which is the highest of any IRD (Suleman, 2025)(*RetNet: Summaries*, Accessed March 2025). Likewise, many different types of variants in one gene can cause multiple retinal phenotypes. A prime example of this is seen with mutations in the *ABCA4* gene where, depending on the location, type of mutation and combination of variants, it can most commonly cause the juvenile onset macular dystrophy, STGD1, or less commonly RP. Phenocopies of STGD1 have also been observed in patients

where their genetic diagnosis indicates variation within the *PRPH2* or *NMNAT1* genes, respectively (Cremers *et al.*, 2020). IRDs can be inherited in autosomal recessive, dominant, X-linked and mitochondrial forms. In rare instances, a digenic inheritance pattern is observed or a mutation can occur *de novo*. In recent years, research has been undertaken to identify and investigate modifier loci involved in IRDs and how they may alter severity, prognosis and onset of disease (Ford and Petersen-Jones, 2025). These varying patterns of inheritance and the existence of potential modifier loci blur the lines between the Mendelian inheritance that we usually see in IRDs, and complex disorders further highlighting the vital need of molecular diagnoses for IRD patients. Such concepts and intricacies will be discussed throughout this PhD thesis.

Macular degenerations (MD) are a subtype of IRDs, and will be highlighted throughout this thesis. They are typically characterised initially by a decline in visual acuity, followed by central vision loss along with the progressive degeneration of the macula and underlying RPE cells. Peripheral vision remains largely retained but can devolve over time depending on the severity of disease and the photoreceptors impacted (Pozo-Valero *et al.*, 2022). Different forms of inherited (i)MD have varying clinical presentations and have high levels of genetic heterogeneity, with many genes being implicated in disease. Such iMDs include; Best Vitelliform Macular Dystrophy (MD-BV), Stargardt disease (STGD1), CCRDs, North Carolina Macular Dystrophy (NCMD), Pattern dystrophy, autosomal Dominant drusen and X-Linked retinoschisis, among others (Audo, Meunier and Sahel, 2022; Georgiou and Michaelides, 2022). There is a variable age of onset, with first symptoms tending to occur within the first two decades of life. Late-onset iMD can be challenging to diagnose solely through a clinical phenotype, as it can look similar to Age-Related Macular degeneration (AMD). In this case, multimodal retinal imaging is studied thoroughly but ultimately a genetic diagnosis is required to distinguish between iMD and AMD clinical diagnoses. iMDs, like most IRDs, are inherited in an autosomal dominant, autosomal recessive, X-linked or mitochondrial fashion. The genes implicated in iMDs tend to function across key visual and photoreceptor pathways, most notably involved in phototransduction, OS morphogenesis and photoreceptor structure, retinal layer homeostasis, neurotransmitter release and Intraflagellar Transport (IFT) (Brotherton and Megaw, 2024). iMDs and cone/cone-rod dystrophies will be the main focus of some of the studies presented within this doctoral thesis, as we try to elucidate the genetic background of such disorders in the Irish population (Chapter 3). Genotype-phenotype correlation studies are of great importance in the study of iMDs, allowing us to have a greater understanding regarding the genetic

pathogenesis and clinical presentation of these disorders as well as potentially identifying novel associations.

iMDs are often closely associated with cone/cone-rod dystrophies (CCRD). Both of these IRD subtypes ultimately lead to central vision deterioration due to the loss of photoreceptors from the macula. Cones are primarily affected in CCRDs leading to symptoms such as photophobia and poor visual acuity, as well as colour vision defects impacting ability to discriminate between the three colour axes. Based on the level of rod involvement, some CCRDs will eventually lead to the deterioration of peripheral vision and nyctalopia (Hamel, 2007). The overarching theme of this PhD thesis is to highlight the rapidly growing importance of providing a molecular diagnosis to IRD patients, in an era of personalised medicine and gene therapies, and the many challenges involved in achieving such diagnoses for IRDs. In the next section, some of the Next Generation sequencing (NGS) methodologies used to reach these genetic diagnoses globally will be outlined, as well as the techniques employed for the studies described in this thesis and other IRD studies that have been carried out in recent years.

1.4 An introduction to NGS and its application to IRDs/iMDs

There are many aspects of IRDs as a group of disorders that make them ideally suited to genetic diagnosis through the use of comprehensive DNA sequencing technologies. While IRDs are extremely heterogenous, they display a high level of Mendelian inheritance, with more complex digenic patterns being relatively rare. As well as this, a vast amount of research has been undertaken in recent decades to implicate over 300 genes in IRDs, leading to an ever-expanding amount of knowledge on the genetic aetiology of disease. Therefore, NGS technologies can allow us to efficiently genotype IRD patients, while also enabling us to identify novel variants and gene associations. As gene-based therapies and clinical trials continue to emerge for IRDs, a molecular diagnosis and DNA sequencing become essential in identifying which patients would be suitable to benefit from these therapeutic strategies. A genetic diagnosis can also provide a more accurate prediction of disease prognosis, risk information for the family and a better understanding for the patient of their own disease through genetic counselling. Genetic testing can also provide clarity in differential diagnosis, allowing for the avoidance of a misdiagnosis and incorrect treatment. As will be discussed later in this thesis, an IRD phenotype can often be a very early symptom in various multisystemic syndromes. An early molecular diagnosis would help to provide clarity on the later development of a syndrome, and allow for proper medical planning and monitoring to be implemented. Many genes that have been implicated in causing iMDs have also been seen

to cause varying phenotypes associated with other subtypes of IRDs, such as RP or Leber Congenital Amaurosis (LCA). This overlap between monogenic IRDs makes giving an accurate diagnosis almost impossible by evaluating the clinical disease phenotype alone. However, this does not diminish the need for deep clinical phenotyping, as this often allows us to refine our search and greatly assists genotype-phenotype studies to further inform the IRD field.

High-throughput NGS technologies enable the sequencing of millions of DNA fragments in parallel, many times over, resulting in a fast and accurate system of sequencing. Sequencing studies often tend to focus on exonic regions of genes, however this approach means that we are typically left with many unresolved patients as much of the genome remains unexplored (Carrigan *et al.*, 2016; Whelan *et al.*, 2020a; Hitti-Malin *et al.*, 2022, 2024; Britten-Jones *et al.*, 2023; Panneman *et al.*, 2023). Of note, traditionally it has been standard practice in IRD genetic diagnosis to utilise either a targeted/panel sequencing approach or a phenotype-based data extraction for a subset of IRD genes from whole exome sequencing (WES). Recent sequencing studies have been focused on sequencing the non-coding as well as the coding regions of IRD associated genes, where possible, in an effort to capture the candidate mutations that are missed using traditional exome-centric sequencing strategies. Disease causing variants that explain the pathogenesis of a very distinct retinal disease phenotype, such as X-linked Retinoschisis or *ABCA4*-associated retinopathies, are more likely to be identified through phenotype-specific gene panels (Khan *et al.*, 2020; Corradi *et al.*, 2023). However, IRD subtypes like iMDs display high levels of genetic heterogeneity and clinical overlap are more difficult to categorise, therefore often display reduced diagnostic rates. A recent meta-analysis suggests that the pooled diagnostic yield of NGS for patients with iMDs or CCRDs is approximately 58%, ranging from 21 to 73% (Britten-Jones *et al.*, 2023). The current landscape of sequencing technologies utilised for IRDs is important to introduce, as well as outlining those chosen for this doctoral thesis below.

Target or panel-based sequencing approaches have been seen in previous studies to be a promising option for preliminary sequencing analysis, as they can be customised to cover specific exonic, intronic and regulatory regions of the genes of interest. One such method, which will be discussed further in Chapter 3 of this thesis, are single-molecule Molecular Inversion Probes (smMIPs), which allow for the sequencing of hundreds of patients at the same time. The aim of a 2020 study was to use smMIPs as a cost-effective, high throughput and comprehensive method of sequencing all 50 exons of *ABCA4*, along with their flanking

splice sites, and 14 choice intronic regions that contain known deep-intronic variants associated with Stargardt disease (Khan *et al.*, 2020). The study sequenced 411 previously unresolved cases, and successfully identified two novel noncanonical splice site variants, and one new deep-intronic variant. Since then, this method has been successfully applied to various IRD focused studies on large patient cohorts with STGD1, MD or RP/LCA clinical phenotypes (Hitti-Malin *et al.*, 2022, 2024; Mc Clinton *et al.*, 2023; Panneman *et al.*, 2023). There are a number of benefits when using smMIPs multiplex targeted sequencing over other NGS sequencing approaches. smMIPs are highly cost-effective as they allow for thousands of loci to be amplified in many samples simultaneously, with a high mean nucleotide coverage of up to 400x (Hitti-Malin *et al.*, 2022). They are designed so that each smMIP targets a distinctive DNA sequence and allow for an extra layer of specificity by using molecular tags that are unique for each patient sample. This means all of the samples can be pooled into a single sequencing run while still differentiating between each sample after amplification. This tagging system combined with high coverage levels allows more confident variant calls to be made when analysing the data (Hiatt *et al.*, 2013). This will be further discussed in depth in Chapter 3 of this thesis, where smMIPs sequencing was employed.

During recent years, a steady decrease in cost of Whole Exome Sequencing (WES) has been observed, in turn increasing its popularity in IRD diagnostic sequencing. WES allows for the sequencing of the whole protein-coding genome. It has been previously reported that around two thirds of IRD cases can be resolved by variants occurring in coding regions (Duncan *et al.*, 2018a), hence the popularity of WES. However, this missing third is a significant concern, as many patients are left without a molecular diagnosis after utilising this sequencing method. WES is not capable of detecting deep-intronic variation (DIV), copy number variants (CNVs) or structural variants (SVs) that are often associated with aberrant splicing and transcription defects. It is often that WES studies, as previously mentioned, extract data for IRD associated genes from the resulting sequencing data and only these data are analysed. This approach was previously undertaken by our research group as part of Target 5000, where the sequencing information of 254-IRD was analysed (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020a). Due to the comprehensive nature of WES, it is possible to return to the sequencing data when novel disease associations are reported, allowing reanalysis without having to re-sequence. This in theory also means that variants can be found within the coding genome even before their relevance has been elucidated.

Whole genome sequencing (WGS), is undoubtedly the most comprehensive form of sequencing as the entire genome is captured. WGS allows for the detection of non-coding variation as well as variants in regulatory regions, CNVs and SVs including deletions, insertions or complex rearrangements. The additional context allows for breakpoints of these CNVs and SVs to be easily identified. While the cost for WGS is becoming more accessible globally, this form of sequencing produces the largest amount of data that requires downstream processing and specialised bioinformatics that may not be as readily available to all genetic testing and research labs. As is also the case with WES, WGS generates a lot of secondary findings that need to be advised on and acted on accordingly, and are often not related to the phenotype being analysed. Due to the vast amount of variation called, more than 3 million variants per sample (Gonzaga-Jauregui, Lupski and Gibbs, 2012; Shen *et al.*, 2013), it can be challenging and very time consuming to filter through these data to identify the pathogenic variation causing the IRD phenotype.

While more comprehensive genetic testing is often the best approach for IRDs, in cases where a condition is typically caused by mutations within one specific gene it can often be beneficial to sequence the entirety of a single gene. This is rarely the case with IRDs, due to their heterogenous nature and vast phenotypic overlap between conditions that are caused by different genes. A good example however of a candidate for single whole gene sequencing is *ABCA4*, where variants in this gene are seen to cause over 90% of STGD1 cases (Ścieżyńska *et al.*, 2020; Al-khuzaei *et al.*, 2021). After the aforementioned study in 2019 carried out by Khan *et al.* established smMIPs as a good method for targeted sequencing, the team expanded these probes to cover the full length of the *ABCA4* gene. Their follow up study using this whole gene sequencing method allowed them to re-sequence over 1000 unresolved individuals with a Stargardt (STGD1) or Stargardt-like phenotype, resulting in a 42.5% solve rate (Khan *et al.*, 2020). This research led to further studies that utilised the same approach, further refining the sequencing methodology and phenotypic criteria, with the most recent study by Corradi *et al.* reporting a 47.1% solve rate when sequencing the whole of *ABCA4* in a cohort of patients with *ABCA4*-associated retinopathies (Corradi *et al.*, 2023). Whole gene sequencing allows us to uncover intricacies about the mutation profile causing a particular disease, as well as enabling us to evaluate connections between mutation type and location, and disease severity or age of onset (Cornelis *et al.*, 2022a). Both of these whole *ABCA4* studies identified a number of novel SVs and DIVs, performing further investigations such as in vitro RNA splice assays where applicable to categorise the pathogenicity of these variants, and hence better inform current knowledge of *ABCA4*-associated disease. This would not be possible without efficient high coverage sequencing

of a single gene, provided by the smMIPs method. A number of patients that will be discussed in Chapter 3 of this thesis have been included in whole *ABCA4* smMIPs sequencing runs in collaboration with the Cremers team in RadboudUMC.

The wide range of diagnostic yields seen across IRD sequencing studies can be attributed not only to the heterogeneity of IRDs, but also to a whole host of other factors. It is not always the case that a patient will undergo WGS as the primary form of sequencing as it is not a feasible practice in many areas of the world. Therefore, as previously stated, targeted sequencing or data extracted from WES is often the first approach. This means that the panel of genes analysed is chosen based on the clinical diagnosis, which could contribute to the variability seen in diagnostic yield. Recent advances in genetic therapeutics have made it crucial for patients who are suspected to have an IRD to receive a complete molecular diagnosis, as this will affect their eligibility for a clinical trial or approved therapy, and will also determine their suitability and whether the therapeutic is likely to benefit them based on their causative genotype. Accurate and early genetic testing in IRDs is not only vital for patients in terms of clinical trial inclusion or access to approved therapies, but it is also imperative in diagnosing syndromic IRDs that could require extraocular interventions before the onset of potential systemic symptoms.

1.5 Mutational mechanisms and splicing in IRDs and iMDs

As it stands today, the genetic diagnostic rate for IRDs is around 50-70%, with an increase in solve rate observed with advancements in NGS (Carrigan *et al.*, 2016; Whelan *et al.*, 2020b; Perea-Romero *et al.*, 2021a; Karali *et al.*, 2022; Schneider *et al.*, 2022; Stöhr and Weber, 2025). Some NGS methodologies, such as the targeted panel pipelines, do not have the ability to detect structural variants or CNVs readily therefore patients harbouring these kinds of variants remain without a genetic diagnosis until more comprehensive sequencing is utilised. Pathogenic SVs and CNVs are estimated to be present in approximately 9% of IRD cases (Zampaglione *et al.*, 2020; Zeuli *et al.*, 2024a). This estimate varies quite widely between studies, and is potentially due to an inability to identify more complex rearrangements, or due to the size of the population used in the study and the phenotypic make-up of the population (Schneider *et al.*, 2022). While it is true that variants in the coding regions of genes account for the majority of disease-causing variation that we have identified, it has become increasingly clear that non-coding regions of the genome must also be interrogated to see the full picture. Intronic variation such as deep-intronic variants (DIV) and near-exonic variants lead to aberrant splicing of the mRNA and often have severe harmful effects on the resulting gene product. Therefore, many of the unresolved IRD cases

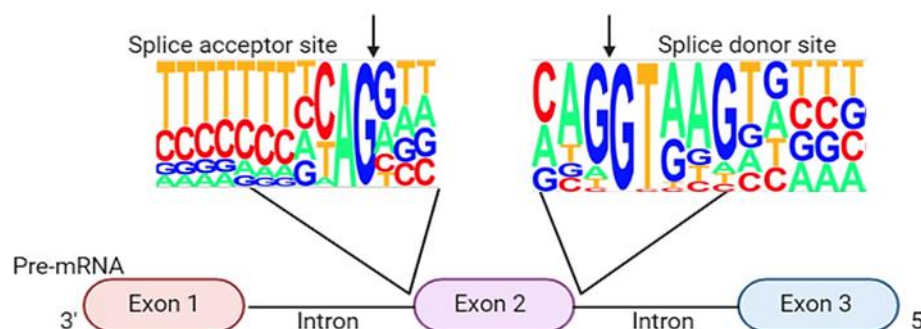
may be likely due to the fact that intronic and regulatory regions are typically not being analysed in most studies.

Splicing occurs during the processing of pre-mRNA into mature mRNA, and involves the excision of introns through the recognition of specific splice motifs, which indicate the location of exons to the spliceosome machinery. These 'splice sites' are widely conserved sequences, with around 99% of all mammalian introns containing the same motifs at exon-intron boundaries (Burset, Seledtsov and Solovyev, 2000). The canonical splice donor site is located at the 5' end of the exon, and is made up of a GT dinucleotide at positions +1/+2 upstream of the exon. The canonical splice acceptor site is located at the 3' end of the exon, and is made up of an AG dinucleotide at positions -2/-1 downstream of the exon (Ma *et al.*, 2015) (Figure 1.1). The less conserved sequences flanking the canonical splice sites (CSS), are referred to as the non-canonical splice site (NCSS) regions and are located between +3/+6 and -3/-14 up and downstream of the exon, respectively, as well as the first and last 3 bases of an exon. Furthermore, the Branchpoint (BP) sequence motif contains an adenosine (yUnAy), located close to the 3' splice site in pre-mRNA (**Figure 5.1** in Chapter 3) (Gao *et al.*, 2008). This motif is also highly conserved, but there is a lot still unknown regarding splicing branchpoints present in humans. However, due to the high levels of degeneracy of the sequences that surround the branchpoint, it can often be difficult to identify and therefore challenging to find mutations that affect the motif. One aspect that has been established is that the 5' splice site is linked with the branchpoint that is next to the 3' end of the intron that is to be spliced out of the mRNA. This link forms the lariat structure, which is recognised by the spliceosome and is subsequently removed to join together the two surrounding exons (Will and Lührmann, 2011). If any of the aforementioned splice motifs are mutated, this can lead to exon skipping, pseudoexon inclusion, and intronic inclusion into the mature mRNA, among other kinds of harmful splicing consequences (Lee and Rio, 2015).

Variants in the CSS that abolish the motif will most commonly lead to complete or partial exon skipping. However, variation in the NCSS is less predictable as the sequence of the NCSS is not as highly conserved, therefore some variants at these positions can be tolerated, while others can lead to multiple splicing alterations. An example of this will be further outlined in Chapter 3 of this thesis, where a splice altering variant at the +5 position of an exon in *ABCA4* causes multiple aberrant splicing consequences. Intronic variation like DIVs or near-exonic variants can lead to the creation of novel cryptic splice sites. This will in turn cause competition for the spliceosome between this novel site and the CSS. Upon the creation of a cryptic site, the splice machinery might preferentially recognise this newly

produced splice site, leading to the partial loss of an exon, and/or possible inclusion of a further intron in the mRNA. Intronic retention occurs most often if this cryptic site is created close to an exon that has weak CSS or an abundance of cis-regulatory elements (Sakabe and de Souza, 2007). Aberrant splicing is not only caused by mutations in the splice motifs, but can also be caused by variation in the splice enhancer and silencer motifs. These are cis-regulatory elements which are involved in promoting or suppressing the use of particular splice sites (Wang and Burge, 2008). Abnormal splicing can also be caused by mutations that occur within coding regions of the gene, as they too can lead to the creation of cryptic splice motifs but within the coding region. Recently, many studies have been able to show that missense and synonymous variation in certain disease genes can also lead to aberrant splicing resulting in an IRD phenotype (Corradi *et al.*, 2023; Kaltak, Corradi, *et al.*, 2023). This will be discussed further in Chapter 5 of this thesis, focusing on *in vitro* functional studies undertaken to elucidate pathogenicity of variants impacting splicing. In recent years, continued applications of NGS technologies has allowed for the identification of deep intronic and near-exonic variants in IRD associated genes (Sangermano *et al.*, 2018a, 2019; Qian *et al.*, 2021a; Corradi *et al.*, 2023; Whelan *et al.*, 2023). Upon identification, experimental functional studies such as Minigene or Midigene RNA splice assays are undertaken in order to determine the functional impact of the variant on splicing.

Figure 1.2. Annotation of canonical splice sites flanking the exon. Splicing machinery recognises the splice sites, in order to remove introns, resulting in the production of mature mRNA. Splice consensus sequences for donor and acceptor sites were adapted from (Ma *et al.*, 2015) (Created using



BioRender).

Additionally, the advances in NGS technologies have resulted in a large number of variants being identified which are classified as ‘Variants of Unknown Significance’ (VUS). These variants represent a significant challenge for diagnosis and development of optimal patient care pathways, as there is insufficient evidence to classify them as pathogenic or benign. Results from the analysis of such variants will be presented in detail throughout Chapters 3-5 of this doctoral thesis. Variants will be classified using the standardised guidelines

published by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (Richards *et al.*, 2015), along with additional recommendations from The Association for Clinical Genomic Science (ACGS) for standardised usage on rare diseases in labs and clinics in the UK and Ireland (Ellard *et al.*, 2020; Durkie *et al.*, 2024). These guidelines and their application are discussed in great detail in Section 6.3, as well as throughout Chapters 3-5.

Modifier loci have been an increasingly popular area of study in IRDs, and are most commonly defined as a variant at a secondary locus that alters the disease phenotype caused by mutations in the primary causative gene. These secondary genetic factors have a potential influence on phenotypic expression in patients, disease prognosis and severity (Meyer and Anderson, 2017). Such modifiers are often very difficult to identify due to the heterogeneity, both phenotypically and genotypically, observed in IRDs. When suspected modifiers occur in isolation, they do not lead to a disease phenotype, but instead impact the expressivity, penetrance, pleiotropy and severity of an IRD that is caused by a different primary gene (Ford and Petersen-Jones, 2025b). Further examples of this will be discussed in Chapter 3 of this thesis, where the potential for modifiers and digenic inheritance patterns are outlined in a sequencing study within the Irish population.

1.6 Target 5000 introduction and workflow

Since 2009, the Trinity College Dublin research team has been involved in establishing the Target 5000 project, an Irish national registry of patients with IRDs (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020b; Stephenson *et al.*, 2023a). This study aims to provide a more complete genetic diagnosis as well as a clinical phenotype for the Irish population of IRD patients, thereby aiding the implementation of optimal care pathways and potentially improving therapeutic options where appropriate. Target 5000 has since been adopted as part of a standardised care pathway for IRD care in Ireland, and has shown significant benefit in providing patients with clinical and genetic diagnoses (KAJ *et al.*, 2021b). Worldwide over 5 million people (Van Schil *et al.*, 2018; Ben-Yosef, 2022a) are affected with an IRD, with a prevalence of between 1 in 2000 and 1 in 4000 people (Britten-Jones *et al.*, 2023; Hanany *et al.*, 2024). Whether it is a form of RP or visual deterioration with macular involvement, IRDs are now considered the leading cause of vision loss in people of working age (Liew, Michaelides and Bunce, 2014; Duncan *et al.*, 2018b; Heath Jeffery *et al.*, 2021). The most recent Irish Census reports just over 296,000 people living with a form of blindness or visual impairment, making up 6% of the total Irish population (CSO, 2023. Accessed March 2025). Target 5000 endeavours to determine how many of

these cases are IRDs of genetic origin, and it aims to classify in depth the candidate variants identified thereby enabling patients to have greater clarity on disease diagnosis, prognosis, progression and inheritance.

The first step of the Target 5000 workflow is for the patient to be seen by a clinician from an affiliated recruitment hospital: Royal Victoria Eye and Ear Hospital (Dublin, Ireland), the Mater Misericordiae University and Mater Private Hospital (Dublin, Ireland), The Belfast Health and Social Care Trust Hospital (Belfast, Northern Ireland) (**Figure 1.3**). It is of paramount importance that the original detailed patient history of symptoms including; field of vision, visual acuity and ability to see in various intensities of light is taken into account when investigating the genetic cause of disease. Such clinical profiles can be an accurate indicator of what cells of the retina are affected. These details help to direct the approach employed for genetic diagnosis towards each patient. Hence, targeted forms of sequencing with specific panels of retinal genes can be utilised more efficiently as a primary form of screening. While this may be true for some IRDs, one must be mindful of the high levels of genetic heterogeneity observed among IRDs as well as the phenotypic overlap seen between genotypes, making it challenging to implicate a single gene during clinical diagnosis.

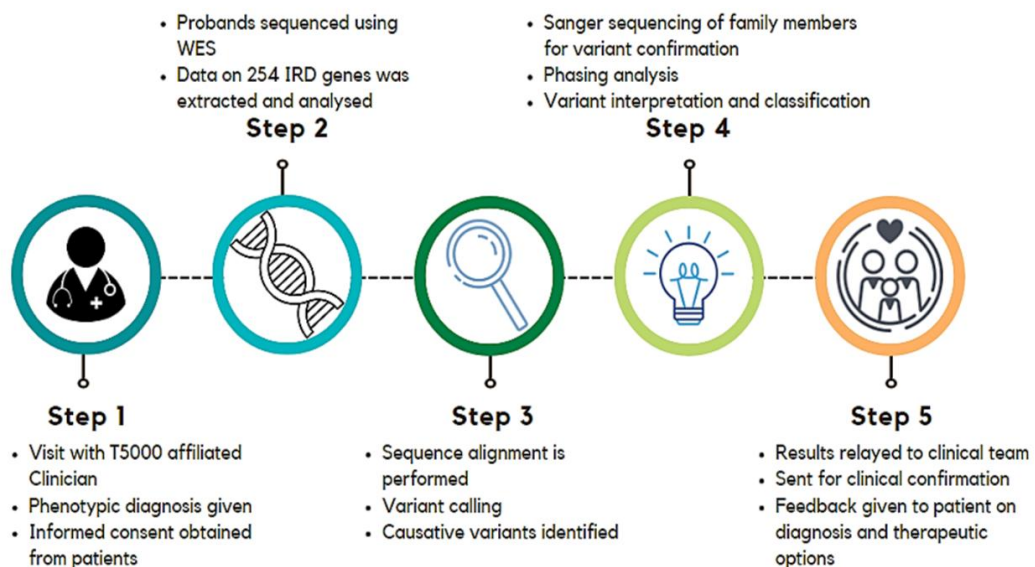


Figure 1.3. Outline of the Target 5000 workflow. This is a schematic representation of the steps that are undertaken once a patient with an IRD visits one of the Target 5000 affiliated hospitals.

Approximately 2500 patients from 1054 pedigrees have received NGS and their results have been analysed as part of Target 5000, thus far. Overall, a combination of pathogenic, likely pathogenic or VUS variants believed to be causative of disease have been detected in

approximately 74% of pedigrees analysed (**Figure 1.4**). Most pedigrees that have been genetically resolved have a form of Stargardt disease (STGD)/Fundus Flavimaculatus or autosomal recessive RP. Previously, Target 5000 employed target capture NGS, with an initial panel covering the exonic regions of over 250 IRD genes, as well as a few intronic regions that have confirmed splice variants. This panel was used as a preliminary form of sequencing for patients and provided accurate results with high levels of coverage (Carrigan et al., 2016). When panel sequencing provided no promising candidate variants, further sequencing methods were employed such as targeted smMIPs, WES or WGS (KAJ et al., 2021). Subsequently, additional in vitro studies are undertaken to resolve the pathogenicity of variants initially classified as ‘variants of unknown significance’ (VUS) when applicable and possible. Once research grade results are verified by a clinically accredited laboratory, an in-depth genetic diagnosis can be given to patients by the clinical teams. The work described and completed as part of this doctoral thesis aims to contribute to the growing landscape of IRD variants in Ireland, through the use of targeted smMIPs sequencing technologies to complete the first ever Irish Macular dystrophy landscape study, as well as utilise in vitro functional assays, such as Midigene splice assays, to categorise novel splice-altering variants that have been identified within the Target 5000 cohort for the first time globally. The Irish population is a fairly homogeneous population that has been shaped by historical and geographic structure. These distinct features often have an influence on the distribution and frequency of pathogenic variants, with population-specific patterns potentially observable within the IRD subpopulation. While the current analysis within the scope of this doctoral thesis does not explicitly examine geographic stratification due to the sample sizes used, the scale and nationwide recruitment of the Target 5000 project provide a unique opportunity for future work aimed at investigating these patterns.

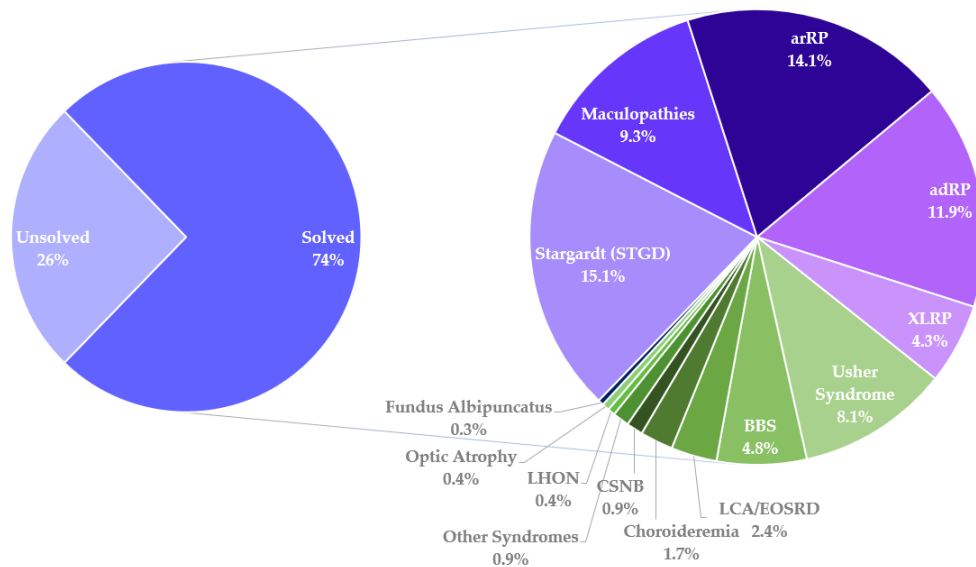


Figure 1.4. Breakdown of Target 5000 cohort (by pedigree). (a) Percentage of pedigrees that have been genetically resolved vs. unresolved through NGS as part of Target 5000. (b) Distribution of resolved pedigrees into IRD subgroups refined after genetic diagnosis. (ar; autosomal recessive, ad; autosomal dominant, BBS; Bardet-Biedl syndrome, CSNB; Congenital stationary night-blindness, LCA/EOSRD; Leber congenital amaurosis/ early-onset severe retinal dystrophy, LHON; Leber Hereditary Optic Neuropathy, RP; Retinitis Pigmentosa, XL; x-linked)

1.7 Aims and Objectives of this thesis

The research undertaken and described within this doctoral thesis broadly serves to expand our knowledge on the genetic basis of disease in the IRD population of Ireland, as part of the Target 5000 project. In order to shed light on the genetics underlying IRDs in Ireland, targeted sequencing technologies have been employed to screen a multitude of macular variant filtering of sequence data has been undertaken to highlight candidate disease-causing variants. Subsequently, cascade analyses to classify variants identified within Target 5000 pedigrees were undertaken, as well as *in silico* and wet laboratory-based functional studies to investigate the effects of novel and known variants.

The first research Chapter reports the results of a smMIPs targeted sequencing study, utilising a panel of 105 genes, covering all exonic and well-known intronic regions carrying pathogenic variants, to identify candidate variants in 223 unresolved Irish macular dystrophy probands (Chapter 3). This project represents the first genetic landscape study of iMDs in Ireland, and resulted in the identification of 9 novel variants and a ~33% solve rate, demonstrating the effective use of targeted sequencing methodologies as a preliminary genetic screening technique. Sub-Chapter 3.B focuses more closely on the first family identified in Ireland where candidate variants in the BBS7 gene have been implicated in a syndromic form of RP. Two null variants in BBS7, one of which is novel, have been

thoroughly categorised utilising the ACMG/AMP guidelines along with the ACGS recommendations (Richards *et al.*, 2015; Durkie *et al.*, 2024).

In the second research Chapter, a collection of family studies within Target 5000 are presented, encompassing a wide variety of IRD phenotypes and genes implicated in disease (Chapter 4). Data was collated and filtered and candidate variants were classified with the overarching aim of contributing to the growing understanding of disease causation and mechanisms. The study highlights the diversity of variants implicated in IRD-associated genes. Data sharing and collaboration are vital in such research, as deep-phenotyping is needed to inform these classifications, which in turn contribute to comprehensive molecular diagnoses for IRD patients.

The final research chapter illustrates the significant value of *in vitro* functional assays (midigene studies) to shed light on the effects of potential splice-altering variants that are found within this Irish IRD patient cohort (Chapter 5). A coding variant in *RPGR* was identified and characterised, and found to lead to the creation of a branchpoint within an exon. This is the first instance of such a variant identified in an IRD gene, where an aberrant branchpoint is created within the coding region. Similarly, a novel *ABCA4* variant, identified through the smMIPs study presented in Chapter 3, was also categorised by midigene splice assay and found to lead to a multimodal splice defect.

Ultimately the aim of the research presented in this doctoral thesis is to contribute to the expanding landscape of IRD variants globally, and elucidate the basis of disease in the IRD population of Ireland recruited via the Target 5000 study. Providing a deeper understanding the pathogenesis of IRDs is important for ocular researchers, clinicians and patients, thereby enabling more comprehensive genetically informed care for patients nationally and globally.

Summary of objectives:

1. Utilise an MD-smMIPs panel of 105 genes to sequence 223 Irish probands in order to elucidate the genetic architecture of iMDs in Ireland.
2. Investigate candidate variants implicated in disease in multiple IRD families recruited via Target 5000, employing variant filtering, in silico modelling, cascade analyses, and deep-phenotyping, thereby providing additional layers of evidence towards the classification of variants as pathogenic, VUS or benign.
3. Employ *in vitro* functional assays to categorise the impact(s) of candidate splice-altering variants in IRD genes, to provide evidence of disease causality.

2. General Materials and Methods

Detailed Materials and Methods are described within each Chapter respectively, with general methods utilised across all results Chapters described within the following section.

2.1 Ethical approval

Prior to the beginning of this study, ethical approval was sourced from the local ethics committees of the Royal Victoria Eye and Ear Hospital (Dublin, Ireland), the Mater Misericordiae University and Mater Private Hospital (Dublin, Ireland), The Belfast Health and Social Care Trust Hospital (Belfast, Northern Ireland), Trinity College Dublin (Dublin, Ireland) and the Radboud University Medical Center (Nijmegen, The Netherlands). This has allowed for patients from both the Republic of Ireland and Northern Ireland to be included in the Target 5000 study. In agreement with the tenets of the Declaration of Helsinki, informed consent was obtained by all patients before participating in the Target 5000 study.

2.2 Clinical Assessment

Once informed consent was given by each patient to participate in the study, a combination of tests were run depending on the suspected phenotype. This cohort of tests has been described in previous studies, but will briefly be outlined here for reference (Dockery *et al.*, 2017; KAJ *et al.*, 2021a). To assess best corrected visual acuity, the revised 2000 Early Treatment Diabetic Retinopathy Study (ETDRS) charts were utilised (Precision Vision, IL, USA). Colour vision defects were analysed under standardised lighting conditions, using the Lanthony desaturated D-15 panel (Gulden Ophthalmics, PA, USA). Visual fields were assessed using Goldman perimetry, where the IV4e, I4e and 04e targets were measured (Haag-Streit AG, Switzerland). Multimodal retinal imaging was taken for each patient where possible, with Fundus colour and autofluorescence photos being taken using either a Topcon CRC50DX (Topcon Great Britain Ltd., England) or Optos Daytona (Optos plc, Scotland). Optical coherence tomography readings and images were obtained using a Cirrus HD-OCT (Carl Zeiss Meditec, Germany). Full-field electroretinograms were also performed where possible, and were completed using a Roland Consult RETI-port retiscan (Brandenburg an der Havel, Germany). While some of the patient sites mentioned above may have updated or changed their equipment, the standard of these tests and the thresholds used remains the same. Along with these comprehensive tests which allow for deep-phenotyping, a thorough family and disease history is taken to inform the clinical diagnosis, inheritance pattern and disease progression.

2.3 Patient Cohort and DNA procurement

Patients included in this study have all received a clinical diagnosis from ophthalmologists in either the Royal Victoria Eye and Ear Hospital, the Mater Misericordiae University Hospital, or the Belfast Health and Social Care Trust Royal Victoria Hospital as described above. There was a particular focus placed on patients diagnosed with iMDs for the work presented in this thesis, but a few of the case studies presented were completed as part of Target 5000 where other IRD phenotypes have been included. Specific phenotypes for each cohort are described in the Materials and Methods sections for each respective chapter. Once it was determined that the patient presents with an IRD, and informed consent was obtained, DNA samples were obtained from patients and family members at one of the affiliated hospital sites nationwide. DNA samples are taken in the form of blood or saliva, depending on what is appropriate and comfortable for each patient. A 5ml sample of blood would be taken, or alternatively a 3ml sample of saliva. BD Vacutainer collection tubes were used to collect blood samples, and saliva was collected in DNA Genotek ORAcollect DNA tubes, with both types of samples being stored at -20°C.

2.4 DNA isolation

Initially, the anonymised blood or saliva samples were sent to a Trinity College Dublin affiliated laboratory in St. James's Hospital (Dublin, Ireland) in order to isolate an appropriate concentration of DNA. The Qiagen/Gentra "Salting out method" was followed to manually extract DNA, using the Qiagen Gentra Autopure LS Automated DNA Purifier. Ideally, 2ml of blood/saliva was sent for each sample. Once isolated, the resulting genomic DNA concentrations were quantified on a Qubit Fluorometer using both the Qubit dsDNA BR and HS assay kits (Thermo Fisher, US), according to the manufacturer protocol. The above DNA isolation method was used for all samples sent for a form of NGS as part of this thesis. An alternative method, the Qiagen DNA Blood Maxi Kit (Hilden, Germany), was used for the purpose of isolating patient DNA from blood for PCR amplification and Sanger sequencing confirmation if there was no isolate leftover from the initial run, as well as for isolating DNA from family members for cascade analyses. This kit was used as per the manufacturer's protocol.

2.5 Next Generation Sequencing

Patients participating in the Target 5000 project have undergone various forms of NGS sequencing, with many of the patients in this study having undergone multiple forms of pre-screening. Initially, all patients recruited to Target 5000 underwent Target capture sequencing (TCS) of the exonic and choice intronic regions of a panel of 254 IRD genes, as

previously described in detail (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020b). A choice few individuals with Stargardt 1 clinical diagnoses underwent smMIPs sequencing of the whole ABCA4 gene (Khan *et al.*, 2020). Whole Exome and Whole Genome sequencing were performed on some unresolved cases at BGI-Europe (Copenhagen, Denmark) and BGI (Hong Kong, special administrative region of China) respectively, as described previously (Fadaie *et al.*, 2021). As part of this study, to focus on the unresolved cases of iMD in an Irish cohort, 223 patients were sent for smMIPs sequencing using a panel of macular-associated genes (Hitti-Malin *et al.*, 2022). This panel and sequencing type will be described in more detail in the Materials and Methods section and main results body of Chapter 3.

3. Novel findings from a smMIPs-based sequencing study of 223 individuals from an Irish cohort with inherited macular degeneration

3.1 Introduction

High-throughput NGS technologies are now being used extensively to interrogate the genetic causes of both monogenic and multifactorial diseases, as has been outlined in the General Introduction (Section 1.4). Indeed, NGS has been employed extensively to elucidate the genetic pathogenesis of monogenetic and multifactorial IRDs. NGS allows sequencing of millions of DNA fragments in parallel, many times over, while also being a fast and accurate system of sequencing. While WGS and WES provide sequence of larger genomic regions, it is often the case that clinicians and research labs use custom designed targeted panel sequencing as a form of first tier sequencing for patients who have not received a genetic diagnosis. Studies often tend to focus on exonic regions of genes when looking for pathogenic variants, however this approach means that frequently many patients remain unresolved as much of the genome remains unexplored. It has been previously reported that around two thirds of IRD cases can be resolved by variants occurring in coding regions (Duncan *et al.*, 2018a; Perea-Romero *et al.*, 2021a; Zeuli *et al.*, 2024b). This ‘solve rate’ highlights the importance of sequencing both the coding, non-coding and regulatory regions of IRD genes, with the potential of solving some of these elusive unresolved cases.

Target or panel-based sequencing approaches have been seen in previous studies to represent an effective preliminary sequencing strategy, as such panels can be customised to cover specific exonic, intronic and regulatory regions of genes of interest. An example of a cost-effective sequencing technology rising in popularity is termed single molecule molecular inversion probe (smMIPs) based sequencing (**Figure 3.1**). This method involves multiplex target capture sequencing, in which libraries of sequences are pooled and sequenced simultaneously. This results in an efficient workflow, and allows for faster sequencing times with hundreds of samples being analysed in a single run. Multiplexing has proven to be useful when sequencing targeted or smaller genomic regions. This, along with cost-efficiency, are the reasons why smMIPs have been chosen as an excellent first tier sequencing methodology when analysing unresolved iMD cases in this study (Hiatt *et al.*, 2013; Hitti-Malin *et al.*, 2022). Indeed, smMIPs have been used successfully to provide a genetic diagnosis for IRD patients in previous studies, specifically for *ABCA4*-associated Stargardt disease (Khan *et al.*, 2019, 2020; Corradi *et al.*, 2023). Furthermore, MIPs have

been used to study autism disorders (O’Roak *et al.*, 2012), Parkinson’s disease (Shu *et al.*, 2019) and neurodevelopmental disorders (Stessman *et al.*, 2017).

Of note, smMIPs were designed with a number of ‘key characteristics’ in mind including; improving diagnostic yield with minimal error rates, targeting multiple specific sequences, simultaneous sequencing of many samples and a manageable cost-effective workflow. The smMIPs design concept is a combination of MIP target capture that has been previously described, and a single molecular tagging system (Hiatt *et al.*, 2013). Each smMIP, according to Molecular Loop Biosciences Inc’s design criteria, targets a specific 225 nucleotide capture region of sequence (**Figure 3.1**). This capture region is flanked on either side by probe arms. In addition, there are 5 nucleotide randomers attached to either probe arm enabling the unique barcoding system that differentiates smMIP molecules from each other, and the detection of both unique and duplicate reads. Furthermore, dual index primers are added to act as the patient barcoding system to allow the creation of uniquely tagged libraries for each patient.

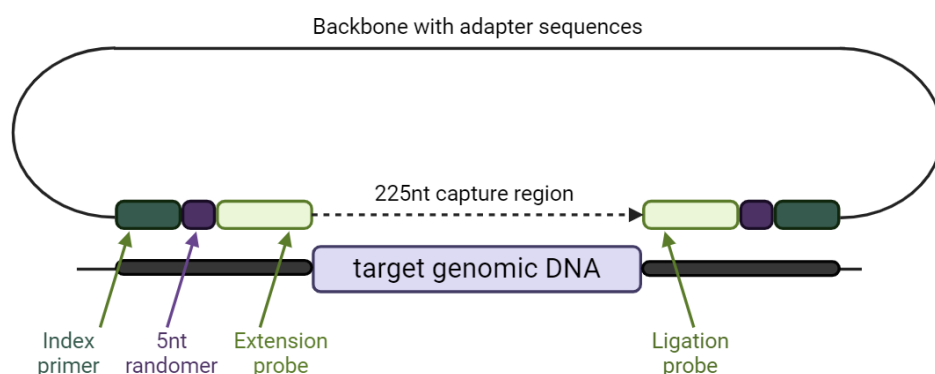


Figure 3.1. Molecular Loop Biosciences Inc. smMIPs design schematic. A smMIP is a single-stranded sequence of genomic DNA, that includes a 225 nucleotide (nt) capture region, flanked by; probe arms, 5nt randomers and index primers. The dual index primers are then linked together by a single loop backbone that contains adapter sequences and other motifs needed for sequencing (Image created using BioRender.com, adapted from (Hiatt *et al.*, 2013; Hitti-Malin *et al.*, 2022)).

In this study, smMIPs based sequencing has been utilised to sequence a cohort of 223 iMD patients in Ireland in an effort to demonstrate the effectiveness of targeted sequencing as a preliminary form of sequencing methodology, as well as to elucidate the genetic architecture of iMDs in Ireland. The study is focused on analysing the solve rate achieved when sequencing predominantly coding regions and choice intronic regions, where known pathogenic IRD-associated variants have previously been identified. The MD-smMIPs panel utilised in the following study was created by Hitti-Malen and colleagues, with this project being undertaken in collaboration with their team (Hitti-Malin *et al.*, 2022). 17,394 smMIPs

were designed for this panel to cover exons and selected intronic regions of 105 iMD and AMD-associated genes, as well as some deep-intronic iMD and AMD risk factor single-nucleotide polymorphisms (SNPs) (**Supplemental 3.8** provides a list of the 105 iMD genes and loci evaluated in this study). This previously tested MD-smMIPs panel is able to detect single nucleotide variants (SNVs), small insertions and deletions (indels) and copy number variation (CNVs). Previous studies using this MD-smMIPs panel, with on average eight smMIPs covering each nucleotide distributed across both DNA strands, have achieved average nucleotide coverages of ~431x (Hitti-Malin et al., 2022) and ~680x (Hitti-Malin et al., 2024) respectively. This is comparable to other smMIPs studies, such as the aforementioned whole *ABCA4*-smMIPs study, where an average nucleotide coverage of ~700x was achieved, with most nucleotides being covered by two smMIPs (Khan et al., 2020).

The primary objective of the work undertaken in this study was focused on employing smMIPs based-methods to sequence 223 patients with an iMD or CCRD clinical diagnosis in an Irish IRD cohort. We aimed to highlight candidate disease causing variants in this patient cohort to advance our knowledge of the landscape of iMDs in Ireland. Furthermore, for all candidate variants, additional studies were performed where possible, *inter alia*, cascade analyses in additional family members to phase variants, interrogate segregation and inform on the pathogenicity of the variant. Once research grade results resulting from this study are verified by a clinically accredited lab and a multidisciplinary team (MDT) meeting held, an in-depth genetic diagnosis may be provided to patients by clinicians (Conway et al., 2024a). The results to date are presented below. Candidate variants believed to be causative of disease have been identified in approximately 33% of cases in this iMD patient cohort of 223 individuals. Notably, 9 novel variants identified within the genetically resolved patient cohort are highlighted, as well as the additional work undertaken to allow classification of these variants. Case studies of particular interest identified during this iMD sequencing project are also described herein.

3.2 Materials and Methods

3.2.1 Patient cohort

Patients that were included in this particular study all received a clinical diagnosis from senior referring ophthalmologists in either the Royal Victoria Eye and Ear Hospital (RVEEH), or the Mater Misericordiae University Hospital based on the patient's medical history, family history and detailed ophthalmological exams as described in General Materials and Methods Section 2.2. Patient inclusion criteria focused on iMD patients and patients with a predominant cone-led phenotype. All probands chosen for this study had received a clinical diagnosis within one of the following categories; Stargardt Disease (STGD1) or Stargardt-like phenotype, an MD diagnosis with central vision defects, a cone or cone-rod dystrophy, or an opsin-associated phenotype with colour vision defects or Achromatopsia (**Figure 3.2**)(Hitti-Malin et al., 2022). Patients with mild extraocular symptoms were included only when these features occurred as part of a recognized IRD syndrome covered by the iMD panel, and if the macular phenotype represented the predominant or initial clinical manifestation. Probands recruited for this study were typically above the age of 18 at recruitment, as was set out by the parameters of the broader Target 5000 project. Additionally, the age of onset threshold set for this study was ≤ 55 years of age, to allow for a more inclusive sequencing study while taking into account the potential for late-onset iMD phenotypes where a strong family history is noted for the proband. A number of patients in this cohort had undergone various forms of pre-screening (see below and also **Supplemental 3.7**). Blood or saliva samples were obtained from patients and family members, following informed consent, in one of the affiliated hospital sites nationwide following the methods outlined in the General Materials and Methods section 2.3 of this thesis.

3.2.2 Pre-screening methods

Patients participating in the Target 5000 project often undergo multiple forms of NGS sequencing, with a few of the patients discussed in this Chapter having undergone pre-screening prior to this MD-specific smMIPs study (**Supplemental 3.7**). 41 of the patients have previously undergone TCS of the exonic and choice intronic regions of a panel of 254 IRD genes (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020b). 4 individuals with Stargardt 1 clinical diagnoses underwent smMIPs sequencing of the whole ABCA4 gene (Khan *et al.*, 2020). WES was performed on 5 cases by BGI-Europe (Copenhagen, Denmark) as described previously (Fadaie et al., 2021). The patients mentioned remained unresolved after these prior forms of sequencing, and were included in this study as the panel

utilised covers regions of the genome not previously analysed and thought to be specific to their respective phenotypes (**Supplemental Table 3.8**).

3.2.3 smMIPs design

Each smMIP is a single-stranded DNA-oligonucleotide that targets a specific 225 nucleotide capture region of sequence (Molecular Loop Biosciences Inc.) (**Figure 3.1**). The capture region is flanked on either side by extension and ligation probes 5' and 3', respectively. In addition, 5 nucleotide randomers are attached to either probe arm allowing for the unique barcoding system that differentiates the smMIP molecules from each other and also detects both unique and duplicate reads. Furthermore, dual index primers are added on to act as the patient barcoding system, allowing for the creation of uniquely tagged libraries for each patient. A panel of 105 genes and risk factor loci for iMD and AMD were compiled. 17,394 smMIPs were designed to tile over the coding regions of these genes as well as the 5'UTRs, known deep-intronic variants and pseudoexons, some select non-coding and regulatory regions where known causative variants have been previously reported, and the whole mitochondrial genome. The MD-smMIPs panel used in this study is described in detail by Hitti-Malin *et al.* in a recent paper on cost-effective sequencing of MD patients (Hitti-Malin *et al.*, 2022), along with a comprehensive list of all loci included in the capture panel (adapted in **Supplemental Table 3.8**). The research described in this section of the thesis was undertaken in collaboration with the Cremers research group that designed this panel; approximately 6 weeks of my doctoral studies were spent in RadboudUMC completing library preparations, carrying out quality controls, learning methodologies, and beginning the process of sequence analysis and variant calling/annotation, along with variant prioritisation and classification (see sections below) (RadboudUMC, Nijmegen, Netherlands).

3.2.4 Sample preparations

DNA concentrations of samples were measured using the Qubit dsDNA BR and HS assay kits (Thermo Fisher, US), according to manufacturer's protocol. Subsequently the DNA was diluted to a concentration of 16.7ng/μL as required by the Molecular Loop Biosciences Inc. protocol version 2.4.1H. 100ng of each patient DNA was loaded onto a 0.8% agarose gel for electrophoresis, along with three different quantifications (25, 50 and 100ng) of uncut lambda DNA for quality control and concentration comparisons. Once compared to the lambda DNA, each patient DNA was labelled as High or Low Molecular weight (MW). Samples were considered as high MW when the fragment was ≥ 23 kilobases (kb) and low when ≤ 23 kb or it appeared as a smear on the gel. DNA samples with high MW were plated

onto a 96-well plate and were incubated at 92°C for 5 min before library preparation, as a form of pre-treatment to denature the DNA fully. Samples with low MW skipped this treatment and progressed straight to library preparation.

3.2.5 Library preparations and sequencing

DNA library preparations and sequencing were carried out in a manner previously described by Hitti-Malin and colleagues (Hitti-Malin et al., 2022). To briefly summarise, DNA libraries for each patient sample were prepped using the High Input DNA Capture Kit, Chemistry 2.3.0H Protocol version 2.4.1H (Molecular Loop Biosciences Inc.), with an incubation time of 18 hours to facilitate smMIP hybridization. Following this, a fill in step was performed and then a clean-up PCR was undertaken. 5µL of each sample library was run on a quality control agarose gel, to confirm the amplifications were all around 400bps in size. After samples were pooled, library purification was completed through bead purification and quantified using a Qubit Fluorometer (dsDNA HS assay kit; ThermoFisher Scientific) and a 2200 TapeStation system (HS D1000 DNA kit; Agilent Technologies) for library concentration and size. A number of library pools were combined in equimolar amounts, to create a 100 µL mega pool of 1.5 nmol (nM). The mega pool was denatured in accordance with Illumina's NovaSeq 6000 System Denature and Dilute Libraries Guide, resulting in 300 pM final libraries. Each library was sequenced by paired-end sequencing on a NovaSeq 6000 Illumina platform.

3.2.6 Variant calling and annotation

For each sequencing run, the resulting sequencing reads were converted into FASTQ raw sequencing data files. A bioinformatics pipeline designed by the team in RadboudUMC, Nijmegen was used to process the raw FASTQ files. This pipeline was described in previous publications in further detail (Khan et al., 2019; Hitti-Malin et al., 2022; Panneman et al., 2023). The random molecular identifiers were trimmed from all sequencing reads and saved with the read identifier to use later on. Paired-end sequencing reads were mapped directly onto the hg19 Human reference genome (GRCh37). Following this, duplicated reads were removed. The ligation and extension arms were trimmed away, along with any overlap between read-pairs. The reads were then grouped on a patient barcode basis and a binary alignment map (BAM) file was generated for each patient. The resulting BAM files were used to visualise variants once they were called, using Integrative Genome Viewer (IGV) version 2.4.

3.2.7 Copy Number Variant (CNV) analysis

The first step of variant analysis for all patient samples was to assess CNVs to detect large deletions, insertions or duplications. This was undertaken using a previously described Excel script method, in which the coverage per smMIP is compared to the normalised coverage across all samples (Khan et al., 2020). The standard used in this analysis is as follows; if six consecutive smMIPs have a normalized coverage across all samples in a sequencing run of ≤ 0.65 , this indicates a suspected deletion. Conversely, if six consecutive smMIPs with a normalized coverage ≥ 1.20 are present, this indicates a suspected duplication.

3.2.8 Variant prioritisation

Variant prioritisation was initially undertaken as part of a collaborative sequencing project with the Cremers group in RadboudUMC, Nijmegen, utilising the same data analysis pipeline for continuity. The pipeline used is described in detail, including cut-offs/thresholds, by Hitti-Malin and colleagues (Hitti-Malin et al., 2022). To summarise, once CNV analysis was completed, we progressed to evaluating single nucleotide variants (SNVs) and indels. The first step was to extract variants in *ABCA4* that have been previously reported in the *ABCA4* Leiden Open (source) Variation Database (LOVD) from the sample data (LOVD; <https://databases.lovd.nl/shared/genes/ABCA4>). These variants included both common pathogenic *ABCA4* variants, and deep-intronic variants (DIVs) that the smMIPs were specifically designed to target based on previous publications (Khan *et al.*, 2020). Next, we looked at homozygous and heterozygous SNVs and indels with a minor allele frequency of $\leq 0.5\%$, and at heterozygous variants in genes associated with autosomal dominant phenotypes with a minor allele frequency of $\leq 0.1\%$. These frequencies were obtained from online databases such as the Genome Aggregation Databases (gnomAD) (Karczewski *et al.*, 2020). Subsequently, variants present in more than 10 unique sequencing reads ($>10\times$ coverage) were the only variants assessed, with variants appearing in $\geq 10\%$ of samples within a given run also being excluded to remove potential sequencing artefacts or errors. Variants with allele frequency variation between 35% and 80% were assumed heterozygous, and when multiple variants were seen in any one specific gene this was assumed compound heterozygous. Variants with allele frequency variation greater than or equal to 80% were considered homozygous variants. Each candidate variant was visualised manually in patient BAM files using IGV version 2.4 to exclude any potential sequencing artefacts from the later interpretation analyses.

All of the remaining variants that have been filtered through the above criteria were then prioritised based on their predicted protein effects and other *in silico* pathogenicity

predictions. The pathogenicity of missense variants was analysed based on the following *in silico* prediction tool thresholds; PhyloP ≥ 2.7 (Pollard et al., 2010), Grantham score ≥ 80 (Grantham, 1974) and CADD PHRED ≥ 15 (Kircher et al., 2014). Variants that exceed all three of these thresholds were prioritised over variants that only met fewer than three of them. For noncanonical splice site variants and DIVs, *in silico* tools were used within the Alamut Visual software version 2.13 (Interactive Biosoftware) to predict the impact on splicing (Fadaie *et al.*, 2019a). The SpliceAI online tool was utilised, through the BROAD Institute web interface (<https://spliceailookup.broadinstitute.org/#>) to predict splicing effects by providing a delta score for 4 different splice site predictions (acceptor loss, acceptor gain, donor loss and donor gain). Variants with a predicted SpliceAI score of ≥ 0.2 for at least one of the effects were considered as potential splice-altering variants.

3.2.9 Variant classification

Preliminary ACMG classifications were obtained for each candidate variant from the Franklin Genoox platform (<https://franklin.genoox.com>, Version 84). Novel candidate variants genetically resolving a patient that were identified during the prioritisation step have been reclassified in-house according to the ACMG/AMP classification guidelines outlined by Richards et al. (Richards et al., 2015). Recommendations and updates from the Association for Clinical Genomic Science (ACGS) and ClinGen Sequence Variant Interpretation (SVI) group have also been adopted into the classification pipeline (Rehm *et al.*, 2015; Durkie *et al.*, 2024). Variant classifications utilised phasing analysis data (where applicable), as well as data from previously published or newly generated *in silico* pathogenicity predictors; MetaLR (Dong *et al.*, 2015), M-CAP (Jagadeesh *et al.*, 2016) and REVEL (Ioannidis *et al.*, 2016). The thresholds for MetaLR and M-CAP are 0.5, 0.025 respectively, with updated recommendations for the REVEL score cutoff being applied (Pejaver *et al.*, 2022). The AlphaMissense *in silico* prediction tool has been incorporated into the Trinity in-house pipeline, using cutoff thresholds recommended by (Kurtovic-Kozaric *et al.*, 2024; Nguyen *et al.*, 2024; Zhuo, Aggarwal and Liao, 2024). Only variants with a preliminary Franklin classification of variant of unknown significance (VUS), likely pathogenic or pathogenic were considered further as candidate variants, these are known as class 3, 4 and 5 variants respectively (Richards et al., 2015). Benign (class 1) and likely benign (class 2) variants are not considered. All candidate variants were subsequently investigated in online public databases such as gnomAD (v2.1.1 for GRCh37) (Karczewski *et al.*, 2020), LOVD.nl (Fokkema *et al.*, 2021), ClinVar (Landrum *et al.*, 2014) and VarSome (Kopanos *et al.*, 2019). Where applicable, novel variants were annotated according to the

Human Genome Variation Society (HGVS) nomenclature guidelines (based on GRCh37 hg19 genomic coordinates).

Where it was not possible for family studies to be completed, patients could only be classified as “very likely solved”, “possibly solved”, or “unsolved” as phasing data would be required to fully complete the ACMG/ACGS classification analysis. These ‘diagnostic statuses’ were assigned to patients based on the variant criteria for the MD-Panel, outlined here (Hitti-Malin et al., 2022). These statuses were used for all patients throughout this report, regardless of whether phasing data was available, for consistency and clarity.

3.2.10 Confirmational Sanger sequencing and phasing analysis

Sanger sequencing was performed on each ‘genetically resolved’ patient’s DNA sample to confirm the presence of the candidate variants identified. Where family members have consented to participate in the study, Sanger sequencing was performed to determine phasing. Sanger sequencing was carried out through Eurofins Genomics Tubeseq service (Heidelberg, Germany). The final volumes of samples and primers were sent according to Eurofins desired guidelines, with the upper limit of PCR product concentration being sent when possible. Once variants are confirmed through Sanger sequencing, pedigree trees are created for the available families, in order to make phasing calls for each variant.

3.2.11 CDH3 deletion breakpoint identification

As part of this study, a large deletion of 2 exons was observed in the *CDH3* gene homozygously in one patient (CI68). BAM files for patient CI68 were visualised using IGV version 2.4 to confirm the absence of reads across exons 12 and 13 of *CDH3* (**Figure 3.9(a,b)**). Primers were designed to probe the intronic regions surrounding these 2 exons on *CDH3* in order to determine the breakpoints of the deletion, allowing for accurate classification and annotation of the variant. Primer pairs were chosen within exons 11 and 14 based on the previous identification of the c.(1570+1_1571-1)_(2002+1_2003-1)del *CDH3* deletion (Halford et al., 2012; Karti et al., 2017; Ellingford et al., 2018). Similarly, multiple primer pairs were also chosen flanking the deletion to ensure no structural mutations such as inversions are flanking the deletion. Primers were also designed closer to the breakpoints once they were identified in order to confirm the wildtype sequence is present only in the carrier mother and control DNA and not in the affected patients within the family. The primers utilised can be found in **Supplemental 3.12**.

3.2.12 Variant confirmation in pedigree with *OPN1LW* variant

Due to the high level of amino acid sequence homology between the LW and MW cone opsin genes, primers that bind preferentially in one or the other gene were designed as proof-of-concept. Benchling (Biology software; <https://benchling.com>, accessed 2025) was used to align the *OPN1LW* gene with the green cone opsin genes (*OPN1-MW*, *-MW2*, *-MW3*). Primer pairs were designed in regions where the *OPN1LW* and green opsin genes differed in multiple bases, in order to promote the preferential binding of the primers. This was undertaken to demonstrate that the variant identified in patient CJ17 was present in the *OPN1LW* gene in this pedigree and not in any of the green opsin genes, and in an effort to visualise whether a hybrid gene may have occurred in this pedigree, to the best of our ability. The primers utilised here can be found in **Supplemental 3.13**.

3.3 Results

A total of 223 unresolved Irish probands with a CCRD or iMD-associated clinical diagnosis have been sequenced as part of this study. Once smMIPs based sequencing of these probands was completed, along with the positive controls included in each run, as outlined in the original proof-of-concept paper (Hitti-Malin *et al.*, 2022), SNV and CNV analysis were undertaken to prioritise candidate variants for each patient as described in the Materials and Methods section 3.2.6-3.2.8 of this Chapter. All of the previously known variants were successfully identified for each positive control sample. While DNA samples from 223 people have been sequenced as part of this study, 7 of these patients remained unresolved after smMIPs sequencing, and upon reanalysis by affiliated clinicians were deemed to not have an IRD and their ocular conditions were attributed to other causes. Therefore, the results presented herein are from the remaining 216 patients (**Figure 3.2**). This MD-smMIPs targeted sequencing method enabled a diagnostic yield of ~33% for this iMD cohort, where 57 patients were given a ‘Very Likely solved’ status and 15 a ‘Possibly solved’ status, depending on the combination of variants identified (**Figure 3.3**). Genetically solved patients are listed along with their identified variants in **Table 3.1**. Two of the samples failed two rounds of smMIPs sequencing due to poor DNA quality or insufficient concentrations obtained from blood samples. New samples must be obtained and DNA reisolated from these patients where possible in the future. It is of note, that this particular iMD cohort did not include proportionally as many STGD1/*ABCA4* patients as would be expected as the majority of these patients from Target 5000 were analysed in a prior study on *ABCA4*-associated disease (Khan *et al.*, 2020) (**Figure 3.2**). Therefore, the actual solve rate is estimated to be higher if all iMD cases, including these prior STGD1/*ABCA4* patients were included, as will be discussed in the General Discussion section 6. of this PhD thesis.

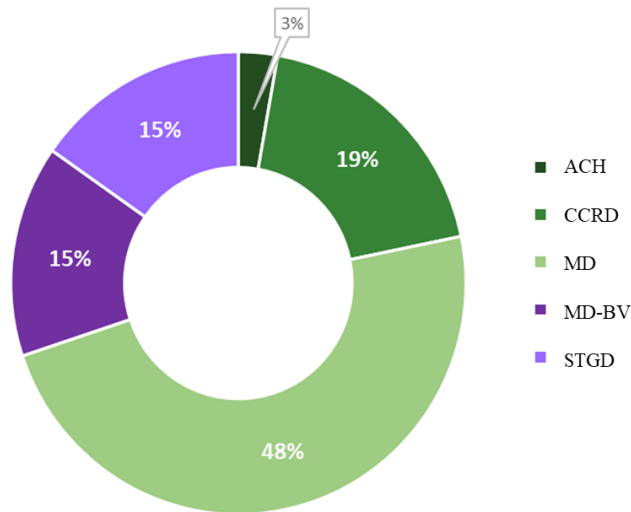


Figure 3.2. Phenotypic breakdown of 216 patients sequenced using the Macular smMIPs panel approach. This breakdown utilises the initial clinical diagnosis that the patient received prior to this study. Patient inclusion criteria are based on macular involvement or central vision defects, including cone/cone-rod dystrophies. (ACH; Achromatopsia, CCRD; Cone/Cone-Rod dystrophy, MD; macular degeneration, MD-BV; Best Vitelliformis, STGD1; Stargardt disease).

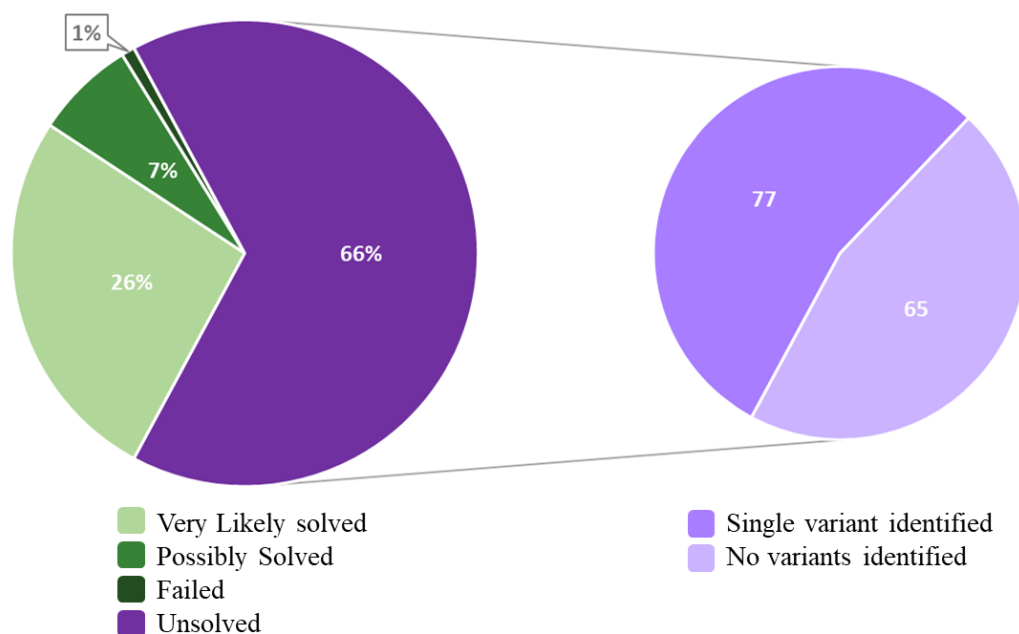


Figure 3.3. Overall diagnostic yield, including breakdown of unresolved cohort. The first chart depicts the percentage of people thought to be genetically resolved by the MD-smMIPs panel broken down into 'diagnostic status'. Note: 1% of the cohort (2 patients) are labelled as 'Failed', as multiple rounds of sequencing were not successful. The second chart highlights the unresolved cohort, showing the proportion of patients not solved by the MD-smMIPs panel where either single/monoallelic variants were identified or no candidate variants were identified.

Table 3.1 Probands considered genetically resolved (Records divided into 2 tables below, 1/2)

Patient barcode	Submitted Phenotype	Gender	Status	Gene	Allele 1 cDNA	Allele 1 protein	Franklin ACMG (Allele 1)	Allele 2 cDNA	Allele 2 protein	Franklin ACMG (Allele 2)	Proposed Inheritance
1462	MD-BV	M	Very Likely Solved	ABCA4	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	Pathogenic	c.[4222T>C;4918C>T]	p.[Trp1408Arg; p.Arg1640Trp]	Pathogenic	AR
CJ13	MD-BV	F	Very Likely	BEST1	c.73C>T	p.Arg25Trp	Pathogenic	+	+		AD
CC33	MD-BV	F	Very Likely	BEST1	c.886A>C	p.Asn296His	Pathogenic	+	+		AD
CI51	MD-BV	M	Very Likely	BEST1	c.652C>T	p.Arg218Cys	Pathogenic	+	+		AD
CH13	MD-BV	F	Possibly	CACNA1F	c.3146G>T	p.Arg1049Leu	Likely	+	+		XL
CC31	MD-BV	M	Possibly solved	BEST1	c.203A>G	p.Tyr68Cys	VUS/Likely pathogenic	+	+		AD
CI31	MD-BV	F	Very Likely	BEST1	c.886A>C	p.Asn296His	Pathogenic	+	+		AD
CF78	MD-BV	F	Possibly solved	BEST1	c.203A>G	p.Tyr68Cys	VUS/Likely pathogenic	+	+		AD
CA48	MD-BV	M	Very Likely	BEST1	c.652C>T	p.Arg218Cys	Pathogenic	+	+		AD
CI4	MD-BV	F	Very Likely	ABCA4	c.4540-2A>G	p.?	Pathogenic	c.5882G>A	p.Gly1961Glu	Pathogenic	AR
CC5	MD-BV	F	Very Likely	BEST1	c.103G>A	p.Glu35Lys	Pathogenic	+	+		AD
R68	MD-BV	M	Very Likely	BEST1	c.652C>T	p.Arg218Cys	Pathogenic	+	+		AD
CJ25	MD-BV	M	Very Likely	BEST1	c.294G>T	p.Glu98Asp	Likely	+	+		AD
CC78	CCRD	M	Very Likely	OPN1LW	c.283del	p.Leu95TrpfsTer6	Likely	Y	Y		XL
CH67	CCRD	M	Very Likely	CFAP410	c.218G>C	p.Arg73Pro	Pathogenic	c.460_469del	p.Gly154TyrfTer	Likely	AR
CJ17	CCRD	M	Very Likely	OPN1LW	c.607T>C	p.Cys203Arg	Likely	Y	Y		XL
CG80	CCRD	F	Very Likely	KCNV2	c.1381G>A	p.Gly461Arg	Pathogenic	c.1381G>A	p.Gly461Arg	Pathogenic	AR
R25	CCRD	F	Very Likely Solved	CFAP410	c.33_34insAGCTGCACAGCGTGCA	p.Ala12SerfsTer60	Pathogenic	c.355C>T	p.Gln119Ter	Pathogenic	AR
CI64	CCRD	F	Very Likely	CRB1	c.2129A>T	p.Glu710Val	Pathogenic	c.2129A>T	p.Glu710Val	Pathogenic	AR
CI26	MD	F	Very Likely	BEST1	c.73C>T	p.Arg25Trp	Pathogenic	+	+		AD
G59	MD	F	Possibly	PROM1	c.1339C>G	p.Leu447Val	VUS	+	+		AD
H72	MD	F	Possibly	KCNV2	c.257C>G	p.Pro86Arg	VUS	c.1148G>T	p.Arg383Leu	VUS	AR
1666	MD	NN	Very Likely	PRPH2	c.457A>G	p.Lys153Glu	Pathogenic	+	+		AD
1688	MD	M	Very Likely Solved	ABCA4	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	Pathogenic	c.4139C>T	p.Pro1380Leu	Pathogenic	AR
CG48	MD	M	Very Likely Solved	DRAM2	c.700_706del	p.(Ser234Glyfs*9)	Likely pathogenic	c.700_706del	p.(Ser234Glyfs*9)	Likely pathogenic	AR
CI79	MD	F	Very Likely Solved	ABCA4	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	Pathogenic (;) VUS	c.3322C>T	p.Arg1108Cys	Pathogenic	AR
CI68	MD	F	Very Likely Solved	CDH3	c.1570+229_2003-860del	p.(?)	Likely pathogenic	c.1570+229_2003-860del	p.(?)	Likely pathogenic	AR
CG42	MD	F	Very Likely	PROM1	c.1117C>T	p.Arg373Cys	Pathogenic	+	+		AD
CH14	MD	M	Very Likely	GUCA1A	c.296A>G	p.Tyr99Cys	Pathogenic	+	+		AD
CH71	MD	F	Possibly	RS1	c.327G>C	p.Gly109=	VUS	+	+		XL
CI56	MD	M	Possibly solved	EYS	c.2822G>T	p.Gly941Val	Likely pathogenic	c.4991C>T(;);c.6632C>T	p.Thr1664Ile (;) p.Ser2211Leu	VUS	AR
CF34	MD	M	Very Likely	CFAP410	c.218G>C	p.Arg73Pro	Pathogenic	c.218G>C	p.Arg73Pro	Pathogenic	AR
CI18	MD	F	Very Likely	RP1L1	c.133C>T	p.(Arg45Trp)	Likely	+	+		AD
CD45	MD	F	Very Likely	PRPH2	c.584G>A	p.Arg195Gln	Pathogenic	+	+		AD

The genotype for each patient is displayed, along with the Franklin ACMG classification. (Phenotypes: ACH; Achromatopsia, CCRD; Cone/Cone-Rod dystrophy, MD; macular degeneration, MD-BV; Best Vitelliformis, STGD1; Stargardt disease. Inheritance: AR; autosomal recessive, AD; autosomal dominant, XL; X-linked.)

Table 3.1 Probands considered genetically resolved (table continued from above, 2/2)

Patient barcode	Submitted Phenotype	Gender	Status	Gene	Allele 1 cDNA	Allele 1 protein	Franklin ACMG (Allele 1)	Allele 2 cDNA	Allele 2 protein	Franklin ACMG (Allele 2)	Proposed Inheritance
CJ31	STGD	M	Very Likely Solved	ABCA4	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	Likely pathogenic	c.735T>G	p.Tyr245*	Pathogenic	AR
CH50	STGD	F	Very Likely Solved	ABCA4	c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	Pathogenic	c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	Pathogenic	AR
CH32	STGD	F	Very Likely Solved	ABCA4	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	Pathogenic	c.4577C>T	p.Thr1526Met	Pathogenic	AR
CH42	STGD	F	Possibly	ABCA4	c.5882G>A	p.Gly1961Glu	Pathogenic	c.5882G>A	p.Gly1961Glu	Pathogenic	AR
CG77	STGD	F	Very Likely Possibly	ABCA4	c.1805G>A	p.Arg602Gln	Pathogenic	c.1805G>A	p.Arg602Gln	Pathogenic	AR
CH43	STGD	M	Very Likely solved	ABCA4	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	Pathogenic	c.5584+5del	p.?	VUS	AR
CH33	STGD	F	Very Likely	PRPH2	c.394del	p.Gln132LysfsTer7	Pathogenic	+	+		AD
CH30	STGD	F	Very Likely Solved	ABCA4	c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	Pathogenic	c.5882G>A	p.Gly1961Glu	Pathogenic	AR
CJ35	STGD	F	Very Likely Solved	ABCA4	c.3323G>A	p.Arg1108His	Pathogenic	c.[5461-10T>C;5603A>T]	p.[Thr1821Aspfs*6,Thr1821Valfs*6]	Likely pathogenic	AR
CJ47	STGD	F	Very Likely Solved	ABCA4	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	Pathogenic/VUS	c.4326C>A	p.Asn1442Lys	Pathogenic	AR
CH46	STGD	F	Very Likely	ABCA4	c.3323G>A	p.Arg1108His	Pathogenic	c.6229C>T	p.Arg2077Trp	Pathogenic	AR
CB72	STGD	F	Very Likely	NMNAT1	c.53A>G	p.Asn18Ser	Pathogenic	c.769G>A	p.Glu257Lys	Pathogenic	AR
CH35	MD-BV	F	Very Likely	BEST1	c.652C>T	p.Arg218Cys	Pathogenic	+	+		AD
CH63	MD-BV	F	Very Likely	PRPH2	c.903_906del	p.Ser301ArgfsTer2	Pathogenic	+	+		AD
CH28	MD-BV	M	Very Likely	ABCA4	c.3323G>A	p.Arg1108His	Pathogenic	c.3323G>A	p.Arg1108His	Pathogenic	AR
CG35	MD-BV	M	Very Likely	CNGA3	c.101+1G>A	p.?	Pathogenic	c.967G>C	p.Ala323Pro	Pathogenic	AR
CF76	MD-BV	M	Possibly	IMPG2	c.392G>A	p.Arg131His	VUS	+	+		AD
CA64	ACH	F	Very Likely	CNGB3	c.1148del	p.(Thr383Ilefs*13)	Pathogenic	c.1148del	p.(Thr383Ilefs*13)	Pathogenic	AR
CC21	ACH	M	Very Likely	CNGB3	c.1148del	p.Thr383IlefsTer13	Pathogenic	c.1148del	p.Thr383IlefsTer13	Pathogenic	AR
CJ60	STGD	F	Very Likely	ABCA4	c.1253T>C	p.Phe418Ser	Pathogenic	c.3056C>T	p.Thr1019Met	Pathogenic	AR
CJ56	CCRD	F	Very Likely	CRB1	c.2639A>G	p.(Asn880Ser)	Pathogenic	c.2639A>G	p.(Asn880Ser)	Pathogenic	AR
CJ75	MD	F	Possibly	RP11L	c.2107C>T	p.Arg703*	Pathogenic	+	+		AD
CJ78	STGD	M	Possibly solved	ABCA4	c.872C>T(;c.1531C>T)	p.Pro291Leu (;)	VUS/Pathogenic	c.3482G>A(;c.5882G>A)	p.Arg1161His (;)	Pathogenic	AR
CJ79	STGD	F	Very Likely Solved	ABCA4	c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	Pathogenic	c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	Pathogenic	AR
CK2	MD	F	Very Likely	BEST1	c.475C>T	p.(Gln159*)	Pathogenic	+	+		AD
CK6	STGD	F	Very Likely	ABCA4	c.5371dup	p.(Ala1791Glyfs*9)	Likely pathogenic	c.5882G>A	p.Gly1961Glu	Pathogenic	AR
CK13	STGD	F	Very Likely	RDH12	c.698T>A	p.Val233Asp	Pathogenic	c.869T>G	p.Val290Gly	VUS	AR
CK18	MD	F	Very Likely	BEST1	c.722C>A	p.(Thr181Asn)	Likely pathogenic	+	+		AD
CK68	MD	F	Very Likely	CRB1	c.2129A>T	p.Glu710Val	Pathogenic	c.3997G>A	p.Glu1333Lys	Likely	AR
CK38	MD	F	Very likely	TULP1	c.1496-6C>A	p.?	Pathogenic	c.1496-6C>A	p.?	Pathogenic	AR
CK40	MD	F	Very likely	PRPH2	c.903_906del	p.Ser301ArgfsTer2	Likely pathogenic	+	+		AD
CK41	MD	M	Possibly solved	ABCA4	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	Likely pathogenic	c.5603A>T	p.Asn1868Ile	VUS	AR
CK50	MD	F	Very likely	CDHR1	c.297+1G>A	p.?	Pathogenic	c.783G>A	p.Pro261=	Likely	AR
CK51	MD	F	Very likely	ABCA4	c.1805G>A	p.Arg602Gln	Likely pathogenic	c.3322C>T	p.Arg1108Cys	Likely	AR
CK56	ACH	M	Very likely	KCNV2	c.1354G>C	p.Ala452Pro	VUS	c.1381G>A	p.Gly461Arg	Likely	AR
CL2	MD	M	Very likely	CFH	c.1291T>A	p.Cys431Ser	Likely pathogenic	+	+		AD
O14	ACH	M	Possibly	ATF6	c.479A>G	p.Lys160Arg	VUS	c.950G>A	p.Arg317Gln	VUS	AR
P24	STGD	F	Possibly	PROM1	c.2436G>A	p.Ala812=	VUS	+	+		AD

In summary, 156 unique variants with predicted ACMG/AMP classifications of classes 3, 4 or 5 were identified in 43 genes associated with MD and CD/CRD (**Table 3.2, Supplemental 3.1**). In **Figure 3.4(a)** we observe that the proportion of VUS/class 3 variants called in the unresolved cohort is significantly higher, with 65/78 total VUS present in genetically unresolved individuals. Among the probands thought to be genetically ‘solved’, 77 variants were identified in 24 genes thought to be disease-causing (**Table 3.2**). 44 of the variants are predicted to be class 5 (pathogenic), 20 class 4 (likely pathogenic) and 13 class 3 (variant of unknown significance; VUS) (**Table 3.2, Figure 3.4(a)**). The majority of candidate variants identified within the ‘solved’ cohort appear to be missense variants, and only one CNV was identified via this analysis pipeline (**Figure 3.4(b)**). These preliminary ACMG/AMP classifications were obtained from the Franklin Genoox platform (*Franklin by Qiagen*, accessed May 2025). All of the variants identified that are thought to be causative of disease in this cohort were confirmed by PCR amplification and Sanger sequencing. One exception to this is the *CFAP410* c.33_34insAGCTGCACAGCGTGCA variant identified in patient R25, which we were unable to Sanger sequence due to the repetitive nature of this region of the gene. Where family members had consented to partake in the study, phasing data was obtained through segregation analyses. As this was not possible for some patients, we acknowledge that for these cases a phase of *in trans* was assigned for rare compound heterozygous variants, however further work-up is required to confirm this. For example, future studies using long reading sequencing should help to establish phase for some cases where additional family members are not available, as will be discussed later within this thesis chapter. Out of the 72 resolved cases, 25 patients had family members participating that allowed us to carry out segregation studies (**Supplemental 3.2**).

Of the 142 genetically unresolved cohort (**Supplemental 3.3**), 40 individuals were monoallelic for a class 3-5 variant in genes associated with autosomal recessively inherited MDs (**Supplemental 3.4**). There were no variants identified for 65 individuals, with 28 of these patients having had pre-screening prior to this study (**Supplementals 3.6 and 3.7**). For the rest of the unresolved individuals where a mono-allelic class 3-5 variant has been identified, the inheritance pattern is unclear and further assessment of these few patients and their relatives may provide further insight into these interesting cases (**Supplemental 3.4 and 3.5**). For 17 of the individuals from the unresolved cohort, a mono-allelic class 3-5 variant has been identified in more than one gene (**Supplemental Table 3.5**). Some of these variants are in genes that are associated with both an autosomal dominant and recessively inherited MD, however the location and nature of the variants suggest they would follow a

recessive inheritance pattern in the patients analysed based on previous reported findings, and so they remain genetically unresolved. Additional analysis and functional studies for such variants could elucidate more about the pathogenesis of mutations in such genes.

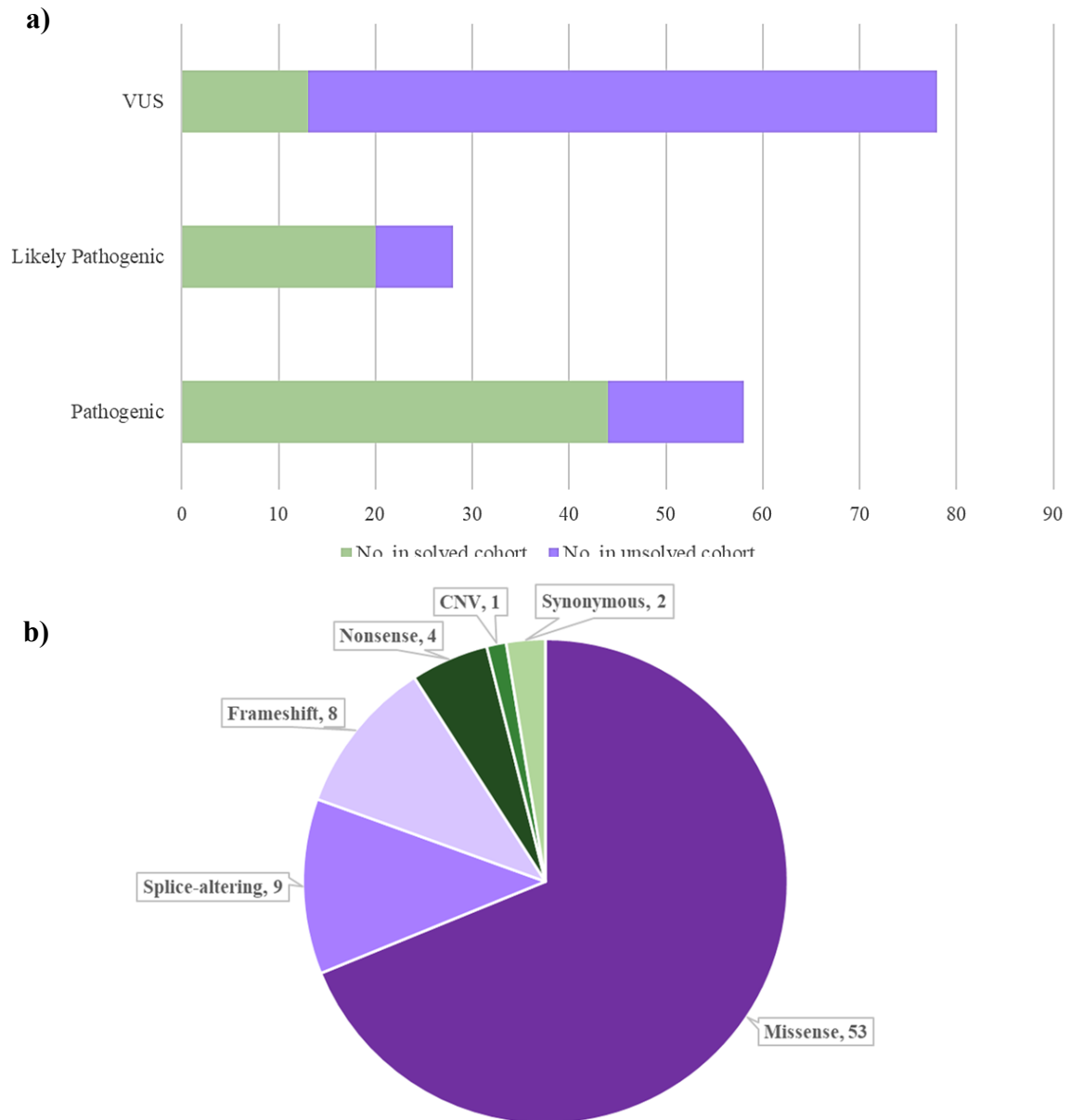


Figure 3.4. Outline of variants identified within the Macular cohort based on classification and variant type. (a) Distribution of variants based on their classification (class 3-5) that have been identified in both the solved and unsolved cohort. **(b)** Types of variants identified in the 72 genetically resolved patients. The number of variants for each category is indicated in the callouts.

Table 3.2. All candidate variants identified in the 72 patients considered genetically solved. Total gnomAD allele frequency is noted for each variant along with the ACMG/AMP classification as appears on Franklin and the allele count within this study.

Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALL AF	Franklin ACMG Classification	Allele count
ABCA4	c.[1622T>C;3113C>T]	1:g.[94528806A>G; 94508969G>A]	p.[Leu541Pro; Ala1038Val]	0.016265% (46) / 0.17546% (496)	Likely pathogenic	5
ABCA4	c.[2588G>C;5603A>T]	1:g.[94517254C>G; 94476467T>A]	p.[Gly863Ala; Gly863del; Asn1868Ile]	0.42951% (1214) / 4.2191% (11928)	Likely pathogenic	4
ABCA4	c.[4222T>C;4918C>T]	1:g.[94496583A>G; 94486896G>A]	p.[Trp1408Arg; Arg1640Trp]	0.0015913% (4) / 0.0031819% (8)	Likely pathogenic	1
ABCA4	c.[5461-10T>C;5603A>T]	1: g.[94476951A>G; 94476467T>A]	p.[Thr1821Aspfs*6; Thr1821Valfs*13]	0.021971% (62) / 4.2191% (11928)	Likely pathogenic	1
ABCA4	c.161G>A	1:g.94577135C>T	p.[Cys54Serfs*14; Cys54Tyr]	0.0035363% (10)	Pathogenic	4
ABCA4	c.735T>G	1:g.94564383A>C	p.Tyr245*	not in gnomad	Pathogenic	1
ABCA4	c.872C>T	1:g.94546261G>A	p.Pro291Leu	0.0582% (164)	Likely pathogenic	1
ABCA4	c.1253T>C	1:g.94544249A>G	p.Phe418Ser	0.001193% (3)	Likely pathogenic	1
ABCA4	c.1531C>T	1:g.94543269G>A	p.Arg511Cys	0.016296% (46)	Likely pathogenic	1
ABCA4	c.1805G>A	1:g.94528265C>T	p.Arg602Gln	0.0021257% (6)	Pathogenic	3
ABCA4	c.3056C>T	1:g.94509026G>A	p.Thr1019Met	0.0031844% (8)	Pathogenic	1
ABCA4	c.3322C>T	1:g.94508323G>A	p.Arg1108Cys	0.012733% (36)	Pathogenic	2
ABCA4	c.3323G>A	1:g.94508322C>T	p.Arg1108His	0.0015915% (4)	Pathogenic	4
ABCA4	c.3482G>A	1:g.94506805C>T	p.Arg1161His	0.0021214% (6)	Likely pathogenic	1
ABCA4	c.4139C>T	1:g.94496666G>A	p.Pro1380Leu	0.023363% (66)	Pathogenic	1
ABCA4	c.4326C>A	1:g.94496010G>T	p.Asn1442Lys	0.0017675% (5)	Likely pathogenic	1
ABCA4	c.4540-2A>G	1:g.94490606T>C	p.?	0.00039765% (1)	Pathogenic	1
ABCA4	c.4577C>T	1:g.94490567G>A	p.Thr1526Met	0.0063631% (18)	Pathogenic	1
ABCA4	c.5371dup	1:g.94480188dup	p.(Ala1791Glyfs*9)	not in gnomad?	Pathogenic	1
ABCA4	c.5584+5del	1:g.94476813del	p.?	not in gnomad	VUS	1
ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS	1
ABCA4	c.5882G>A	1:g.94473807C>T	p.Gly1961Glu	0.45643% (1291)	Pathogenic	4
ABCA4	c.6229C>T	1:g.94467467G>A	p.Arg2077Trp	0.00039772% (1)	Pathogenic	1
ATF6	c.479A>G	1:g.161761322A>G	p.Lys160Arg	not in gnomad??	VUS	1
ATF6	c.950G>A	1:g.161789463G>A	p.Arg317Gln	0.00081097% (2)	VUS	1
BEST1	c.73C>T	11:g.61719351C>T	p.Arg25Trp	0.00040216% (1)	Pathogenic	2
BEST1	c.103G>A	11:g.61719381G>A	p.Glu35Lys	not in gnomad?	Pathogenic	1
BEST1	c.203A>G	11:g.61722629A>G	p.Tyr68Cys	not in gnomad?	Likely pathogenic	2
BEST1	c.294G>T	11:g.61723236G>T	p.Glu98Asp	not in gnomad?	Pathogenic	1
BEST1	c.475C>T	11:g.61723417C>T	p.(Gln159*)	0.0013917% (2)	Pathogenic	1
BEST1	c.652C>T	11:g.61724874C>T	p.Arg218Cys	not in gnomad?	Pathogenic	4
BEST1	c.722C>A	11:g.61725625C>A	p.(Thr181Asn)	not in gnomad?	Pathogenic	1
BEST1	c.886A>C	11:g.61726988A>C	p.Asn296His	not in gnomad?	Pathogenic	2
CACNA1F	c.3146G>T	X:g.49072932C>A	p.Arg1049Leu	0.0011604% (2)	Likely pathogenic	1
CDH3	c.1570+229_2003-860del	16:g.68706158_68741622del	p.(?)	not in gnomad?	Likely pathogenic	2
CDHR1	c.297+1G>A	10:g.85956407G>A	p.?	0.00039763% (1)	Pathogenic	1
CDHR1	c.783G>A	10:g.85962879G>A	p.Asp214_Pro261del	0.30516% (863)	Likely pathogenic	1
CFAP410	c.218G>C	21:g.45753071C>G	p.Arg73Pro	0.032545% (90)	Pathogenic	3
CFAP410	c.33_34insAGCTGCACACGCTGCA	21:g.45759044_45759045insTGCACGCTGTCAGCT	p.Ala12Serfs*60	0.0081269% (10)	Pathogenic	1
CFAP410	c.355C>T	21:g.45752934G>A	p.Gln119*	0.0031865% (1)	Pathogenic	1
CFAP410	c.460_469del	21:g.45751804_45751813del	p.Gly154Tyrf*5	not in gnomad?	Likely pathogenic	1
CFH	c.1291T>A	1:g.196659324T>A	p.Cys431Ser	0.00039851% (1)	Likely pathogenic	1
CNGA3	c.101+1G>A	2:g.98986540G>A	p.?	0.02386% (67)	Pathogenic	1
CNGA3	c.967G>C	2:g.99012600G>C	p.Ala323Pro	0.0095445% (27)	Pathogenic	1
CNGB3	c.1148del	8:g.87656009del	p.(Thr383Ilefs*13)	0.175% (493)	Pathogenic	4
CRB1	c.2129A>T	1:g.197396584A>T	p.Glu710Val	0.0021268% (6)	Pathogenic	3
CRB1	c.2639A>G	1:g.197397094A>G	p.(Asn880Ser)	not in gnomad?	Pathogenic	2
CRB1	c.3997G>A	1:g.197411414G>A	p.Glu1333Lys	0.001591% (4)	Pathogenic	1
DRAM2	c.700_706del	1:g.111660877_111660883del	p.(Ser234Glyfs*9)	0.0028189% (7)	Likely pathogenic	2
EYS	c.2822G>T	6:g.65612030C>A	p.Gly941Val	0.0081402% (15)	Likely pathogenic	1
EYS	c.4991C>T	6:g.65300769G>A	p.Thr1664Ile	0.01967% (36)	VUS	1
EYS	c.6632C>T	6:g.64776324G>A	p.Ser2211Leu	0.047324% (89)	VUS	1
GUCA1A	c.296A>G	6:g.42146112A>G	p.Tyr99Cys	not in gnomad?	Pathogenic	1
IMPG2	c.392G>A	3:g.101023099C>T	p.Arg131His	0.048815% (138)	VUS	1
KCNV2	c.257C>G	9:g.2717996C>G	p.Pro86Arg	not in gnomad?	VUS	1
KCNV2	c.1148G>T	9:g.2718887G>T	p.Arg383Leu	0.034479% (96)	VUS	1
KCNV2	c.1354G>C	9:g.2719093G>C	p.Ala452Pro	not in gnomad??	VUS	1
KCNV2	c.1381G>A	9:g.2729470G>A	p.Gly461Arg	0.012379% (35)	Pathogenic	3
NMNAT1	c.53A>G	1:g.10032184A>G	p.Asn18Ser	0.0017696% (5)	Pathogenic	1
NMNAT1	c.769G>A	1:g.10042688G>A	p.Glu257Lys	0.069488% (196)	Pathogenic	1
OPN1LW	c.283del	X:g.153416298del	p.Leu95Trpfs*6	not in gnomad?	Likely pathogenic	1
OPN1LW	c.607T>C	X:g.153420077T>C	p.Cys203Arg	not in gnomad?	Likely pathogenic	1
PROM1	c.1117C>T	4:g.16014922G>A	p.Arg373Cys	0.00040137% (1)	Likely pathogenic	1
PROM1	c.1339C>G	4:g.16008276G>C	p.Leu447Val	0.0017971% (5)	VUS	1
PROM1	c.2436G>A	4:g.15982098C>T	p.Ala812=	0.0055408% (11)	VUS	1
PRPH2	c.394del	6:g.42689680del	p.Gln132Lysfs*7	0.00039789% (1)	Pathogenic	1
PRPH2	c.457A>G	6:g.42689616T>C	p.Lys153Glu	not in gnomad?	Pathogenic	1
PRPH2	c.584G>A	6:g.42672347C>T	p.Arg195Gln	not in gnomad?	Pathogenic	1
PRPH2	c.903_906del	6:g.42666171_42666174del	p.Ser301Argfs*22	not in gnomad?	Pathogenic	2
RDH12	c.698T>A	14:g.68195947T>A	p.Val233Asp	0.0012048% (3)	Pathogenic	1
RDH12	c.869T>G	14:g.68200483T>G	p.Val290Gly	0.0084853% (24)	Likely pathogenic	1
RP1L1	c.133C>T	g.10480579G>A	p.(Arg45Trp)	0.0020281% (5)	Likely pathogenic	1
RP1L1	c.2107C>T	8:g.10469501G>A	p.Arg703*	0.0010699% (3)	Pathogenic	1
RS1	c.327G>C	X:g.18662745C>G	p.Gly109=	not in gnomad?	VUS	1
TULP1	c.1496-6C>A	6:g.35466243G>T	p.?	0.0082098% (23)	Likely pathogenic	2

Of the 216 probands being described, 46 had undergone at least one previous form of pre-screening as part of the Target 5000 study (**Supplemental Table 3.7**). 38 patients had been sequenced with a target capture panel covering the exonic regions of 254 IRD-associated genes (Target 5000 panel) (Carrigan *et al.*, 2016). 4 patients had WES, where 3/4 remained unresolved. 3 patients had been sequenced by both the Target 5000 panel and smMIPs sequencing of the entire *ABCA4* gene due to a previous queried STGD1 clinical diagnosis (Khan *et al.*, 2019, 2020). Additionally, one patient was sequenced via both whole-*ABCA4* smMIPs sequencing and WES, with no candidate variants identified. For some of these cases, it may be that their conditions are in fact not hereditary and they do not have an IRD but instead present with a non-genetic retinopathy and were mistakenly diagnosed. It is important for these patients to be reviewed and for their clinical phenotype to be refined before they undergo WGS, which would routinely be the next step if preliminary targeted sequencing is unsuccessful.

It is interesting to note that of the variants identified that were considered to genetically solve probands, 9 are novel variants that have not been previously reported in the literature, to the best of our knowledge (**Table 3.3**). The thresholds used to filter pathogenic variants were in line with those set by current datasets for what is deemed to be pathogenic (Material and methods section 3.2.8); however, these parameters can be modified and indeed this will likely change over time as more data on IRDs is amassed. Each of the 9 novel variants identified as part of this study have been reclassified through the application of the ACMG/AMP criteria. Due to the substantial size of the resulting table, this classification data is listed and discussed in greater detail within **Supplemental 3.9**, in order to maintain clarity and not overwhelm the main text. Phasing was completed for novel variants where possible, thereby enabling the use of certain ACMG/AMP criteria where appropriate (phasing detailed in **Supplemental 3.2**). 2 of the identified novel variants have a VUS classification, where no further evidence is available at present to upgrade this to class 4 or 5. This highlights the importance of the use of functional assays and *in silico* modelling to enable more confident classification of pathogenicity for such novel variants. These concepts will be further outlined in Chapters 4 and 5 of this PhD thesis.

Table 3.3. Novel variants identified within solved cohort. Total gnomAD allele frequency is noted for each variant, if they were present in the database. Both the ACMG/AMP classification as appears on Franklin and the revised in-house classification are listed (further details in **Supplemental 3.9**).

Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALL AF	Franklin ACMG Classification	In-house Classification
ABCA4	c.5584+5del	1:g.94476813del	p.?	not in gnomad?	VUS	Likely Pathogenic
ATF6	c.479A>G	1:g.161761322A>G	p.Lys160Arg	not in gnomad?	VUS	Likely Benign
BEST1	c.203A>G	11:g.61722629A>G	p.Tyr68Cys	not in gnomad?	Likely pathogenic	VUS
BEST1	c.294G>T	11:g.61723236G>T	p.Glu98Asp	not in gnomad?	Likely pathogenic	Likely Pathogenic
CFAP410	c.460_469del	21:g.45751804_45751813del	p.(Gly154Tyrfs*5)	not in gnomad?	Likely pathogenic	Pathogenic
DRAM2	c.700_706del	1:g.111660877_111660883del	p.(Ser234Glyfs*9)	0.0028189% (7)	Likely pathogenic	Likely Pathogenic
KCNV2	c.1354G>C	9:g.2719093G>C	p.Ala452Pro	not in gnomad?	VUS	Likely Pathogenic
OPN1LW	c.283del	X:g.153416298del	p.(Leu95Trpfs*6)	not in gnomad?	Likely pathogenic	Likely Pathogenic
RS1	c.327G>C	X:g.18662745C>G	p.Gly109=	not in gnomad?	VUS	VUS

Within the genetically resolved cohort, 37% of probands were proposed to be affected with an autosomal dominant IRD and only 6% with an X-linked IRD (**Figure 3.5(a)**). Whereas in 57% of the probands an autosomal recessive inheritance pattern has been predicted. *ABCA4* and *BEST1* are the two genes that are most frequently implicated in disease in this MD cohort, with 21 solved cases associated with *ABCA4* candidate variants, and 14 cases with *BEST1* candidate variants (**Figure 3.5(b,c)**). *ABCA4* was implicated in 50% of all autosomal recessive cases analysed in this study. Cases with ‘solved’ status, and their associated candidate variants are listed in **Table 3.1**. Cascade analysis of participating family members has allowed us to confirm a dominant inheritance pattern in 3/29 suspected dominant pedigrees, and an X-linked inheritance pattern in both of the *OPN1LW* pedigrees and an *RS1* female carrier that will be discussed below (**Figure 3.5(b,d)**, **Supplemental 3.2**). 14/24 genes implicated in the solved cohort harboured causative variants in only 1 patient.

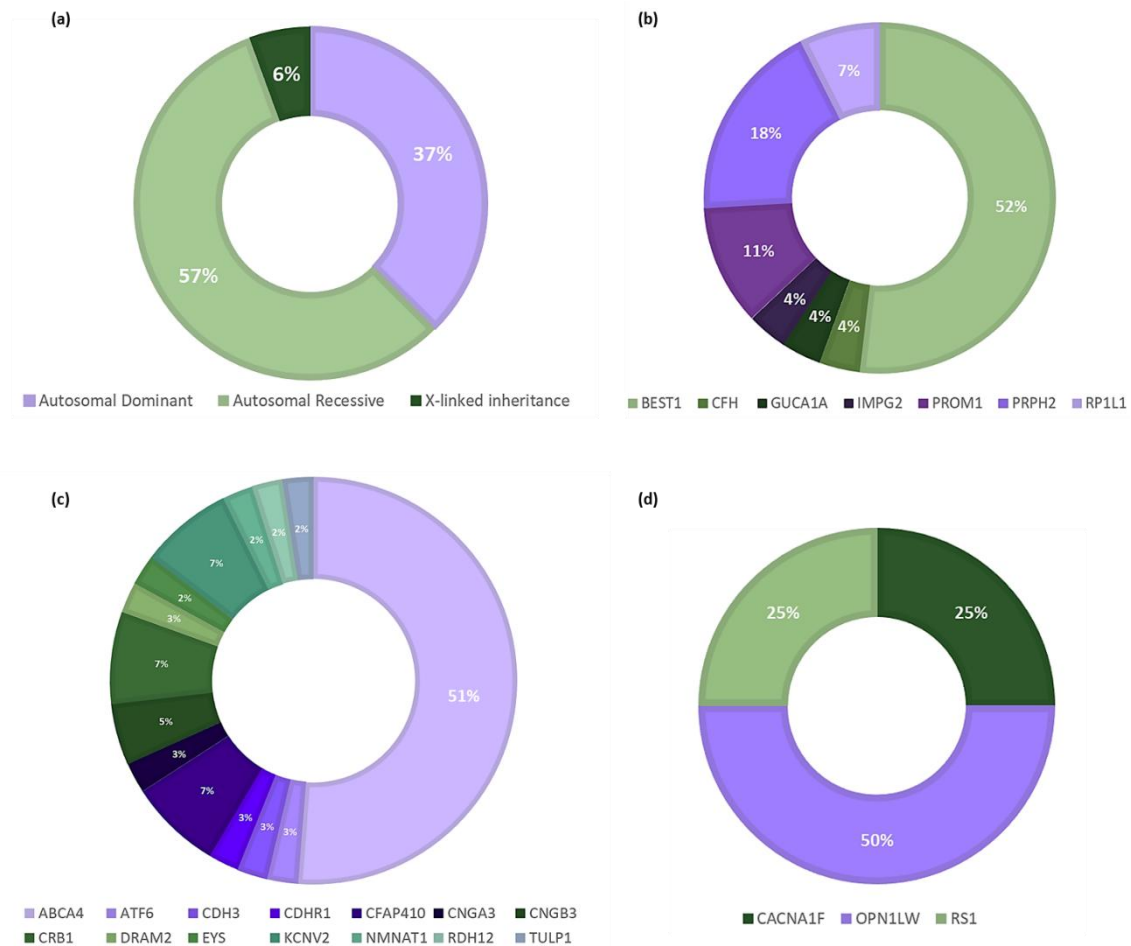


Figure 3.5 Inheritance modes and genes implicated in disease within the solved cohort. (a) Breakdown of the proposed inheritance modes for 72 patients considered genetically solved. The three remaining charts indicate the genes implicated in the solved cohort, based on the inheritance pattern as follows; **(b)** autosomal dominant, **(c)** autosomal recessive and **(d)** X-linked.

Having provided an overview of the overall results from this Irish Macular smMIPs study (Table 3.1-3.3, Figures 3.2-3.5) in which 72 patients are considered to be genetically resolved, we will now explore some of the more specific findings from particularly noteworthy cases in greater detail thereby illuminating some of the implications of the study overall.

From our patient cohort, 7 IRD cases have been presumed solved with candidate variants found in *ABCA4*, however these patients did not have an initial clinical diagnosis of STGD1. In fact, 3 of these individuals had initial diagnoses of Best vitelliform MD (MD-BV) (1462, CI4, CH28). One novel *ABCA4* variant has been identified in patient CH43 via this smMIPs sequencing study; c.5584+5del (Table 3.3). An additional functional assay has been undertaken to analyse the impact this variant has on splicing, and will be described in Chapter 5 of this doctoral thesis, along with a detailed variant classification. 10/18 patients

that remain unresolved with an *ABCA4* variant after this sequencing study have been found to be mono-allelic for the common variant c.5603A>T (**Supplemental 3.3 – 3.5**). *ABCA4* has some very commonly occurring complex alleles, where two variants that are seen to occur together in a patient will almost always be *in cis*. 4 such complex alleles have been seen in 9 of our IRD patients; c.[2588G>C;5603A>T], c.[1622T>C;3113C>T], c.[5461-10T>C;5603A>T] and c.[4222T>C;4918C>T] (**Table 3.1**). The *ABCA4* complex allele c.[1622T>C;3113C>T] appears homozygously in two of our patients (CH50 and CJ79). When a patient sample with 3 or 4 candidate variants in one gene is seen, such as the *ABCA4* gene, unless the variants have previously been reported to occur in complex alleles, we cannot tell the allelic distribution of the variants until cascade analysis of additional family members or long-read sequencing is completed. There is only one case within our solved *ABCA4* cohort (patient CJ78) where 4 variants have been identified and the phasing has not been confirmed, nor do they harbour a known complex allele, therefore it is acknowledged that the phasing of the 4 variants remains unknown in this patient, but that both alleles are likely to contain an *ABCA4* mutation (**Table 3.1**). This also highlights the potential finding of a novel *ABCA4* complex allele within this patient, and may warrant further genotype-phenotype analysis. One STGD1 patient (CH42), has been observed to have the common *ABCA4* variant c.5882G>A in a homozygous state (**Table 3.1**). This homozygous variant is believed to resolve this patient. It is typically the case that for *ABCA4* patients the combination of two mild variants or one mild and one moderate variant would not be expected to lead to an *ABCA4*-related phenotype (Cremers *et al.*, 2020; Cornelis *et al.*, 2022b; Lee *et al.*, 2022a). Therefore, although the c.5882G>A variant is classified as a mild variant, it has been seen to go against the trend outlined above. Furthermore, similar cases have been previously reported where patients seem to only carry this variant (Lee *et al.*, 2021a; Corradi *et al.*, 2023). However, as we have not sequenced *ABCA4* in its entirety, we cannot rule out the possibility of another variant being present, therefore we graded this patient as ‘Possibly solved’ and recommend progression to whole-*ABCA4* gene sequencing including exons, introns and flanking sequences in the future.

In another proband (CG35), an initial clinical diagnosis of MD-BV was provided. Interestingly, this patient is considered ‘solved’ by two variants in the *CNGA3* gene, which has been associated with recessive achromatopsia and cone-rod dystrophies, and not MD-BV, to the best of our knowledge (Sun and Zhang, 2019). Similarly, some patients presenting with a STGD1-like phenotype were solved with variants not in *ABCA4* but in other iMD-associated genes (**Table 3.1**). While typically over 90% of STGD1 patients harbour variants

in *ABCA4*, there are some instances of STGD1 phenocopies (Cremers *et al.*, 2020; Al-khuzaei *et al.*, 2021). For 4 patients in this iMD cohort with a STGD clinical diagnosis, potential causal variants in *PRPH2*, *NMNAT1*, *RDH12* and *PROM1* have been found (**Table 3.1**). Patient CH33 has been solved by a presumed dominant acting mutation in the *PRPH2* gene. In this regard, mutations in the *PRPH2* gene have been found to cause pseudo-Stargardt pattern dystrophy (Boon *et al.*, 2007). Another patient with a STGD1-like clinical presentation has biallelic heterozygous variants in *NMNAT1* (patient CB72); c.53A > G, p.(Asn18Ser) and c.769G > A, p.(Glu257Lys). This combination of *NMNAT1* variants has been reported previously in a study undertaken on two patients with CCRD clinical phenotypes; in this study these two variants were also classified as pathogenic (Nash *et al.*, 2018). The macular degeneration seen in our patient could have been initially diagnosed as STGD1, with similar misdiagnoses being seen in patients with LCA caused by *NMNAT1* mutations. The presence of these two variants along with macular atrophy suggests that the variants in *NMNAT1* may be causative of the CCRD phenotype in our patient. Similarly, patient CK13 also presenting with a STGD1 clinical phenotype was resolved with one pathogenic variant and one VUS in the *RDH12* gene; c.698T>A, p.Val233Asp and c.869T>G, p.Val290Gly respectively. This result is in fact comparable to previous literature, where patients presenting with STGD1-like phenotypes with peripapillary-sparing and heavy macular involvement have been solved with variants in the *RDH12* gene (Scott *et al.*, 2020; Amaral *et al.*, 2023). Scott *et al.*, found that *RDH12* variants located within the catalytic domain of the encoded protein, responsible for the conversion of all-*trans*-retinal to all-*trans*-retinol (the substrate for *ABCA4*), were implicated in a STGD1-like phenotype. The c.698T>A *RDH12* variant that we have identified in patient CK13 is located within this domain, further reinforcing our confidence in considering this patient as genetically resolved. Phasing analysis was possible for this patient (**Supplemental 3.2**), to confirm that the variants are acting *in trans*. Genotype-phenotype and family history studies, such as Scott *et al.*, and as will be outlined in Chapter 4 of this thesis, provide vital information when categorising variants in IRD genes, as well as expanding our knowledge of possible modifiers of disease. While *ABCA4* and *RDH12* have not been confirmed in the literature to be acting as modifiers of disease, in the context of patient CK13, we cannot rule out potential *trans*-modifiers of disease located in genes that act within the same biochemical pathway as *RDH12*, such as *ABCA4*, that we were not able to detect in our study (Cremers *et al.*, 2020; Amaral *et al.*, 2023). Conversely, much research has been carried out on *PROM1* and *ABCA4* and the interplay between these genes, where *PROM1* phenotypes can be modified by variants in *ABCA4* and dominant *PROM1* mutations have been found to cause a Stargardt-

like phenotype (Lee *et al.*, 2019; Amaral *et al.*, 2023; Ricca *et al.*, 2023). Patient P24 presents with such a STGD1 phenotype, where a presumed dominant-acting VUS in *PROM1* has been identified; c.2436G>A, p.Ala812=, with a high SpliceAI score of 0.6 suggesting that it leads to aberrant splicing. To confirm the inheritance mode and pathogenicity of this variant, further cascade analysis and *in vitro* functional studies are required, however we do consider that in principle this *PROM1* variant may fit the disease phenotype, as referenced above, if it is indeed confirmed to have an impact on splicing.

28 of the 105 genes sequenced using this MD-panel have been associated with both MD and RP phenotypes (**Supplemental Table 3.8**). These 28 genes overlap with the RP/LCA smMIPs panel designed and utilised by our collaborators in Nijmegen (Panneman *et al.*, 2023). A number of patients from our iMD cohort have been found to have mutations in IRD genes which can typically lead to an RP or LCA phenotype. This is somewhat surprising, as the initial clinical diagnoses for these patients did not indicate such a phenotype; phenotypic criteria used is outlined in the Materials and Methods section 3.2.1 and (Hitti-Malin *et al.*, 2022). For example, patient CI56 has a clinical diagnosis of iMD, however 3 *EYS* variants have been identified and are thought to potentially be disease-causing; c.2822G>T, c.4991C>T and c.6632C>T with the first variant being likely pathogenic and the latter two being VUS (**Table 3.1**). Phasing of these *EYS* variants has not been confirmed as no family members were available for segregation analysis. Once again, this patient may benefit from long-read sequencing in the future to complete such phasing analysis and allow for a potential upgrade of the 2 VUS candidate variants in *EYS*. Multimodal imaging reveals a sparing of the macula, predominantly evident in the colour fundus and FAF images (all imaging displayed in **Supplemental 3.11**). It is clear in the FAF that there is a patchy area of hypo- and hyperfluorescence surrounding the maculae in both eyes. The OCT reveals a loss of photoreceptors in same area as the hypofluorescent spot observed in the FAF of the left eye. While there is a slight thinning of the retinal layers in the OCT, they remain largely intact centrally. Another patient, CK79, has a broad iMD clinical diagnosis, where two variants have been identified in *EYS*; c.6079-8_6079-3del and c.9299_9302del (**Supplemental Table 3.5**). Upon further assessment of their phenotype with the clinical team, it was concluded that these *EYS* mutations did not seem to fit the phenotype as the macula was much more compromised than would typically be observed for an *EYS* patient, albeit we cannot rule out *EYS* as contributing to disease as increasingly diverse genotype-phenotype correlations are being observed in IRDs. While the *EYS* c.9299_9302del variant is categorised as ‘likely pathogenic’, the second variant c.6079-8_6079-3del is an intronic

variant and has much poorer predictions with two ClinVar submissions classifying it as likely benign, as well as a likely benign and VUS classification on Varsome and Franklin Genoox respectively. Therefore, this patient currently remains unresolved.

Patients CI64 and CJ56 have both been solved with homozygous *CRB1* mutations; c.2129A>T and c.2639A>G respectively (**Table 3.1**). A third patient CK68, has also been solved with biallelic variants in *CRB1*; c.2129A>T and c.3997G>A. This is interesting as patient CI64 had an initial clinical diagnosis of CRD, patient CJ56 had a clinical presentation of dominant cystoid macular dystrophy and patient CK68 received a macular dystrophy clinical diagnosis. In the case of CJ56, the observation of a mutation in a gene typically causative of RP would seem to be somewhat contradictory to that anticipated given the initial macular phenotype diagnosis (**Table 3.1**) (Daich Varela, Georgiou, *et al.*, 2023). However, it is clear from this study and indeed many studies globally that there is a great diversity in clinical phenotypes observed even within a single IRD gene, that is, different mutations in the same IRD gene can give rise to very different ocular disease phenotypes (Del Pozo-Valero *et al.*, 2019a; Cremers *et al.*, 2020; Perea-Romero *et al.*, 2021a; Karali *et al.*, 2022; Hitti-Malin *et al.*, 2024; Liu *et al.*, 2024a). Therefore, in the field of IRDs we have learned to expect the unexpected as exemplified by patient CK68 with biallelic *CRB1* variants.

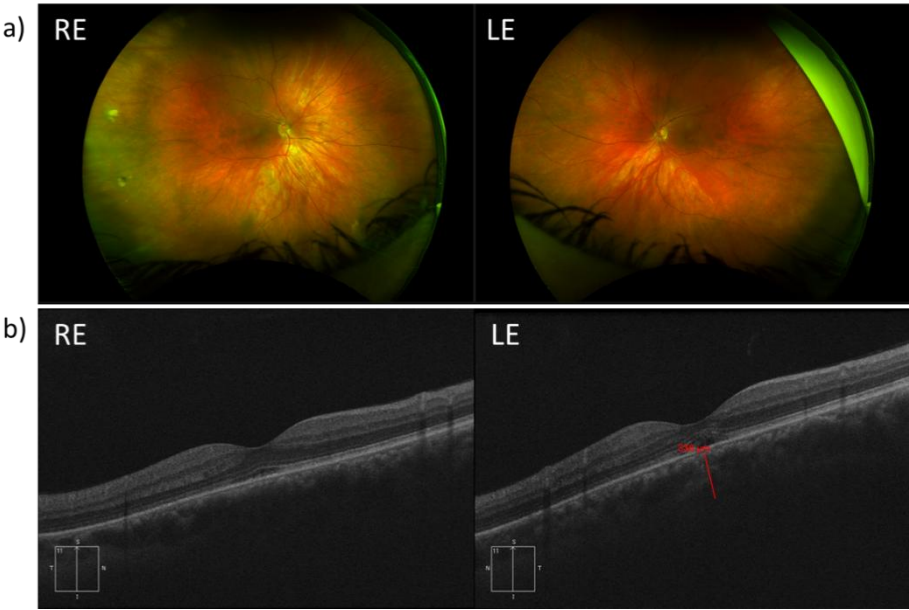
RS1 Female carrier case study

Among the novel variants identified within the resolved cohort, the synonymous variant in *RS1*; c.327G>C, p.Gly109= was called heterozygously in a female patient with a compelling iMD phenotype, Patient CH71 (**Tables 3.1 and 3.3**). *RS1* is associated with X-linked retinoschisis, which had been found to be the most common cause of juvenile iMD in males (Sikkink *et al.*, 2007). It is very rarely the case that a carrier female will exhibit clinical features, however it has been reported in rare cases (Kirkby *et al.*, 2023). It is imperative to be cautious with such a case, as the presumed causative variant in *RS1* is a novel variant that has not been previously reported as associated with an IRD, to the best of our knowledge. For this reason, this patient is labelled as ‘Possibly solved’. To further confirm our suspicions of this variant, the clinical team was consulted and it was concluded that the OCT and fundus imaging indicate a potential X-linked carrier phenotype (**Figure 3.6**). Colour fundus for patient CH71 (**Figure 3.6(a)**) reveals a thinning of the RPE nasal to the optic disc, symmetrical in both eyes suggesting a genetic cause. Punched out defects are also noted peripherally, predominantly in the right eye. Optical coherence tomography (OCT) traces for this patient evidence a disturbance in the photoreceptor outer segments as it has a feathery appearance, once again more prominent in the right eye (**Figure 3.6(b)**). Patient CH71 has

an unaffected sister (CI73), that has been confirmed to not carry the RS1; c.327G>C variant (**Supplemental Table 3.2**). Their mother was affected with a macular dystrophy, but is not available for genotyping at present.

Figure 3.6. (a,b) Multimodal imaging for patient CH71, with potential retinoschisis carrier phenotype. (a) Pseudocolour widefield fundus photos and (b) Optical coherence tomography (OCT) images. (RE=right eye, LE= left eye).

Typically, in retinoschisis we would see a depletion of the ERG b wave in comparison to the



a wave (we have not noted the a wave amplitudes in **Table 3.4**), however this is not the case here as the b wave is still double the amplitude of the a wave, where all responses seem normal in timing. A multifocal ERG was also carried out, where the parafoveal segments appear attenuated in both eyes, with some foveal segments attenuated in left eye. These readings typically reflect photoreceptor dysfunction at the fovea and immediate surroundings within the macula. This patient initially received a clinical diagnosis of MD-BV. However, the ERG results noted in **Table 3.4** are normal, which would argue against an MD-BV diagnosis. Therefore, further phenotyping and functional assays of the RS1 synonymous variant are required to provide conclusive results for this patient.

Table 3.4. Clinical data table displaying Visual acuity (VA) and Electroretinogram (ERG) readings for patient CH71. (OD; right eye, OS; left eye).

Patient	Photophobic? (yes/no, age of onset)	Visual Acuity (VA)		Electroretinogram (ERG)					
		Visual Acuity - OD (right)	Visual Acuity - OS (left)	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)
CH71	Yes, 1 yr	6/9.5	6/24	553	486	195	183	173	140

Mutation in CFH AMD-risk factor gene

Patient CL2 presents as an interesting case, as the patient was initially considered to be unresolved. Upon closer inspection of the candidate variants identified, a variant in the *CFH* gene was highlighted; c.1291T>A, p.Cys431Ser (**Table 3.1**). This variant has been classified previously as pathogenic in a study where it appears in a heterozygous manner in an affected individual with early-onset macular dystrophy with drusen (Taylor *et al.*, 2019). This same variant has been confirmed to be present in our patient and their affected mother who also presents with a similar phenotype (Sanger confirmation performed by a clinically accredited lab as per clinicians' request) (**Supplemental Table 3.2**). The patient CL2 presents with early onset macular drusen; a phenotype similar to that described in Taylor et al 2019 (**Table 3.5**). Patient CL2 remains asymptomatic in terms of visual problems, with their visual acuity being largely normal at 6/6 and 6/7.5. The ERG shows a normal cone response with only slight deviations observed to the rod response amplitude and timing. A diffuse colour error is recorded bilaterally for patient CL2, suggesting a generalised colour vision loss rather than on only one specific axis. Colour fundus for patient CL2 displays clear evidence of macular drusen, with the OCT traces also revealing drusen deposits amongst the retinal layers, heavily focused at the macula as well as a general disorganisation of the layers (**Figure 3.7**). The drusen causes the layers of retina to become raised and can begin to distort central vision over time. The FAF is fascinating as patchy areas of hyperfluorescence are noted to be in a net like distribution. Patchy hypo- and hyperfluorescence is seen at both posterior poles, with the hypofluorescent spots alluding to the death of photoreceptor cells caused by the drusen deposits.

Table 3.5. Clinical data table displaying Visual acuity (VA), Electroretinogram readings (ERG) and Colour vision abnormality details for patient CL2. (OD; right eye, OS; left eye).

Patient	Clinical phenotype	Visual Acuity (VA)		Electroretinogram (ERG)						Colour vision		
		Visual Acuity OD	Visual Acuity OS	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)	Axis of colour dysfunction - OD (protan, deutan, tritan)	Axis of colour dysfunction - OS (protan, deutan, tritan)	Lanthonys Desaturated 15 hue test Error score
CL2	Early onset macular drusen	6/6	6/7.5	338	237	174	138	145	121	diffuse	diffuse	100 OD, 154 OS Upper limit normal is 76 for patient age



Figure 3.7. (a-c) Multimodal imaging for patient CL2, with early onset macular drusen phenotype. (a) Pseudocolour widefield fundus photos (b) Fundus autofluorescence (FAF) and (c) Optical coherence tomography (OCT) images. (RE=right eye, LE= left eye).

Opsin gene cluster: A brief case study

Two cases have been solved by variants identified in *OPNILW*. For male patient CC78, the novel variant c.283del was identified and confirmed through Sanger sequencing (**Tables 3.1 and 3.3**). The second male patient, CC17, presents an interesting case where variant c.607T>C,p.Cys203Arg was identified in *OPNILW* (**Table 3.1**). *OPNILW* is an opsin gene located on the X-chromosome encoding the red/L-wavelength sensitive opsin. This variant is often also seen in the green/M-wavelength opsin protein *OPNIMW*, which is a homologue of the red opsin protein. These two genes occur together in a gene cluster, and pathogenic variation in this region can often be missed using short-read sequencing due to high sequence homology. These two genes can also often be seen to create a hybrid gene together due to homologous recombination, in which this variant has been thoroughly established as pathogenic (Gardner *et al.*, 2014). The encoded cysteine amino acid (203) is seen to be conserved across red and green opsin proteins, and is located in a disulfide bond. Its mutation in the green cone opsin has been studied in depth with functional studies suggesting it leads to misfolding of the resulting protein and its retention in the endoplasmic reticulum (Kazmi, Sakmar and Ostrer, 1997).

In the case of patient CJ17, the c.607T>C variant was initially identified in only the *OPNILW* gene through smMIPs sequencing, and therefore we believed this variant was only present in the red opsin gene, but it merited further study. To date, only one family was identified to carry this variant in the red opsin gene only, to the best of our knowledge (Nathans *et al.*, 1993). Using PCR amplification and Sanger sequencing (with primers outlined in the **Supplemental 3.13**), a preliminary result allowed us to speculate that the variant may be present in both the *OPNILW* and *OPNIMW* genes, as both sets of gene specific primers amplify DNA fragments containing the c.607T>C alteration. However, using PCR amplification only, we could not infer how many copies of the *OPNIMW* gene were present nor could we establish how many of these gene copies harboured our variant of interest. Since the initial macular smMIPs project, collaborators have begun work on a study to develop a tool to analyse this complex opsin gene cluster. When analysing patient CJ17 with this software tool, they were able to confirm the presence of the p.Cys203Arg mutation in both *OPNILW* and *OPNIMW* and have identified 4 copies in total of the *OPNIMW* gene, with 2 copies carrying the mutation of interest (Unpublished data, Mathieu Quinodoz, Carlo Rivolta).

Patient CJ17 presents with a BCM/cone dystrophy phenotype, with earliest symptoms arising before the age of 5 (**Table 3.6**). ERG results show a normal scotopic response, with

no convincing photopic responses except for the Blue/S-Cone ERG. The Blue/S-Cone ERG displays recordable responses similar to that seen in the light adapted b wave, indicating that only the S-cone response was recordable, allowing phenotype refinement to BCM. Lanthony's Desaturated 15 hue test was carried out, with a deutan defect noted in the right eye and protan in the left. Fundoscopy was normal, which is characteristic of a BCM clinical phenotype, except for a tiny patch of hyperfluorescence near the fovea that was noted, but difficult to analyse further due to poor image quality (**Figure 3.8**). The mother of the proband (CJ18) was confirmed to be a carrier of the mutation in *OPN1LW* (**Supplemental 3.2**), but remains phenotypically normal. Long-read sequencing of the proband and the mother would confirm the presence and organisation of the disease haplotype present in the opsin gene cluster definitively, and will be carried out by collaborators as part of their upcoming study (Unpublished data, Mathieu Quinodoz, Carlo Rivolta). The clinical diagnosis here for Patient CJ17 is in line with the BCM phenotype characteristic of such pathogenic variation previously described in this gene cluster in association with the p.Cys203Arg variant, thus we consider this patient to be solved.



Figure 3.8. (a-c) Multimodal imaging for patient CJ17, with a BCM phenotype. (a) Pseudocolour widefield fundus photos **(b)** Fundus autofluorescence (FAF) and **(c)** Optical coherence tomography (OCT) images. (RE=right eye, LE= left eye).

Table 3.6. Clinical data table displaying Visual acuity (VA), Electroretinogram readings (ERG), Visual fields and Colour vision abnormality details for patient CJ17. (OD; right eye, OS; left eye).

Patient	Clinical phenotype	Age of Onset	Photophobic? (yes/no, age of onset)	Visual Acuity (VA)			Electroretinogram (ERG)						Visual fields		Colour vision			
				Visual Acuity OD	Visual Acuity OS	Age of onset	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)	Peripheral or central field loss?	Horizontal degrees of field IV4e	Abnormality to colour vision (yes/no, age of onset)	Axis of colour dysfunction - OD (protan, deutan, tritan)	Axis of colour dysfunction - OS (protan, deutan, tritan)	Lanthony's Desaturated 15 hue test Error score
CJ17	BCM/ Cone dystrophy	Before 5 yrs	Yes, before 5 yrs	6/48	6/48	before 5 yrs	156	164	6.5	6.1	non recorable	non recorable	Concentric constriction to all targets slightly, predominantly to smallest target 04e	Approx. 100 in both eyes	Yes, before 5 years old	deutan	protan	524 OD, 554 OS Upper limit normal is 55 for patient age

CDH3 homozygous deletion identified through CNV analysis

Many interesting observations have been highlighted by this dataset thus far. CNV analysis proved to be challenging, however the analysis resulted in the identification of a homozygous deletion in the *CDH3* gene in one patient CI68 (**Table 3.1**). The *CDH3* gene (OMIM * 114021) encodes P-cadherin which is expressed in the RPE and hair follicles. Previous literature has linked *CDH3* with hypotrichosis with juvenile macular dystrophy (HJMD), with patients displaying progressive central vision loss from birth, as well as thin and brittle hair (Sprecher *et al.*, 2001). This *CDH3* variant, with original predicted notation (c.(?_1571)_(2002_?)del, p.(?)), leads to the deletion of exons 12 and 13 of the gene (**Figure 3.9(a)**). Originally when found, it was thought that it may be the same deletion as reported previously; c.(1570+1_1571-1)_(2002+1_2003-1)del (Halford *et al.*, 2012; Karti *et al.*, 2017; Ellingford *et al.*, 2018). Due to the manner in which the CNV analysis is undertaken and the short-read nature of smMIPs sequencing, the exact cut off points of the deletion were unknown. A breakpoint analysis was undertaken to provide further resolution regarding the deletion and confirm which regions of the introns were deleted (Materials and Methods section 3.2.11). In the patient trace (**Figure 3.9(b)**) we observe that intron 11 sequence splices directly into intron 13 sequence. In comparison, in the control we observe that the wildtype sequence of intron 11 is present (without the deletion). The deletion was confirmed to be 8,815 base pairs long, giving a final cnotation of: *CDH3* c.1570+229_2003-860del. To the best of our knowledge, this is a novel variant and the second pedigree ever observed to have a homozygous deletion of exons 12 and 13 within *CDH3*, with the only other occurrence reported in a patient displaying the same macular and systemic symptoms as those observed within our Irish pedigree (Halford *et al.*, 2012).

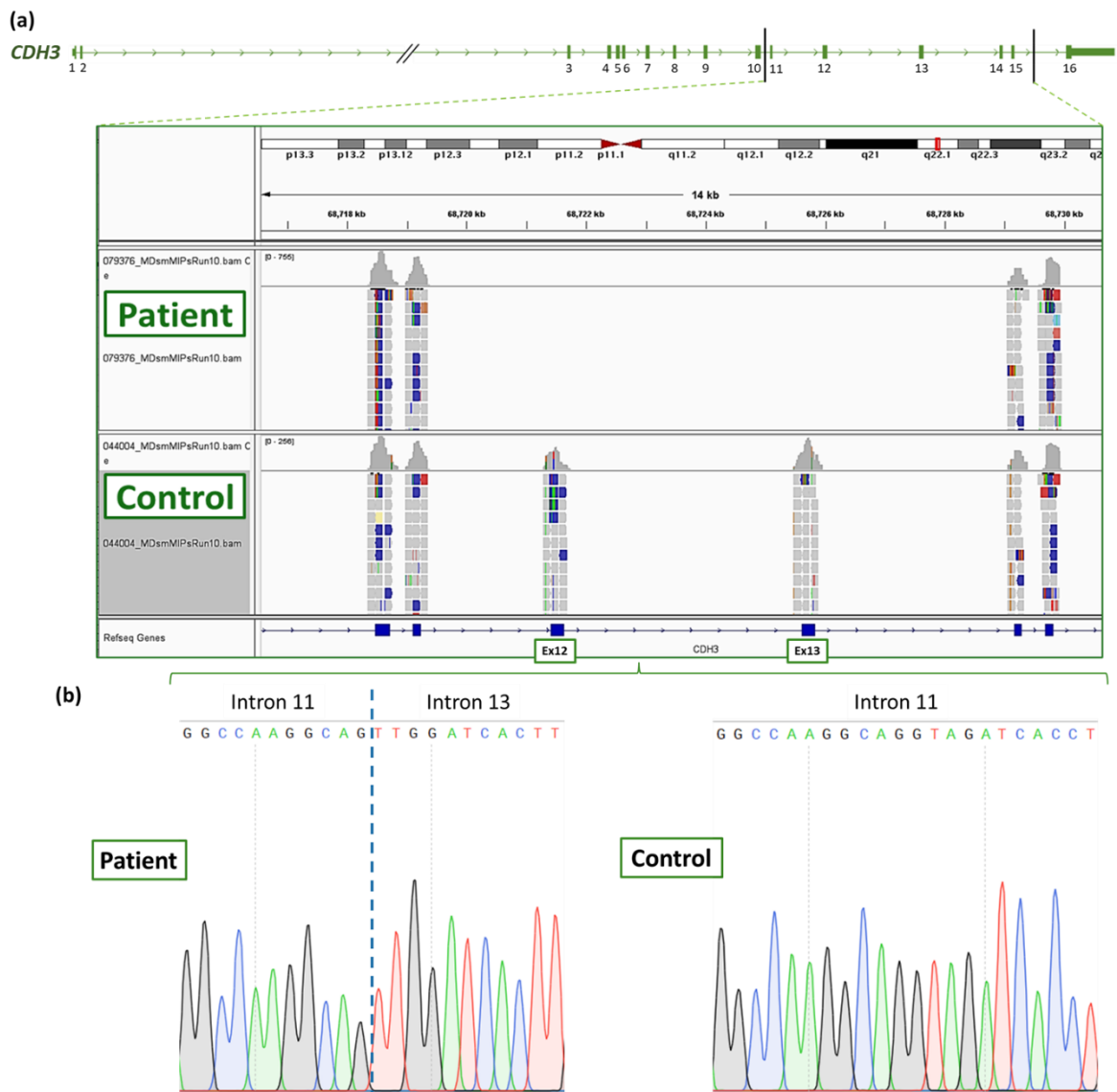


Figure 3.9 (a) A representation of the *CDH3* gene, focused on exons 11 to 15, and displaying sequencing reads across exons using IGV tool. The bottom control track shows clear reads covering each exon, indicating that there is sequence present for these regions. In contrast, in the upper patient track we can see that there are no reads present over exons 12 and 13, suggesting a homozygous deletion of these regions. A breakpoint analysis was undertaken to confirm how much of the introns were deleted. **(b) Sanger sequencing of patient and control DNA.** In the patient trace we can see that the intron 11 sequence splices directly into intron 13 sequence. In comparison, on the right in the control we can see what the wildtype sequence of intron 11 looks like without the deletion. The deletion was confirmed to be 8,815 base pairs long, giving a final c.notation of: *CDH3* c.1570+229_2003-860del.

In this pedigree, the 3 affected siblings with a brittle hair and macular phenotype are highlighted in black, with the arrow indicating the proband (**Figure 3.10(a)**). The unaffected mother (CI67) was found to be a carrier of the 2-exon deletion. All of the affected children are homozygous for the *CDH3* deletion (CI68, CI69 and CI70). 3rd degree consanguinity was observed, as denoted by the double line between the parents (**Figure 3.10(a)**). The father passed away at working age, and had no observable phenotype but his genotype is unknown. All three siblings presented with an ocular phenotype from a very early age, as well as displaying either hair loss, thinning or alopecia from birth (**Table 3.7**). An individual with normal vision would have a visual acuity (VA) score of 6/6, with anything below this value being considered as ‘poor’ or ‘deteriorating’ vision. Due to their severe macular phenotype, the siblings’ central vision and ability to see fine details was impacted, leading to progressive decline in visual acuity (**Table 3.7**). Colour fundus imaging was available for the proband CI68 and sibling CI70, as well as FAF for sibling CI70 (**Figure 3.10(b,c)**). Imaging for the proband reveals extensive pigmentary disturbance with hypo-pigmentation centrally in both eyes. As well as this, a ring of clumpy areas of hyper-pigmentation is seen more peripherally. For sibling CI70, loss of photoreceptor outer segments can be seen in both imaging types. This is indicated by areas of hypopigmentation centrally on the colour fundus which correspond with the areas of hyper-fluorescence on the FAF. This area of loss is surrounded by a more extensive area of hypo-fluorescence, indicating retinal atrophy.

Table 3.7. Synopsis of Visual acuity (VA) and systemic features present in affected family members carrying the *CDH3* homozygous deletion. (OD; right eye, OS; left eye).

Patient	Age of onset	Visual Acuity - OD (right)	Visual Acuity - OS (left)	Abnormality to colour vision (yes/no, age of onset)	Hair phenotype
CI69	8 yrs old	6/38	Counting fingers	Yes, 21 yrs old	Hair loss before 1 yr old Alopecia
CI68	8 yrs old	6/48	6/30	No	Alopecia and Hair thinning
CI70	5 yrs old	6/48	Counting fingers	Yes, 14 yrs old	Hair thinning

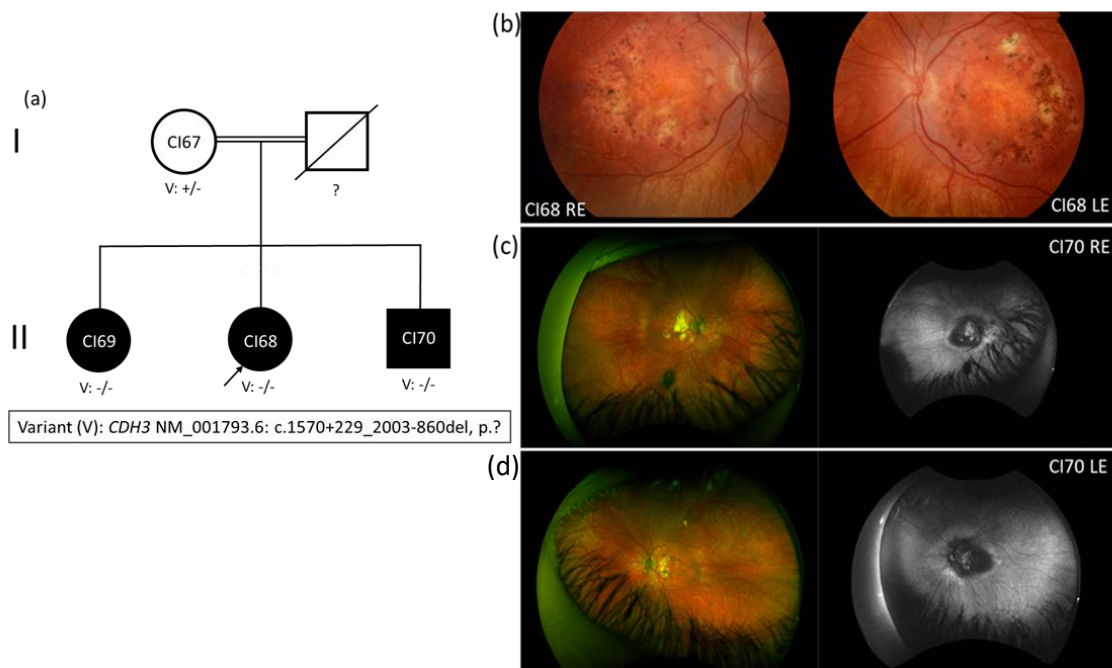


Figure 3.10. (a) Pedigree tree displaying the phasing and segregation pattern of the *CDH3* homozygous deletion in the CI68 family. The segregation patterns of the mutations are shown, with variants indicated under the tree. The arrow indicates the proband. (+ = wildtype allele, – = mutant allele). **(b) Colour fundus for patient CI68, with a focus on the macula. Colour fundus and Fundus autofluorescence (FAF) for (c horizontally) right eye and (d horizontally) left eye of patient CI70.** (RE= right eye, LE= left eye).

Of note, patient CH45 is monoallelic for a novel splice motif variant in *CDH3*; c.247-2A>T which is predicted to be likely pathogenic. Upon phenotypic evaluation it was found that this patient, along with their affected sibling have alopecia suspecting hypotrichosis and juvenile macular degeneration (HJMD) as the clinical diagnosis. Due to the specific phenotype observed, *CDH3* is the most likely candidate, therefore the identification of a single pathogenic variant warrants progression to sequencing of the whole gene (not just exons) to identify further candidate variants.

Here we have described the findings from a cohort of 216 iMD probands, sequenced utilising a smMIPs targeted sequencing panel of 105 iMD and AMD loci as outlined above. In an effort to elucidate towards the genetic architecture of iMDs in Ireland, we have been able to identify many novel disease-causing variants in iMD genes, and describe new potential genotype-phenotype correlations. Similarly, we have classified these novel variants, completing phasing studies where possible to aid in this endeavour.

3.4 Discussion

In recent years, targeted sequencing technologies such as smMIPs have risen in popularity as they have proven to be a cost-effective and high coverage form of first tier sequencing when working with large populations of patients. In this study, a key objective was to utilise a smMIPs panel to sequence a cohort of Irish iMD patients that remain genetically unresolved. The MD-associated panel was designed as previously described (Materials and methods 3.2.3; (Hitti-Malin *et al.*, 2022)), to include the coding regions of 105 genes previously associated with iMD, along with intronic regions that contain known pathogenic variants and a few choice AMD risk loci (**Supplemental 3.8**). In summary, from the 223 Irish iMD probands that were sequenced using this iMD smMIPs panel, 72 patients have potentially been genetically solved, giving a diagnostic yield of ~33%. Our diagnostic yield is consistent with other iMD studies of this kind, with solve rates achieved by the MD-smMIPs panel ranging from 38-39.8% in the Hitti-Malen iMD studies, and 63.2% achieved by the McClinton and Corradi Stargardt study (Hitti-Malin *et al.*, 2022, 2024; Mc Clinton *et al.*, 2023). Furthermore, this iMD diagnostic yield is lower as it excludes a large proportion of the Irish STGD1 cases present in Target 5000 as they formed part of a separate study focused on whole-*ABCA4* gene sequencing (Khan *et al.*, 2020). The diagnostic yield here highlights the efficacy of this form of panel sequencing of 105 iMD genes as a preliminary form of genetic screening. Additionally, as part of this study, we have identified mono-allelic cases with specific phenotypes that potentially do not require full WGS, but could instead benefit from whole gene sequencing of single genes (**Supplemental 3.4**). Genetic screens such as this are of paramount importance in providing patients with curated care pathways, as well as advising on disease prognosis and family genetic risk.

iMDs have a high level of clinical and genetic heterogeneity. This makes an accurate clinical diagnosis challenging and therefore can often result in differential diagnoses or misdiagnoses. Genetic testing and thorough screening methods are utilised to provide an initial informed research-grade genetic diagnosis. Once this preliminary result is validated in a clinically accredited laboratory, the clinical teams can use this information to provide a complete clinical and genetic diagnosis to the patient. This is vital to accurately inform patients of the cause of disease, the potential disease progression, genetic risks, and possible life-style changes that may be useful given the condition. This current iMD study has clearly highlighted the value of genetic screening studies given the identification of likely causative variants in 24 iMD-associated genes. Furthermore, for some patients, likely inheritance

patterns including autosomal dominant, autosomal recessive, and X-linked inheritance, have been proposed (**Figure 3.5(a-d)**).

In line with the protocol utilised by our collaborators in RadboudUMC, variant prioritisation was undertaken using a phenotype-agnostic approach without taking into account the initial disease phenotype and proposed mode of inheritance provided by the clinicians (Materials and methods section 3.2.8). Using this ‘genotype-first’ approach has been shown to be valuable, as after our initial observations regarding genotype from sequencing data are made, IRD patients can be reassessed if needed and their phenotypes refined. Adopting this method means that, in principle, we assess all genetic variants in an unbiased manner and therefore could possibly find new genotype-phenotype combinations that have not been observed before, but that could potentially be causative of disease. Due to the high level of phenotypic overlap between macular dystrophies, refinement of phenotype is probable, as iMD-associated genes can be causative of an array of ocular presentations, as observed with genes discussed in this thesis such as *ABCA4*, *PRPH2*, and *EYS*. Once candidate variants have been identified, as described in the Results section 3.3, the next step was confirmative Sanger sequencing to confirm presence of the variants identified through NGS, and segregation analysis, when additional family members are available, to establish phasing of variants together with the likely predicted mode of inheritance (Materials and methods section 3.2.10). Family segregation analysis was possible to complete for 25/72 patients that are believed to be genetically solved by the MD-smMIPs study (**Supplemental 3.2**). Short-read sequencing techniques often cannot capture the phasing of variants unless the variation is located close enough together to be captured by the same probe. This is very rarely the case, therefore familial testing is needed. For cases where phasing is not possible and biallelic variants have been identified, it could occur that they are mischaracterised as ‘solved’ as the variants identified may not be acting *in trans* where they were thought to be. Further methods to explore this are required, such as long-read sequencing, which should aid in resolving many of these situations where family members are not available to complete cascade analysis. For some patients such as CK68, additional family samples were sent to a clinically accredited lab (given appropriate consents). As outlined in the Results section 3.3, patient CK68 has two variants in *CRB1* which are believed to be acting *in trans*, leading to an autosomal recessive retinopathy (**Table 3.1**). Familial testing completed by Blueprint confirmed that the mother is a heterozygous carrier of one variant *CRB1* c.2129A>T, allowing us to infer phasing of the variants and complete the genetic diagnosis for this family.

Within the solved cohort, 13 variants of unknown significance (VUS) have been called as candidate variants, along with 63 unique VUS identified in the unresolved cohort (**Figure 3.4(a)**). These categorisations have been noted from Franklin and Varsome as a preliminary classification used for variant calling and prioritisation, with further in-house classification required. While open-source search engines are useful tools that provide information focused on human disease variation, they should be utilised with caution as they can often draw evidence from unreliable or duplicated sources. It is, broadly, the overarching goal of sequencing studies such as this one, to provide further information on variation found in iMD patients and enable complete classification of iMDs as the landscape of knowledge expands. Carefully curated databases of variants in IRD-associated genes such as LOVD.nl, are often very valuable in adding confidence to candidate variants identified and aid in their interpretation. Recent large-scale interpretation studies have been completed for all variation listed on LOVD.nl in *ABCA4* (Cornelis *et al.*, 2022b, 2023). Such studies in combination with the ClinGen Curation panels will revolutionise the way we interpret IRD variation, making it more accurate and targeted for each gene. Additionally, publicly accessible archives such as ClinVar and gnomAD v4.1.0 are continually updated, with ClinVar often having variant classifications submitted by clinical laboratories and researchers from all across the globe (Harrison *et al.*, 2016). Variant classification and cascade analyses will continue to be discussed in further detail throughout Chapters 3B, 4 and the General Discussion of this doctoral thesis.

21 patients were resolved with variants in *ABCA4*; ~51% of the patients with a suspected autosomal recessive form of iMD had candidate variants identified in *ABCA4* (**Figure 3.5(c)**). 14/21 of these patients had an initial STGD1 clinical diagnosis (**Table 3.1**). The common *ABCA4*:c.5603A>T, p.(Asn1868Ile) variant was most often identified as part of the c.[2588G>C;5603A>T] complex allele (4/21 patients), with the other 2 instances of this variant being once *in cis* in the c.[5461-10T>C;5603A>T] complex allele and once alone *in trans* with the pathogenic *ABCA4* variant c.161G>A (**Table 3.1**). In STGD1 cases, the *ABCA4* c.[2588G>C;5603A>T] complex allele has been observed in approximately 50% of all *ABCA4* complex alleles (Runhart *et al.*, 2018; Mc Clinton *et al.*, 2023). This has been recapitulated in this study, with 4/10 individuals with suspected and proven complex alleles in *ABCA4* carrying a copy of this allele. It is interesting to note that 2/21 *ABCA4* patients were found to be homozygous for the c.[1622T>C;3113C>T] complex allele, with no presumed relation between the cases. Typically, in cases where two mild variants or one mild and one moderately severe variant are identified in *ABCA4*, these patients would be

considered unresolved as these combinations are not thought to lead to an *ABCA4*-associated phenotype (Cremers *et al.*, 2020; Lee *et al.*, 2022). As an exception and as was detailed above, the mild common hypomorphic variant c.5882G>A, p.(Gly1961Glu) has been identified homozygously in one patient, CH42, where we consider this patient as ‘possibly’ solved due to the nature of their phenotype (**Table 3.1**). This variant has been associated with reduced penetrance, where a study identified both unaffected and affected homozygous individuals, where no other pathogenic variants were identified (Lee *et al.*, 2021a). The *ABCA4* c.5882G>A variant has also been found to occur with the c.769-784C>T variant as a major *cis*-acting modifier, which contributes both to the severity and penetrance of the disease allele (Sangermano *et al.*, 2019). This was not the case for our patient CH42 as this deep intronic variant was covered in our sequencing panel and was confirmed not to be present. It would be beneficial for this individual to undergo whole-*ABCA4* sequencing to ensure there are no other variants in the gene that are potentially contributing to disease, to allow for a complete genetic diagnosis. While it is possible for *cis*-modifiers to be present in such patients, we also cannot exclude the possibility of variants outside of *ABCA4* that could act as modifiers of disease, that were not captured in our panel. Recent studies have revealed that there is a strong association between rare and common variants and haplotypes in the *ROM1* and *PRPH2* genes with *ABCA4*-specific phenotypes and STDG1 disease (Zernant *et al.*, 2022). Rare cases of digenic inheritance have also been reported prior to this study in patients with a *PRPH2*-associated clinical phenotype presenting with additional variants in *ABCA4* or *ROM1* (Poloschek *et al.*, 2010). Furthermore, there are other examples of genetic modifiers influencing the disease course in IRDs, which will be outlined in the General Discussion section of this doctoral thesis.

Within our unresolved cohort, we have noted 60 patients where only one candidate allele in a single gene was identified (**Supplemental 3.4**). 15/60 have a VUS identified in genes that follow either a confirmed or presumed dominant inheritance pattern, 1/60 is a female carrier of a VUS in *CACNA1F* (patient CG33), and the remaining 44/60 are monoallelic for class 3, 4 or 5 variants in a gene associated with autosomal recessive inheritance. Additionally, we highlighted 17 patients that remain unresolved who have potential disease-causing variants in more than one gene (**Supplemental 3.5**). One patient (CF8) was identified to have a single known VUS in *ROM1*; c.793G>A, p.Ala265Thr (**Supplemental 3.4**). This variant currently has a predicted VUS classification, and was found heterozygously in this patient. This was originally suspected to potentially cause a dominant form of RP in this patient where a clinical re-evaluation could appear useful to refine the phenotype. However, there are also a

few other possibilities for this patient. There could be a second *ROM1* variant in an intronic region that was not covered by the current study. It is also a possibility that *ROM1* is not causative of disease. Interestingly, mutations in *ROM1* have been implicated in causing a digenic form of RP in conjunction with *PRPH2* (Dryja *et al.*, 1997), one of which is the c.793G>A variant in *ROM1* present in patient CF8. In principle, this patient could potentially also have a *PRPH2* mutation that escaped identification during filtration steps or was not covered by the MD-smMIPs panel. Digenic or tri-allelic inheritance has been noted to occur with the *CNGA3* and *CNGB3* genes, as the two encode subunits of the cone-specific cyclic nucleotide-gated ion channel (Burkard *et al.*, 2018). Biallelic variants in these two genes are the most common causes of achromatopsia worldwide, but when observed in a digenic pattern the resulting phenotype ranges from a colour vision abnormality to a more severe cone dystrophy. Single variants have been identified in *CNGA3* in 2 patients, and in *CNGB3* in 1 patient (**Supplemental Table 3.5**). All three of these patients have been analysed for variants in the second gene to ensure nothing was missed during initial filtration of candidate genes and digenic inheritance has not been confirmed as none of the 3 patients carry a second candidate variant in the other gene. While this complex inheritance pattern would be interesting to explore, we do not have sufficient numbers in this particular cohort of patients to infer any conclusions regarding potential known or novel digenic patterns or connections between disease genes.

As our collective knowledge on the genetic landscape of IRDs grows, more loci are being identified and associated with various forms of inherited vision loss. As mentioned above, smMIPs sequencing represents an attractive form of multiplex panel-based sequencing, and has proven to be effective in many previous studies of IRDs such as Stargardt disease and other iMDs (Khan *et al.*, 2020; Hitti-Malin *et al.*, 2022, 2024; Corradi *et al.*, 2023; Mc Clinton *et al.*, 2023; Panneman *et al.*, 2023). The smMIPs design, as described by Molecular Loop Biosciences Inc., represents an incredibly versatile sequencing probe that is accompanied by adaptable methodologies (**Figure 3.1**). The panel of loci targeted by smMIPs can be tailored and expanded quite easily, allowing researchers to include more smMIPs to cover new disease-associated or modifier loci that are continually being reported. The smMIPs design and iMD-panel that was utilised for our study has an additional advantage over previous studies of this kind (**Supplemental 3.8**) (Hitti-Malin *et al.*, 2022). Of note, the panel included a higher level of smMIP tiling (8 layers), and so each targeted nucleotide was covered by at least 8 different smMIPs. This is vital, particularly in regions of the genome that are tricky to sequence due to GC-rich content or repetitive motifs, as if

one smMIP drops out or fails to be sequenced correctly, there are others present to capture the same target. This ‘failsafe’ approach can more often than not, eliminate the need for resequencing of complex regions of sequence, as well as allowing for improved confidence in variant calling and likelihood of CNV detection due to the increased number of unique smMIPs covering a single nucleotide. As well as smMIP sequencing being multiplex, thereby allowing for hundreds of samples to be sequenced simultaneously, the Molecular Loop protocol requires only a small quantity of DNA. This is advantageous as it means older and lower quality samples can in principle still be sequenced using this method. The simple lab workflow also allows for cheaper sequencing per sample, and a reduced margin of error. As part of a continuation of this study in collaboration with the Nijmegen team, a new targeted panel of smMIPs has been created that encompasses novel candidate IRD genes, as well as combining all existing panels into one super panel of IRD novel and known loci (unpublished data, Cremers, Roosing, Corradi, Panneman). A list of 80 candidate patients from the Target 5000 cohort has been curated and DNA prepared alongside quality controls assessments (Materials and Methods 3.2.4). These samples were sent to collaborators in September 2024 for library preparation, with preliminary sequencing runs currently underway. 18 of the patients chosen for this candidate gene panel are unresolved patients from the MD-smMIPs study described in this thesis Chapter. Additionally, smMIPs sequencing of the entire *ABCA4* gene has been carried out for 4 unresolved monoallelic-*ABCA4* patients from this MD-study (CH6, P47, R14 and CD40), where unfortunately no additional variants were detected. Possibly there are unidentified variants in genes that could act as Stargardt phenocopies, such as *PRPH2*, *ROM1*, *PROM1*, *CDHR1* and *CRX* (Wolock *et al.*, 2019; Cremers *et al.*, 2020), where certain regions of these genes were not covered by the iMD-panel.

Further WGS is an especially attractive step for some of our unresolved probands, as out of the 142 unresolved patients, 4 of them have previously received WES and yet still remain unsolved. Additionally, 39 unresolved probands have previously undergone Target 5000 target-capture panel sequencing of exons of 254 IRD-associated genes, along with some intronic regions of *ABCA4* and *USH2A* (Carrigan *et al.*, 2016; A *et al.*, 2017; Whelan *et al.*, 2020a). 3 patients that had a form of pre-screening have been solved with the MD-smMIPs panel (**Table 3.1, Supplemental Table 3.7**). Patient P24 previously underwent the Target 5000 target capture panel, and was subsequently sent for whole-*ABCA4* sequencing using smMIPs due to their STGD1 phenotype. Via this iMD smMIPs study we identified a variant in *PROM1*; c.2436G>A that currently appears to have a likely benign/VUS classification on

Franklin and Varsome tools, but has a very high SpliceAI score leading us to hypothesise that it may be a good candidate for disease-causation in this patient given the phenotypic crossover between *PROM1*-associated disease and Stargardt (Paavo *et al.*, 2023). While there is no record of this variant being called in previous targeted sequencing, it could also be that it was filtered out due to poor missense scores. With a SpliceAI score of 0.6 predicting acceptor gain 2 bases away from the variant in the middle of the exon, it is worth exploring this variant's potential pathogenic impact on splicing, therefore we consider this patient 'possibly solved' with the intention of completing a Midigene functional assay, as is described for 2 other variants in Chapter 5.

A second patient worth highlighting is O14, as this patient had previously undergone Target 5000 target capture sequencing prior to this study. Both smMIPs and the previous screening method identified two variants in *ATF6*, including the novel variant c.479A>G (**Table 3.3**). It is believed the novel *ATF6* variant was discounted and filtered out previously due to its likely benign/VUS conflicting classification (**Table 3.1, Supplemental Table 3.9**). This variant has a moderate SpliceAI score of 0.17 which is below our typical cutoff threshold. Upon consultation with clinicians, they suggest that *ATF6* may be the causative gene due to the stark achromatopsia phenotype present in the patient characteristic of disease-causing mutations in this gene. This is not to say that there are no further variants present in regions of *ATF6* that were not covered by either screening method, however we believe that a functional study is warranted for this novel variant before this patient is progressed to further sequencing such as WGS. For probands that have previously undergone genetic screening but still remain unresolved, a key future direction is to progress them to WGS, or to clinically re-evaluate them to ensure that the disease is still thought to have an underlying genetic cause. This scenario was one of the challenges encountered throughout the course of this study, as after clinical re-evaluation and in-depth MDT consideration, 7 patients were decided to not have an IRD but instead have an alternative cause of their ocular phenotype.

While smMIPs appear to have many advantages, there are some pitfalls that should be mentioned. Utilising the *CDH3* deletion that was detected through CNV analysis as an example, the first disadvantage to outline is the lack of clarity on CNV and structural variation (SV) detection, with breakpoints typically not being found. As these smMIPs are a form of short-read sequencing and are designed to target predominantly the coding regions of genes, they are not efficient in identifying the exact breakpoints of CNVs if they occur outside of coding regions; hence additional work is often required as described in the *CDH3* Results section of this thesis chapter. In a similar vein, since only some genes are included

in this panel (as per all targeted panels), smMIPs cannot detect large SVs like inversions, duplications or deletions. So, within this iMD study specifically, CNVs and SVs with breakpoints in non-coding regions, as well as variants in most non-coding regions, were not captured. The analysis pipeline was designed by our collaborators in RadboudUMC. Of note, it is uncertain if such smMIPs pipelines can easily be adopted by other labs with a lesser bioinformatics capability. Therefore, while smMIPs sequencing proves to be effective, this methodology might not be as universally utilised by other researchers in the field as initially anticipated.

While the tools for CNV analysis for the smMIPs study are evolving and are always being improved, such analysis remains a challenge. As mentioned, a homozygous deletion of exons 12 and 13 of the *CDH3* gene in patient CI68 was observed in the CNV analysis (**Table 3.1**). According to scientific literature, to the best of our knowledge, this is the second patient ever recorded to have such a deletion of these two exons homozygously. As mentioned, breakpoint analysis confirmed a deletion of 8,815 base pairs, giving a final c.notation of: *CDH3* c.1570+229_2003-860del (**Figure 3.9(a,b)**). Frequently it is the case that the expanded genetic context provided by more extensive sequencing such as WGS is required to identify the exact size and breakpoints of a deletion, such as this one. This patient (CI68) with a homozygous *CDH3* deletion has very specific clinical presentation, displaying early childhood ocular problems, fragile hair and hair loss at an early age, as well as a dark choroid that is reminiscent of Stargardt disease phenotypes (**Figure 3.10(b)**, **Table 3.7**). Previous literature on *CDH3* mutations have linked the gene with hypotrichosis with juvenile macular dystrophy (HJMD) and ectodermal dysplasia, ectrodactyly and macular dystrophy (EEM), as the gene is involved in encoding the P-cadherin molecule which is largely expressed in the RPE and hair follicles (Sprecher et al., 2001). Both of these syndromes linked to *CDH3* mutations have significant clinical and phenotypic overlap, and can often go misdiagnosed. The clinical presentation of our patient aligns with those of these syndromes, and upon follow up with the clinical team and validation by Sanger sequencing, their clinical diagnosis was refined to HJMD not only for this patient but also for their two affected siblings. In highlighting such cases, we emphasise the importance of assessing all systemic extra-ocular symptoms when diagnosing patients with iMD, and that genotyping of children born with such systemic features might help alert clinicians to the possible development of iMD later in life. Another such syndromic case study is outlined in part B of this thesis chapter, describing the first family in Ireland to be confirmed to have Bardet-Biedel syndrome associated with variants in *BBS7*.

While candidate disease causing variant(s) have been identified in 72 probands within this Irish study, 142 probands still remain unresolved from our iMD cohort with 65 of these patients having no potential candidate variants identified (**Figure 3.3, Supplemental 3.6**). A likely reason for this is the targeted nature of the sequencing methodology employed. Only the 5'UTR, coding regions and some known intronic loci for 105 genes are covered by the MD-smMIPs panel. This leaves most of the non-coding genome uncovered, and as previously referenced, around one third of IRD patients may potentially be solved by variants in the non-coding genome, as well as enabling the detection of large SVs and CNVs (Duncan *et al.*, 2018a; Perea-Romero *et al.*, 2021a; Zeuli *et al.*, 2024b). There are also regions of the coding genome that are highly repetitive or have a high GC content as previously mentioned, and so have proven difficult to sequence, like *RPGR*-ORF15 for example, which has a purine content of 92% (Hitti-Malin *et al.*, 2022). Inefficiency of sequencing in such regions poses a serious challenge for NGS approaches and implementation of optimal care pathways for patients. By way of example, variation in *RPGR* is the most common cause of XLRP with promising clinical trials currently underway, but only for those patients with a definitive genetic diagnosis (see Chapter 5 Discussion section). An interesting case study and functional assay on a novel splice-altering variant identified in *RPGR* is described in Chapter 5 of this thesis.

Additionally, there may be modifier loci/secondary genes at play in any of these iMD cases, some of which will be further discussed in the General discussion section of this doctoral thesis. Undiscovered modifiers could contribute to cases where the initial clinical diagnosis does not seem to match candidate variants found in the patient. In such cases, patients do not fit disease models proposed for a particular disease-gene, albeit given the clinical and genetic heterogeneity in IRDs, we have established that there are few definitive genotype-phenotype correlations. In essence, this means there could be other variants present in patients that were not identified by smMIPs that, while not directly responsible for causing the disease phenotype, may alter severity or age of onset of the phenotype. Such nuances in genetic causality are difficult to identify through targeted sequencing and so for some unresolved IRD patients it would be valuable to consider WGS or long-read sequencing as possible next steps, as this will provide a more complete genetic picture. A good example of this is seen with patient CJ17 where the opsin gene cluster is highly involved in their disease phenotype. Only more comprehensive forms of sequencing can capture both the pathogenic haplotype and the number of gene copies required to provide a complete genetic diagnosis for such patients.

It is notable that 9 novel variants associated with iMD have been identified in this study so far, that have not previously been reported in scientific literature to the best of our knowledge, and that are not present in any of the open-source sequencing databases we have access to including ClinVar and LOVD.nl. Some of these variants are also not present in gnomAD (**Table 3.3**). One novel splice-altering variant has been found in *ABCA4*, associated with STGD1 (**Table 3.3**). *ABCA4* c.5584+5del is a VUS that occurs within a splice motif and may have a potential splice altering effect(s). It has a SpliceAI score of 0.88 predicting loss of donor site. The completed midgene functional assay for this variant will be discussed in Chapter 5, where the results have allowed us to award a ‘Hot’ VUS classification to this variant (**Table 5.1** in Chapter 5). For novel variants such as these in *ABCA4*, we cannot use previous disease models suggested by Cremers et al. efficiently (Cremers *et al.*, 2020), as we do not know the extent of the impact of this variant or the variant combination identified in the patient on disease progression. Upon identification of novel variants, it is useful to track disease progression with the clinical team over a period of time, as it may be possible to identify new genotype-phenotype correlations, especially for widely studied diseases such as *ABCA4*-related Stargardt.

Two novel variants in *BEST1* have been identified; c.203A>G and c.294G>T (**Table 3.3**, **Supplemental 3.9**). Variant c.203A>G was identified in two patients, CC31 and CF78, where no other family are available to confirm that the variant segregates with disease (**Table 3.1**). As this variant in *BEST1* is thought to be associated with an autosomal dominant inheritance pattern, allelic data may not be used to upgrade the classification as per ACMG/AMP guidelines (Richards *et al.*, 2015). Without familial segregation data or functional studies, we cannot upgrade this novel variant, but it was the only putative likely pathogenic variant identified in these two individuals that clinically match an MD-BV phenotype. Interestingly, as of now, the only patients identified with this novel variant are these two Irish individuals that, to the best of our knowledge, appear to be unrelated. The second novel variant in *BEST1*, c.294G>T, has been classified in-house as likely pathogenic, and was found in only one patient in our study, CJ25. Examples such as this one underpin the importance of careful data sharing and curated variant databases such as LOVD.nl and HGMD (Human Genome Mutation Database), as increasing reports of such variants can allow us to infer their impact on human health and disease.

For 4/9 novel variants we have been able to confirm their phasing in the patient, where an autosomal recessive inheritance pattern is followed (**Supplemental Table 3.2**). Therefore, we have been able to apply the PM3 allelic/phasing ACMG/ACGS criterion based on allelic

data to a few of our novel variants. The use of this criterion has allowed us to upgrade *KCNV2* c.1354G>C from a VUS to Likely pathogenic, based on its presence *in trans* with a second pathogenic variant in the proband (CK56) and their sibling (CK57). Their father was found to carry the novel variant heterozygously and was homozygous wildtype for the second pathogenic variant in *KCNV2*, c.1381G>A. Conversely, the mother is homozygous wildtype for the novel *KCNV2* variant and was confirmed to carry the second pathogenic variant c.1381G>A heterozygously. As well as the use of allelic data for this *KCNV2* family, we have been able to apply PP1_Moderate evidence due to the extensive family available for segregation analysis (see **Supplemental Table 3.9** for full in-house classification). As mentioned previously, due to the large resulting tables containing classification data, they have been included in the Supplementals section to allow for easier reading of the main text.

A novel null variant was identified in *CFAP410*, c.460_469del, where phasing data from the family of patient CH67 has supported the upgrade of this variant from Likely pathogenic to Pathogenic (**Table 3.3, Supplemental Tables 3.2 and 3.9**). This 9-base deletion in *CFAP410* leads to the creation of a premature stop codon, where nonsense mediated decay (NMD) is expected to occur. Another novel variant resulting in a premature stop codon was found in *DRAM2*; c.700_706del. This variant was identified in a homozygous manner in patient CG48, where the parents have been confirmed to be first cousins but no genotyping for family members was possible (**Table 3.1, Supplemental Tables 3.9**). This variant occurs in the final exon of the *DRAM2* gene; therefore, it is not predicted to undergo NMD, but this deletion would result in the loss of more than 10% of the protein (approximately 12%). Using the PVS1 null variant criteria decision tree recommended by Abou Tayoun et al., we were able to apply PVS1_Strong criteria where the strength was altered due to the location of the variant in the gene and the amount of protein predicted to be lost (Abou Tayoun *et al.*, 2018).

A novel synonymous variant in *RS1* (c.327G>C) was identified in a female carrier; patient CH71 (**Table 3.3, Supplemental Tables 3.2 and 3.9**). This is an interesting case as *RS1* is an X-linked gene associated with retinoschisis in males, with very rare cases of a female carrier displaying a mild phenotype. A recent case study summarises a young female heterozygous carrier of a novel variant in *RS1* with a severe phenotype along with 10 carrier females that have been previously reported globally as an overview (Kirkby *et al.*, 2023). The novel *RS1* c.327G>C variant we have identified remains a VUS as we do not have sufficient information to upgrade or downgrade the class of the variant, but it does have an intriguing SpliceAI score of 0.29 indicating acceptor gain, surpassing our cutoff threshold and allowing us to apply the PP3 computational criterion (see **Supplemental 3.9** for full in-

house classification). Patient CH71 has an unaffected sister who is involved in the study (CI73), and she was found to not carry the *RSI* variant. As there are no guidelines stipulating the application of segregation evidence in the case of an X-linked carrier female, we cannot infer anything from this information but it is notable that the unaffected sister (of similar age) does not carry the variant of interest. No other variants of interest that may contribute to disease were identified in patient CH71, however it may be the case that the causative variant was not captured by this targeted panel, therefore we classified this patient as ‘possibly solved’. It would be of interest moving forward to undertake a functional assay for this *RSI* variant to analyse its potential impact on splicing, and for the patient to receive X-inactivation testing to facilitate a more thorough genetic diagnosis.

The final novel variant identified in the genetically resolved cohort was in *OPN1LW*; c.283del, in a single patient CC78 (**Table 3.1**). We have confirmed that the mother (CC79), is a heterozygous carrier of this variant (**Supplemental Table 3.2**). We have not been able to classify this variant as anything more than a Class 4/Likely pathogenic variant, as also predicted by Franklin Genoox and Varsome. We have applied PM2 population frequency criterion at a supporting level as suggested by the most updated ACGS/ClinGen recommendations, as the variant is absent from the gnomAD population database (Durkie *et al.*, 2024) (**Supplemental Table 3.9**).

While no trends have been observed within this Irish iMD cohort that could potentially hint to a broader geographic pattern of variation, we have identified 10 novel variants in iMD associated genes that have never been reported before in any other global population. Additionally, we have two seemingly unrelated Irish patients with the same novel variant identified in *BEST1* (c.203A>G), with a similar occurrence seen with two unrelated pedigrees carrying the c.1148del variant in *CNGB3* (**Supplemental Table 3.2**). While these variants are absent in global databases, their recurrence within this Irish population could suggest a local enrichment rather than sporadic occurrence, however we would need a much larger population size for this study to make any more definitive conclusions. We have also identified a deletion of two exons in *CDH3* in multiple affected family members within an Irish pedigree that has not been reported in any other individuals globally. The identification of such novel variants has expanded our knowledge of the genetic background of iMDs in Ireland, where the further use of WGS and long-range sequencing might allow us to investigate the presence of haplotypes within this population and look for founder mutations and IRD variants that are enriched within the Irish population.

Incomplete penetrance has previously been associated with various IRD disease genes, where individuals with the proposed disease genotype do not develop any clinical symptoms. Reduced penetrance can pose a problem when analysing family studies, especially in the case of proposed dominant inheritance as it can be that the candidate variant is present in a parent or sibling of the proband who appears unaffected. An example of a variant displaying reduced penetrance in an autosomal recessive case is the *CDHR1* c.783G>A variant, which we observed in a biallelic manner in patient CK50 (**Table 3.1**). This hypomorphic variant has been classified as pathogenic and is located in the final nucleotide of the exon, impacting splicing and leading to the skipping of exon 8. It is postulated that due to its role in maintaining the integrity and structure of photoreceptor cells, reduced levels of *CDHR1* could lead to an IRD phenotype. As natural levels of exon 8 skipping have been recorded in *CDHR1*, it is believed that the c.783G>A variant exacerbates this leading to further reduced levels of functional protein, causing disease (Sobolewska *et al.*, 2023a). Variation in *CDHR1* leads to a variety of ocular phenotypes. It has been reported that patients who are compound heterozygous for the c.783G>A mutation and a second null variant in *CDHR1* showed an intermediate phenotype with onset occurring after the fourth decade of life, early macular involvement and more widespread atrophy noted from the fundus imagery (Issa *et al.*, 2019a). This is indeed the case with our patient CK50, where the second variant observed in *CDHR1* is c.297+1G>T, which is predicted to be a null allele, as it will abolish the canonical donor splice site at intron 3 of the gene (Ba-Abbad *et al.*, 2020) (**Table 3.1**). The clinical phenotype observed in patient CK50 is similar to that described above, with age of onset occurring in early to mid-40s. We do not have access to any family member samples for patient CK50 and therefore acknowledge that we cannot be sure that the two variants are acting *in trans*. To further highlight cases of particular interest, the *RP1L1* c.133C>T variant has been associated previously with altered disease penetrance. While it has been reported in control individuals, this variant has also been linked with an autosomal dominant occult macular dystrophy phenotype, as the variant is located within a doublecortin domain that is vital in the interaction of RP1L1 with the RP1 protein in the photoreceptor axonemes (Akahori *et al.*, 2010). We believe this variant to be causative of disease in patient CI18 within our cohort (**Table 3.1**). This variant also appears to be present heterozygously in the patient's unaffected mother (CI19), which has been confirmed by in-house Sanger sequencing and a clinically accredited lab (**Supplemental Table 3.2**). Given the detailed reports of this variant's reduced penetrance, we believe this variant may be the most likely cause of disease in patient CI18 despite its presence heterozygously in the mother.

Macular degenerations can be broadly split into two categories; inherited iMDs which have been studied as part of this thesis, and age-related (A)MD. AMD is a clinically heterogeneous disease occurring in the later stages of life, and has been found to be the leading cause of irreversible blindness in the elderly population of the western world (Yonekawa and Kim, 2015; Colijn *et al.*, 2017). Due to the phenotypic overlap between AMD and certain late-onset iMDs, it can often be very challenging to differentiate between AMD and a macular dystrophy that appears to ‘mimic’ AMD solely through clinical observations and multimodal imaging (Dosunmu and Bakri, 2011; Saksens *et al.*, 2014; Kersten *et al.*, 2018). This commonality can often lead to misdiagnoses, therefore genetic screening of patients with such macular phenotypes is paramount for patients and their families to receive an accurate clinical diagnosis. An interesting case study arose as part of this iMD study, where a likely pathogenic monoallelic variant in the *CFH* gene was identified in patient CL2. *CFH* c.1291T>A was the only promising candidate variant identified. It has previously been reported heterozygously as suspected cause of disease in a family with bilateral macular drusen (Taylor *et al.*, 2019) (**Tables 3.1 and 3.5, Figure 3.7**). This variant occurs in complement control protein (CCP) domain 7 of the *CFH*-encoded protein factor H (FH), and its FHL-1 isoform which is expressed exclusively in Bruch’s membrane (Clark *et al.*, 2014; Taylor *et al.*, 2019). Both Taylor *et al.* 2019 and a previous study in 2015 have confirmed this variant leads to loss of a highly conserved cysteine required for correct function of the CCP domain 7, likely disrupting the protein structure and resulting in the lack of FHL-1 secretion (Triebwasser *et al.*, 2015; Taylor *et al.*, 2019). Such functional studies on *CFH* mutations have hypothesised that loss of function or null variants in CCP domains 1 through 7 results in haploinsufficiency of FHL-1, underlying the cause of early onset macular drusen (EOMD) in patients such as CL2. The analysis of AMD-associated loci and their potential contribution to iMD phenotypes is a growing and complex area of study, with rare and common variants in *TIMP3* and *FBN2* being associated with both AMD and iMD (Weber *et al.*, 1994; Chen *et al.*, 2010; Ratnapriya *et al.*, 2014). In this regard, monoallelic variants in *FBN2* have been identified in 4 patients within our study, where 3/4 patients harbour variants that have not previously been reported in affected individuals to the best of our knowledge (**Supplemental Table 3.4**). Given the novelty of the variants and the association of *FBN2* to an iMD phenotype, we will not consider these patients as solved until more is known about pathogenesis of *FBN2*-associated MDs.

This study using an MD-smMIPS panel to evaluate a cohort of Irish iMD patients has produced many informative outcomes to date including the identification of 9 novel variants,

and has allowed us to have a better understanding of the genetics underlying iMDs in Ireland. For some patients, once these research-grade findings have been clinically validated, this has aided in providing a more complete genetic and clinical diagnosis. Studies such as this allow us to deepen our knowledge of the genetic variation present in MD-associated disease genes, enabling more extensive variant classification and genotype-phenotype studies. This in turn will improve diagnosis capabilities, providing patients with accurate overall genetic diagnoses and offering patients greater clarity regarding disease prognosis, progression and access to more comprehensive care and treatment plans, and the potential for future participation in clinical trials/approved therapies, where appropriate. Continued analysis will undoubtedly facilitate the identification of further known and novel variants in MD-associated genes, with further research illuminating the pathogenesis of iMDs. Given the continued development of personalised medicine and novel gene therapies, it is increasingly important that patients receive a genetic diagnosis to allow for the implementation of optimal care plans.

3B. The First Report of BBS7-Associated Bardet-Biedl Syndrome in Ireland: A Clinical and Genetic case study

As presented within the first Chapter of this thesis, large scale sequencing studies are ongoing across the world in different IRD populations. They hold great promise in elucidating the pathogenesis of IRDs and in expanding our knowledge of how these diseases progress and manifest in affected individuals. The following case study is an example of an Irish pedigree from the Target 5000 cohort that is affected with a syndromic form of IRD, Bardet-Biedl syndrome (BBS), with affected individuals presenting with a cone-rod dystrophy in addition to a multitude of systemic features. This family is of particular interest as they are the first family in Ireland, to the best of our knowledge, with candidate variants identified in the *BBS7* gene, that has previously been associated with BBS. In contrast to the previous Chapter, these variants were found using whole exome sequencing (WES) as opposed to targeted smMIPs sequencing, therefore we continue to discuss the merits and pitfalls of various sequencing methodologies.

3B.1 Introduction

IRDs most often occur in isolation as non-syndromic disorders of the eye. However, it can also be the case that IRDs occur as part of a multisystemic condition, with an ocular phenotype being only one of the many symptoms present as part of a syndrome. More than 80 forms of syndromic IRDs have been described thus far, with more potentially left to be categorised (Singh *et al.*, 2020; Karuntu *et al.*, 2025). An ocular manifestation can occur as the first symptom, with the patient developing other systemic features later in childhood. Conversely the opposite can be true, where a patient presents with extraocular features in early to late childhood with an ocular phenotype developing only later in life. The case study presented here is an example of both of these situations occurring within the same family affected by BBS.

BBS is a rare pleiotropic ciliopathy that displays high levels of genetic and clinical heterogeneity. It is an autosomal recessive syndrome that affects multiple systems in the body due to malformation and malfunction of the primary cilia. BBS is characterised by extensive intra and inter-familial variability which will be discussed further in this case report. The prevalence of BBS varies greatly between global populations from ~1:160,000 in North America and Europe (Klein and Ammann, 1969; Beales *et al.*, 1997; Forsythe and Beales, 2013), to ~1:13,500 and ~17,500 in multiple isolated communities with higher observed rates of consanguinity (Farag and Teebi, 1989; Moore *et al.*, 2005). Characteristic

clinical symptoms of BBS include IRD, polydactyly, truncal obesity and kidney abnormalities among others (Niederlova *et al.*, 2019a). These are considered 'Primary' features, with 'Secondary' features being less common such as neurodevelopmental defects, situs inversus and hypogonadism (P L Beales *et al.*, 1999; Dollfus *et al.*, 2024a).

To date, loss of function variants in more than 24 genes have been implicated in BBS (Tsang, Aycinena and Sharma, 2018; Forsyth and Gunay-Aygun, 2023; Tian, Zhao and Zhou, 2023). The genes most commonly implicated in BBS encode proteins that together form the BBSome complex, involved in the biogenesis of the primary cilia and regulation of ciliary transport and signalling pathways (*BBS1*, *BBS2*, *BBS4*, *BBS5*, *BBS7*, *BBS8/TTC8*, *BBS9*, and *BBS18/BBIP1*) (Nachury *et al.*, 2007; Loktev *et al.*, 2008; Chou *et al.*, 2019). The second most common group of genes involved in BBS encode proteins that form a chaperone complex responsible for BBSome assembly (*BBS6*, *BBS10* and *BBS12*) (Seo *et al.*, 2010). Other implicated genes have varying roles in regulating the activation and entry of the BBSome into the cilia (*BBS3/ARL3*) (Seo *et al.*, 2010), as well as in intraflagellar transport (IFT) complexes IFT-A and IFT-B that interact with the BBSome and are responsible for bidirectional transport along the cilia axial filaments (Bujakowska *et al.*, 2015; Zhou *et al.*, 2022). Primary cilia are present in almost every cell type and are responsible for cell homeostasis by sensing extracellular stimuli and transmitting this information into cells, as well as regulating intracellular signalling (Nishimura *et al.*, 2019). Thus, the disruption of the BBSome and associated pathways of ciliary signalling and development lead to widespread multi-systemic features observed in BBS and such related ciliopathies. More specifically for this study, we will focus on the *BBS7* gene. *BBS7* is expressed in both rod and cone photoreceptor outer segments (OS), where the OS are specialised primary sensory cilia involved in mediating phototransduction (Zhang *et al.*, 2013; Bujakowska, Liu and Pierce, 2017). Genes that encode proteins involved in OS disc morphogenesis, homeostasis and function are implicated in various syndromic and non-syndromic IRDs, such as BBS (Manley *et al.*, 2023). The *BBS7* gene was first identified in 2003 (Badano *et al.*, 2003). *BBS7* has been implicated in approximately 2-6% of cases of BBS in various populations worldwide (Mockel *et al.*, 2011; Forsythe and Beales, 2013; Khan *et al.*, 2016), with some outliers such as a Russian population where the rate of disease-causing variants was observed to be much higher at around 24% in a recent study (Orlova *et al.*, 2024). To date, various databases have reported over 100 mutations identified in *BBS7*; 109 on LOVD.nl, 139 on HGMD and 142 on ClinVar (accessed March 2025: (Fokkema *et al.*, 2011; Stenson *et al.*, 2013; Landrum *et al.*, 2014). It is of note that one single database might not capture all of

the variation reported in a gene, but these three databases were updated most recently in 2024, with only slight deviations between them.

Here we report on two Irish siblings affected with BBS who are compound heterozygous for variants in the *BBS7* gene, with a particular focus on the ocular phenotype. WES performed prior to this doctoral thesis led to the identification of two null variants, one of which is believed to be novel, in *BBS7*. Here we describe the work since their identification, in order to confirm a BBS genetic diagnosis. Each variant was confirmed to be present in the patients and participating probands through Sanger sequencing, allowing for cascade analysis. Additionally, each variant was classified using ACMG/AMP guidelines along with ACGS recommendations for completeness. Clinical data was discussed with clinicians and gathered in order to collate information regarding genotype-phenotype comparisons. As this is the first Irish family where we have identified variants in *BBS7*, establishing and reporting on the disease phenotype is crucial in understanding the retinal pathology associated with this combination of genetic mutations. Sample acquisition, clinical assessment and WES sequencing were carried out prior to this doctoral thesis, as per the General Materials and Methods (Sections 2.1-2.5). Phasing analysis, Sanger sequencing and variant classification were carried out as per Materials and Methods described in sections 3.2.9 and 3.2.10 (Chapter 3), with all primers used listed in **Supplemental 3.14**.

3B.2 Results

The clinical and genetic profiles of two patients in this Irish *BBS7* pedigree are presented below. Patient B1, a 28-year-old female, was referred to the RVEEH with a presentation of a cone-rod dystrophy in addition to various severe syndromic symptoms. She was prescribed glasses at an early age (2 years old) for severe myopia and began to show a more progressive ocular phenotype in her late teens/early twenties. Upon further ocular examination, her recorded visual acuity (VA) was 6/60 and 6/36 in her right and left eyes respectively, with a normal lens appearance in both. As mentioned previously, an individual with regular vision would have a visual acuity score of 6/6. This reduced VA was first observed at age 20, with abnormalities in colour vision experienced around the same time period. This patient displays a protan defect implicating the long-wavelength 'L'-cones, meaning there is difficulty distinguishing between red and green colours. Photophobia, or 'light-sensitivity', was first noticed in the patient from age 19. This is a significantly later presentation than seen in Patient B2 who experienced photophobia since birth. Patient B1 began to experience central visual field defects at age 22. For Patient B1, while their visual fields showed concentric constriction to all 3 targets tested (targets IV4e, I4e and 04e), she still exhibited

reasonably good field retention. The horizontal degrees of field IV4e was approximately 110 for both eyes. Colour fundus photography, FAF and OCT imaging were used, as well as Full-field flash electroretinography (ffERG) testing. The ffERG for Patient B1 showed no convincing cone responses, with the light adapted 30 Hz and cone b wave amplitudes being non-recordable. It is important to note that while this does not necessarily indicate that there is no cone response, it could simply mean that the diminished cone responses remain below the threshold of the equipment. The dark-adapted rod isolated b wave amplitude in both eyes is at the lower limit of normal at 281uV in the right eye and 253uV in the left eye. This pattern of results from the ffERG is indicative of a cone-rod dystrophy.

Patient B2, a 30-year-old female, is the biological sister of Patient B1 and was referred to the RVEEH also with a presentation of a cone-rod dystrophy but with fewer systemic features than Patient B1. Unlike Patient B1, an ocular phenotype developed in very early childhood for Patient B2, with earliest photophobia symptoms being apparent since birth. A protan defect was observed as early as 5 years old, as well as reduced VA with recordings of 6/30 in the right eye and 6/19 in the left eye. Patient B2 experienced peripheral visual field defects at the same age, with concentric constriction to all three fields, similar to Patient B1. The horizontal degrees of field IV4e is approximately 100 degrees for both eyes, once again indicating reasonably good visual field retention. The ffERG revealed similarly affected cone responses as seen in Patient B1, with no recordable value for the light adapted 30 Hz and cone b wave amplitudes. A reduction in the dark-adapted rod isolated b wave was recorded in both eyes; 191uV in the right eye and 223uV in the left, as well as reduction in the a wave also seen in both eyes suggesting that although there is substantial rod activity, it is moderately decreased from the standard. This ffERG suggests a moderately more severe cone-rod dystrophy phenotype in Patient B2, with a much earlier age of onset. This information is displayed in tabulated format below (**Table 3B.1**).

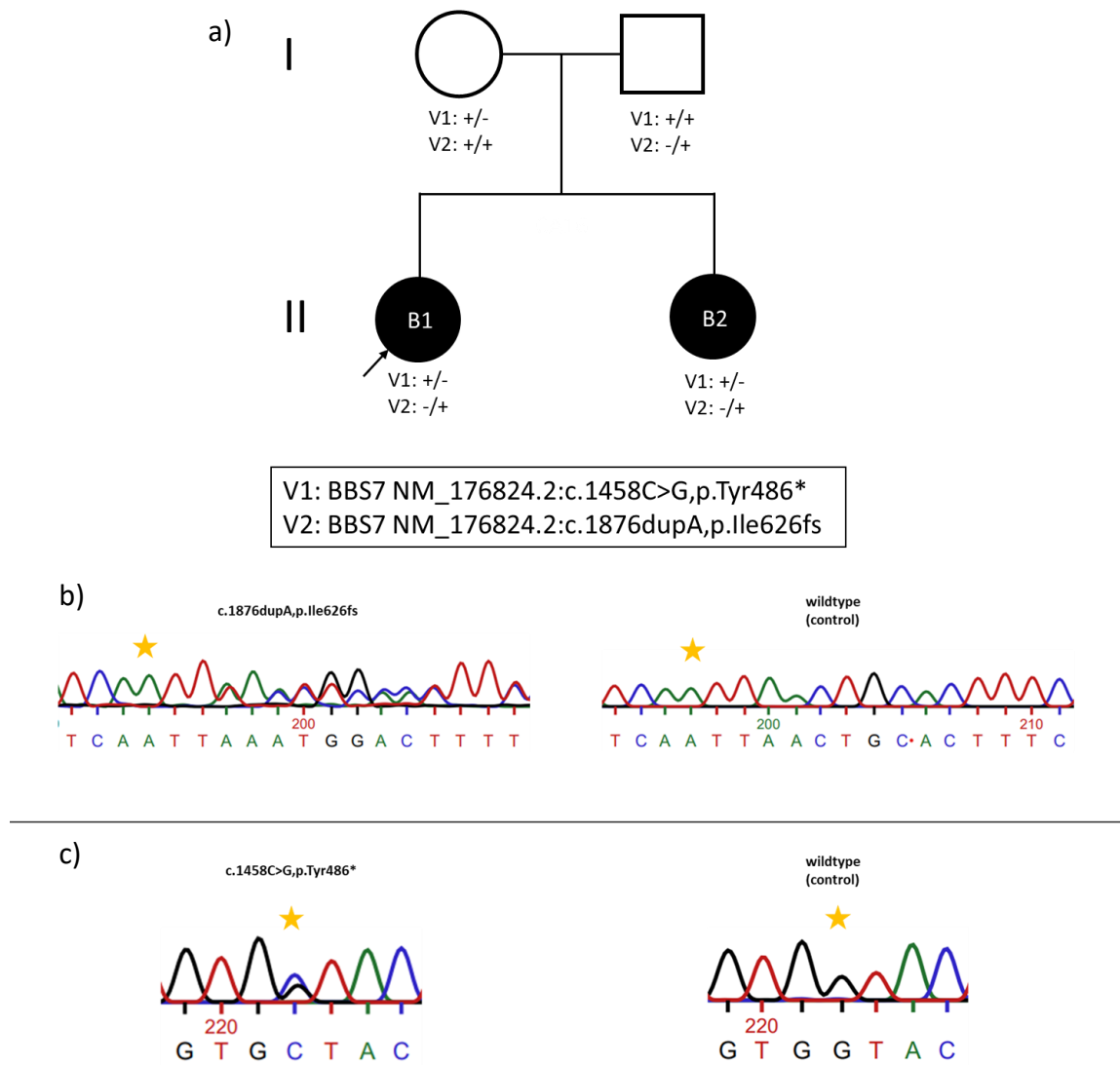


Figure 3B.1. *BBS7* Case study outline of pedigree and allelic data. (a) Irish pedigree tree displaying phasing of *BBS7* variants detected. The segregation pattern of the two recessive null *BBS7* mutations is shown here. Two siblings B1 and B2 are highlighted in black, with the arrow indicating the proband. (+ = wildtype allele, - = mutant allele). Sanger sequencing traces for *BBS7* variants (b) c.1876dupA,p.Ile626fs and (c) c.1458C>G,p.Tyr486*, where the yellow star denotes the location of the altered bp. Sequences were read from the sense strand and *BBS7* is located on the antisense strand.

Table 3B.1. Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision for *BBS7* patients B1 and B2. (OD; right eye, OS; left eye). (Records divided into 2 tables below, 1/2).

Patient	Clinical phenotype	Visual Acuity (VA)			Electroretinograph (ERG)					
		Visual Acuity - OD (right)	Visual Acuity - OS (left)	Age of onset	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz OD (uV)	Light adapted 30 Hz OD (uV)
B1	Cone-rod dystrophy	6/60	6/36	20 yrs	281	253	non recordable	non recordable	non recordable	non recordable
B2	Cone-rod dystrophy	6/30	6/19	5 yrs	191	223	non recordable	non recordable	non recordable	non recordable

Table 3B.1. (Records continued, 2/2).

Patient	Electroretinograph (ERG)	Visual fields		Colour vision		
	Any other details/comments on ERG	Peripheral or central field loss? (age of onset)	Any other details/comments on Visual fields	Abnormalities to colour vision (yes/no, age of onset)	Axis of colour dysfunction (protan, deutan, tritan)	Any other details/comments on colour vision
B1	Amplitude of rod isolated b wave is at the lower limit of normal in both eyes. No convincing cone responses either. Very similar ERG to sister, but slightly better	Central (22 years)	The fields showed concentric constriction to all 3 targets, IV4e, I4e and O4e. Similar pattern to sister, with slightly larger fields. Central visual field defects since age 22.	Yes, since late teens	protan defect	Has cone function and can discriminate colours
B2	Moderately reduced rod activity. Cone responses are non recordable. Not to say there is no cone activity it is just below equipment threshold.	Peripheral (5 years)	Similarly concentrically constricted on all targets.	Yes, since 5 years old	protan defect	Has cone function and can discriminate colours

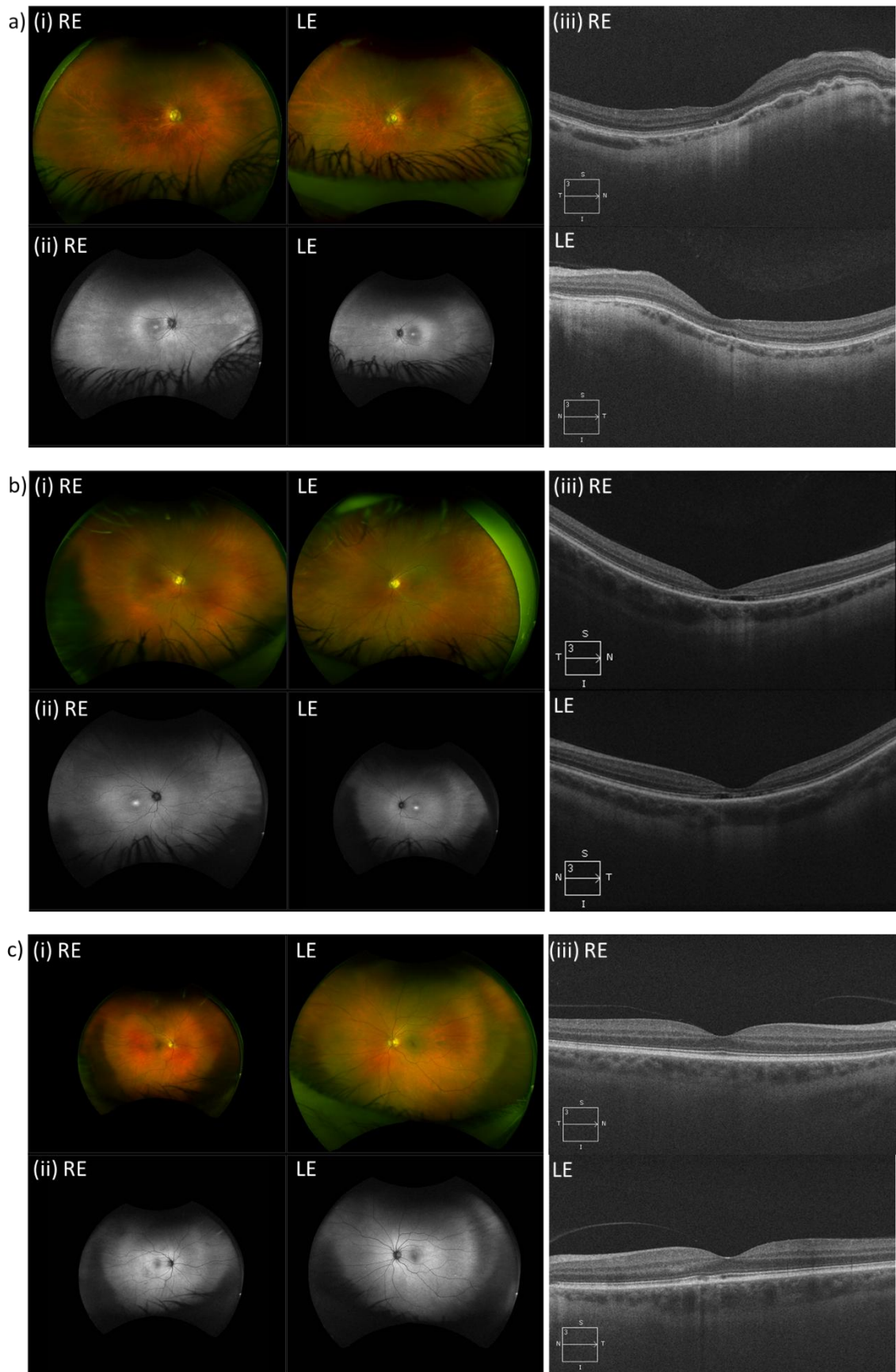


Figure 3B.2. Multimodal Retinal imaging for *BBS7* Patients. (a) B1 and (b) B2, and (c) their unaffected mother including; (i) Pseudocolour widefield fundus (ii) Fundus Autofluorescence (FAF) and (iii) Optical-coherence tomography (OCT) of both eyes (RE- right eye, LE- left eye).

The ocular imaging for both sisters is presented in **Figure 3B.2(a,b)**, along with images obtained from their mother who is phenotypically normal for both ocular and systemic features (**Figure 3B.2(c)**). The colour fundus for Patient B1 reveals very subtle attenuation of the blood vessels with a slight pigmentary disturbance at the fovea, while the periphery appears normal (**Figure 3B.2(a.i)**). In the FAF there are areas of hyperfluorescence at the right and left macula (**Figure 3B.2(a.ii)**). Patient B1's OCT imaging recorded a central retinal thickness of 221 and 197 microns in the right and left eyes respectively. The expected normal range is in the upper 200s. Extensive thinning of the photoreceptor layer, and loss of photoreceptors is observed centrally in both eyes (**Figure 3B.2(a.iii)**). Patient B1 is seen to have an irregularity of the RPE nasally, mostly apparent in the right eye. The imaging between the two sisters differs greatly, this is especially seen in the OCT images. The optic disc appears to be slightly pale in the colour fundus for Patient B2, which is not seen in her sister (**Figure 3B.2(b.i)**). Of note, in the colour fundus of Patient B2, there is stippling observed on the right macula with a slight pigmentary disturbance, while the blood vessels appear of good calibre and the periphery appears normal. In the FAF, areas of hyperfluorescence are observed on the macula in both eyes, with these areas corresponding to atrophic changes observed in the fovea of both eyes on the OCT image (**Figure 3B.2(b.ii, iii)**). Patient B2 has a recorded central retinal thickness of 156 and 157 microns in the right and left eyes respectively, which is below the standard threshold. The OCT also shows the loss of photoreceptors at the fovea of both eyes, denoted by subfoveal hyporeflective regions, while the photoreceptor layer appears relatively healthy peripherally.

These siblings were referred not only because of their ocular phenotype, but also due to the presence of extensive systemic phenotypes that could be characteristic of a syndrome (**Table 3B.2**). Their genetic diagnosis aligns with the systemic features observed, especially in Patient B1 who has extensive and severe extra ocular features. Typically, BBS can have a highly variable presentation even within affected families, with complex multisystemic involvement. Physical examination of Patient B2 indicates that she was obese from early childhood, displaying patterns of female baldness and alopecia. She also presents with the absence of one breast, which is speculated to be a developmental defect, but the cause has not been confirmed. Conversely, Patient B1 presents with a multitude of extraocular features from a very young age. Past surgical history indicates the presence of polydactyly on the patient's hands, as well as chronic liver disease that led to full transplant surgery in early teens. Heart, renal and hepatic anomalies were indicated in notes from Patient B1's referring doctors, as she presented with an atrial septal defect, renal dysplasia and extra hepatic biliary

atresia. Dyspraxia is also noted, indicating delayed motor development. A few of the systemic features observed in Patient B1, such as situs inversus, polysplenia and the bowel anomaly, are indicative of developmental anomalies, and are less commonly seen in BBS than the primary features. These features will be outlined further in the discussion section of this subchapter.

Table 3B.2. A comparison of characteristic systemic manifestations of BBS between two siblings with same *BBS7* genotype. (// = not present).

	Digit anomaly	Obesity	Hypertension	Motor skills	Heart defect	Renal anomaly	Hepatic anomaly	Hair phenotype	Developmental features
Patient B1	Polydactyly	Yes	Yes	Delayed - Dyspraxia	Atrial septal defect	Renal dysplasia, Nephritis	Extra hepatic biliary atresia, chronic liver disease	//	Situs inversus, polysplenia, malrotation of bowel
Patient B2	//	Yes	//	//	//	//	//	Female pattern baldness / Alopecia	Unilateral absence of breast

Biallelic null *BBS7* variants; c.1458C>G, p.Tyr486* and c.1876dupA,p.Ile626fs, were identified via WES of Patient B1 (OMIM*607590). Sanger sequencing confirmed their presence in Patient B2, with cascade analysis revealing that the parents are heterozygous carriers of these variants thus confirming the variants are acting *in trans* in the affected siblings (**Figure 3B.1(a-c)**). Given that one variant results in the generation of a premature stop codon and the other results in a frameshift, radical effects on the encoded protein are predicted, as follows. Both variants have been classified as Pathogenic variants according to the ACMG/AMP guidelines, while incorporating the updates and recommendations published by ACGS) as well as the ClinGen Sequence Variant Interpretation (SVI) working group (Richards *et al.*, 2015; Durkie *et al.*, 2024) (**Table 3B.3**) To the best of our knowledge, *BBS7* c.1876dupA,p.Ile626fs*2 is a novel variant that has not been previously reported in literature or on databases such as ClinVar, LOVD.nl or gnomAD. The observed change for c.1876dupA is a thymine insertion after a duplicated adenine at position chr4:121828416 (GRCh38). This change leads to a frameshift in the hairpin domain of the gene where the resulting mRNA is predicted to undergo nonsense mediated decay (NMD). The *BBS7* c.1458C>G, p.Tyr486* variant has not been reported in the literature previously in a patient affected with BBS to the best of our knowledge, however there are 3 prior likely pathogenic and pathogenic classifications on ClinVar associated with a BBS phenotype. This variant leads to the substitution of a guanine for a cytosine at position chr4:121835197 (GRCh38). The substitution occurs within the gamma-adaptin ear (GAE) domain of *BBS7* which is responsible for heterodimerisation within the BBSome (Singh *et al.*, 2020). The c.1458C>G variation leads to the creation of a premature stop codon, where the resulting mRNA is also predicted to undergo NMD.

Table 3B.3. Detailed overview of the classification of two *BBS7* null variants, according to ACMG/AMP guidelines along with SVI ClinGen Splicing group recommendations.

Gene	Transcript	Variant		Population data			Computational and predictive data			Segregation data			Allelic data			PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	Points Total: classification
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score									
<i>BBS7</i>	NM_176824.2	c.1458C>G	p.Tyr486*	Absent or extremely low frequency in population databases	Total allele frequency is 0.000004337 gnomAD v4.1.0 with no homozygotes. Suggested cutoff threshold is 0.00077 based on most frequent pathogenic variant. 3 reports on ClinVar of likely pathogenic/pathogenic, and not in LOVD.nl.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in exon 14/19 creating a premature stop codon, where the resulting mRNA is predicted to undergo NMD. LOF is a known mechanism of disease.	PVS1	N/A	More family members required	N/A	N/A	Confirmed in trans with novel variant where classification has not been validated	N/A	8	0	0	1	0	0	0	9	Likely pathogenic
<i>BBS7</i>	NM_176824.2	c.1876dupA	p.Ile626fs*2	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in exon 17/19 creating a premature stop codon 2 amino acid residues downstream, where the resulting mRNA is predicted to undergo NMD. LOF is a known mechanism of disease.	PVS1	N/A	More family members required	N/A	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with a likely pathogenic variant in a single proband (B1), and one related sibling. Only single proband from the pedigree can be used to award points (1pt)	PM3	8	0	2	1	0	0	0	11	Pathogenic

Each coloured heading represents a different group of data used. Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per ACMG/AMP guidelines and ACGS updated recommendations. The classification is listed in the final column. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria). See Materials and Methods section 3.2.9 for *in silico* tool thresholds.

3B.3 Discussion

BBS is a heterogeneous ciliopathy which can be characterised by retinal dystrophy, obesity, renal and hepatic malformations, and polydactyly, as well as a multitude of secondary features caused by the malfunction of the primary cilia throughout multiple systems of the body. Two loss-of-function variants in the *BBS7* gene have been found in the pedigree described in this case report. To the best of our knowledge, this is the first reported Irish family affected with BBS where the causative variants have been identified in the *BBS7* gene. As well as this, both variants identified are null *BBS7* variants that have not previously been reported in affected individuals in the literature. We have expanded on the spectrum of *BBS7* variation by describing a novel variant found in an Irish pedigree within this case report, adding to the genetic landscape of BBS. The *BBS7* c.1876dupA,p.Ile626fs results in a frameshift leading to the creation of a premature stop codon. This mutation occurs within the C-terminal α -helix region which forms a coiled-coil. In *BBS7*, this coiled-coil domain does not bind another subunit, but instead comes into contact with the helical neck of the BBSome that joins together the two lobes of the complex (Singh *et al.*, 2020). A premature stop codon in this region is predicted to create an mRNA transcript that will undergo NMD, thereby preventing creation of aberrant protein. The second variant, *BBS7* c.1458C>G, p.Tyr486*, also creates a premature stop codon, but this time in the GAE domain of the gene. This domain is responsible for the heterodimerisation of the BBSome, and allows the BBS2 subunit to dimerise with BBS7 in the first step of BBSome formation (Singh *et al.*, 2020). This variation in *BBS7* is also predicted to produce mRNA that will undergo NMD. The presence of these two nonsense variants in Patient B1 and B2 heterozygously suggests that no healthy wild type *BBS7* protein is produced.

The complete profile of BBSome function and structure is still not fully understood, and because of this neither is the pathogenesis of BBS and other such ciliopathies. While this is true, it is clear from animal studies and the analysis of the mutational profile of the BBSome, that the protein complex is necessary for primary cilia and multi-organ homeostasis, as well as facilitating their developmental processes, as seen from renal and hepatic abnormalities (Tian, Zhao and Zhou, 2023). In the eye specifically, it has been shown that the BBS genes and their encoded proteins are required for OS maintenance and renewal. Various BBSome component knockout mice have been seen to have photoreceptor OS morphogenesis anomalies, BBSome cargo mislocalisation and reduced retinal function in early life (Hsu *et al.*, 2017). This occurs during development as well as in adult mice, indicating that the BBSome does not only have a role in development but is important throughout life. The rate

of retinal degeneration observed varied depending on the gene that was knocked out, with *Bbs2* and *Bbs7* knockout mice showing a relatively slow degeneration rate (Hsu, Seo and Sheffield, 2021). A study using knockout *Bbs7* mice has shown that the BBS7 protein is required for the stability of the BBS2 protein, and its absence prevents correct formation of the BBSome (Zhang *et al.*, 2013). Additionally, when *BBS7* is absent, an abnormal buildup of dopamine D1 receptor (D1R) in the ciliary membrane was observed. This suggests that *BBS7* has a central role in the transportation of specific ciliary proteins to and from the plasma membrane. In other BBS mouse models the proteins responsible for metabolic homeostasis and vesicle trafficking in photoreceptors, usually localised to the IS, are seen to accumulate in the OS, ultimately leading to a depletion of these proteins in the cell body where they are required, causing photoreceptor degeneration (Datta *et al.*, 2015). Loss of BBSome components leads to rhodopsin mis-localisation in rod photoreceptor IS and cell bodies, leading to a depletion of rhodopsin in the OS where it is required for phototransduction, in turn causing photoreceptor death or apoptosis. Notably, BBS gene products have an essential role in cones as well as rods (Dilan *et al.*, 2018). The loss of *BBS8* in cone photoreceptors led to the accumulation of IS fusion proteins in the OS of cones only, and their isolated progressive degeneration. This is a good indicator that the cones are degenerating in BBS due to the impact of the BBSome variants themselves. While these observations remain true, it is still unclear why severe cone dysfunction and milder rod involvement are reported in some BBS patients such as those analysed within this thesis chapter, rather than a more rod-involved phenotype, as both have been associated with *BBS7* variants previously (Niederlova *et al.*, 2019; Chandra *et al.*, 2022; Xin-Yi, Yang-Li and Ling-Hui, 2023; Orlova *et al.*, 2024).

BBS gene products have also been shown to be important in the correct positioning and density of synapses between the photoreceptors and other neurons of the retina (Hsu *et al.*, 2020). Another study has underpinned the role of BBS proteins in the correct development of the RPE, as mice display irregular RPE layer maturation when *BBS8* is knocked out, with a down regulation of mature RPE markers such as *RPE65* (May-Simera, *et al.* 2018.) These results demonstrate that variants in the genes encoding BBS proteins have impacts in other cells of the retina outside of the photoreceptors. It has been suggested that the loss of the endothelial BBSome contributes to abnormalities observed in retinal development (Jiang *et al.* 2021), and that therefore it may be of importance when developing gene therapies for BBS-derived vision loss that a broad spectrum of retinal cell types is targeted.

Curiously, the affected siblings in this Irish pedigree display varying systemic and ocular phenotypes while sharing the same *BBS7* genotype. This further illuminates the extremely variable intra-familial severity of BBS and ciliopathies as a group of disorders. This case report places particular emphasis on the ocular phenotype presented, given the retinal focus of the studies within this doctoral thesis. Both affected sisters present with a cone-rod dystrophy phenotype with differing ages of onset and progression. While Patient B1 experienced myopia at an early age, her ocular phenotype did not fully manifest until her late teens/early 20s, displaying a progressive decline. Conversely, Patient B2 presented with photophobia since birth with colour and visual acuity defects recorded as early as 5 years of age. Even though Patient B2 has lost a substantial amount of vision, it has seemingly stabilised in the third decade. The ocular phenotypes observed in BBS are highly heterogeneous as both rod and cone photoreceptors are seen to degenerate to different degrees in individuals even with the same genotype (Mockel *et al.*, 2011; Grzybowski *et al.*, 2025). Additionally, global retinal degenerations with RPE involvement have also been reported. In the patients described here, they have both presented with a cone-rod dystrophy with profound vision loss occurring before and during the second decade of life. In addition, the two patients in the case study present with a wide range of systemic phenotypes. Taking the ocular phenotype into consideration, the only other physical symptom observed to be shared between both sisters is an early childhood difficulty with weight gain and obesity. Obesity is thought to be the second most common feature of BBS, with over 70% of children under 5 years of age, and over 90% of patients in mixed populations aged 5 and above presenting with this phenotype (Mockel *et al.*, 2011; Pomeroy *et al.*, 2020; Xin-Yi, Yang-Li and Ling-Hui, 2023).

Post-axial polydactyl was observed only in Patient B1, with this being another common feature of BBS seen in approximately 69% of cases (Mockel *et al.*, 2011). Renal abnormalities are one of the primary features used to diagnose BBS, with patients commonly displaying a variable kidney phenotype (Dollfus *et al.*, 2024b). Patient B1 presents with renal dysplasia and fluid filled cysts on the kidneys, indicating a structural and developmental abnormality of the kidneys and renal system. The hypertension noted in Patient B1 is thought to be due to these renal defects. Gastrointestinal abnormalities are one of the secondary features of BBS, where liver disease is often characterised by some bile duct malformation. Chronic liver disease was observed in Patient B1, with extrahepatic biliary atresia meaning an absence or narrowing of the bile ducts where a full transplant was required. Approximately 30% of BBS patients present with some form of hepatic abnormality

(Branfield Day *et al.*, 2016). Delayed motor development in Patient B1 is seen, with the patient's family reporting dyspraxia and clumsiness in the patient. Cardiovascular and thoraco-abdominal defects are less common and are considered secondary features. An atrial septal defect was noted by previous consulting doctors in Patient B1, but was seen to have spontaneously closed and therefore was assumed to be very small in size. Heart anomalies are seen in less than 30% of all BBS patients (Niederlova *et al.*, 2019b), but are almost always present when laterality defects are present. Some of the thoraco-abdominal anomalies observed in Patient B1 can be classified into a subcategory of defects termed 'laterality' defects. These include situs inversus, polysplenia and malrotation of the bowel, all of which are consequences of incorrect development due to malfunction of the primary cilia in the embryo. Such abnormalities are very rare, and have been seen to occur in ~1.6% of patients with BBS (Olson *et al.*, 2019).

As the variants identified in this pedigree in *BBS7* are both null alleles that lead to NMD, it is likely that no functional protein is produced. *BBS7* and *BBS2* within the BBSome depend on each other to maintain stability of the protein complex and to ensure the membrane proteins that they transport are localised correctly to the cilia. As mentioned previously, animal models have shown that the absence of *BBS7* does not completely eliminate the function of the BBSome, but that it does lead to the buildup of specific proteins such as D1R and Smo (Zhang *et al.*, 2013). D1R is a dopamine receptor which is vital in regulating cognitive function and appetite (Arnsten, Wang and Paspalas, 2015; Jones-Tabah *et al.*, 2022), where potentially its abnormal buildup in the neuronal cilia due to *BBS7* knockout could lead to the behavioural anomalies, cognitive impairment and obesity symptoms often experienced by BBS patients. Similarly, Smo is a component of the sonic hedgehog signalling pathway and studies on knockout *BBS7* mice have shown that there is an increase in Smo localisation to the cilia in comparison to wildtype mice, and have hypothesised that this may be due to the lack of the BBSome ability to transport Smo out of cilia efficiently in the absence of *BBS7* (Zhang *et al.*, 2012). The primary cilia are integral to regulating the hedgehog pathway, whose function is to regulate organogenesis, cell positioning and cell fate during early development (Zhuang, 2025). Thus, both dysregulation of the primary cilia and the accumulation of Smo due to lack of *BBS7* or correct BBSome function likely lead to the developmental defects and the diverse symptoms experienced by BBS patients. As mouse models often cannot recapitulate the intricacies of all human BBS symptoms, to analyse kidney anomalies in more detail a recent study from Min *et al.*, (in pre-proof) described their use of patient-derived iPSCs from an individual with biallelic *BBS7* variants

to establish a kidney lineage model to analyse the impact of *BBS7* mutations on kidney development and anomalies (Min *et al.*, 2025). Indeed, they found that the pathogenic biallelic variants in *BBS7* led to reduced expression of both *BBS7* and *BBS2* which led to dysregulated cilia formation and structural defects in the kidney-like tissue, due to disruptions to the hedgehog pathway, required for correct kidney development. Functional studies using animal and system models as described, while both time consuming and resource intensive, would continue to provide direct evidence regarding the interplay in BBS pathogenesis of the ciliary signalling systems and BBS/IFT genes such as *BBS7*.

While Patient B2 presents with much fewer systemic features, they are much rarer and seem to be associated with more general disruptions of the cilia caused during development. This patient presents with alopecia, which is not widely reported in BBS. Despite this, it has been shown that primary cilia play an important role in the signalling pathways needed for correct morphogenesis of hair follicles (Lehman *et al.*, 2008). This study described BBS patients presenting with various dermatological abnormalities such as seborrheic dermatitis and some skin conditions associated with obesity, suggesting an impact of BBS on skin health (Lehman *et al.*, 2008). Patient B2 also presents with a suspected congenital anomaly where there is a unilateral absence of one breast or ‘amastia’. While we know that correct primary cilia function is vital for the development and function of many organs of the body, it is not clear whether this absence of a breast is due to the ciliopathy genotype present in this patient or whether there is a parallel undiagnosed disorder here. Developmental problems and issues during puberty can occur due to endocrine dysfunction that have been noted in ciliopathies such as BBS (Mujahid *et al.*, 2018).

While BBS is inherited in an autosomal recessive fashion, secondary genetic modifiers and an oligogenic inheritance pattern have been previously reported in rare cases where three disease-causing variants have been found at two different BBS loci within affected individuals (Bandano *et al.*, 2003). It is hypothesised by many that the second-site genetic mutations act as modifiers to severity of disease, which could account for the high levels of intrafamilial variation seen in BBS phenotypes, among other syndromes. This has been noted in family studies and large cohort analyses, where a third mutation in a second gene was seen to correlate with early onset phenotypes or more severe rates of disease progression, with second-order modifier loci now being proposed (Bandano *et al.*, 2003; Eichers *et al.*, 2004; Perea-Romero *et al.*, 2022). While such studies examining secondary modifiers of BBS are still emerging, significant strides have been made in highlighting the contribution of the mutational load within the entire ciliary proteome on BBS prognosis and progression,

as well as disease severity and phenotypic variability. Linstrand *et al.*, re-sequenced 17 patients with a form of BBS, where their primary causal biallelic variants had already been identified in a single causal BBS gene, in order to see if any additional CNVs or SNVs are present in the other BBS and IFT genes, along with a few other loci that have been shown to contribute to ciliopathies (Linstrand *et al.*, 2016). 12/17 probands carried 1-4 additional pathogenic mutations in one or more BBS-associated genes (*BBS1-16*, *IFT27/BBS19*, or *NPHP1*) as well as the biallelic mutations in their primary disease BBS gene, indicating oligogenic inheritance and the potential contribution of mutations outside of the primary causal locus to disease presentation. Re-analysis and employment of more comprehensive sequencing methodologies for individuals who have already received a primary causal genetic diagnosis may continue to prove fruitful in identifying protective and additive modifier loci. Individuals presenting with milder BBS symptoms, with no anomalies indicated in certain organ systems characteristic of ciliopathies may even be informative in identifying protective variant combinations. More recently, a large-scale study on 99 BBS patients was carried out to assess allelic overload in BBS genes as a modifier (Perea-Romero *et al.*, 2022). It was found that family members with only biallelic variants in a single gene indeed presented with a milder BBS phenotype in comparison to members of the same family who carried an additional variant in a secondary gene. It was also noted that in one family presenting with biallelic *BBS1* variants as the primary causal genotype, that one family member carrying only these primary variants presented with non-syndromic RP whereas family members carrying an additional null variant in *BBS5* (p.(Arg207His)) presented with more severe syndromic BBS. It has been found that BBS proteins that are involved in specific complexes are more often implicated as modifiers than others, where genes that encode for the chaperonin-like complex (*BBS10*, *BBS12*, and *MKKS*) tend to cause a more severe phenotype and do not appear to be modified by secondary loci. While there are indeed exceptions to this, it is interesting to note that the components of the BBSome and other ciliary signalling proteins that rely on the activity of the chaperonin-like complex appear to modify disease more frequently (Castro-Sánchez *et al.*, 2017; Niederlova *et al.*, 2019a; Perea-Romero *et al.*, 2022). The potential interplay between primary genetic mutation(s) and secondary genetic and environment drivers of ocular disease, is a topic of increasing interest and has indeed been expanded on in recent years. Fully elucidating the pathogenesis of conditions such as BBS will require interrogation of possible modifier loci, among other drivers of disease. It is important to consider this potential for a non-Mendelian inheritance pattern when planning clinical diagnosis and management of BBS.

Up until recently, BBS diagnostic criteria used in clinical practice was based solely on clinical features and the analysis of physical symptoms observed in the patient (Beales *et al.*, 1999). A BBS clinical diagnosis is given based on the presence of either four primary features, or three primary and two secondary features, as described by the cited modified guidelines (Forsythe and Beales, 2013). In recent years, with the improvement of NGS and widespread availability of genetic testing, a confirmed molecular finding has become a necessary part of the diagnostic pathway in syndromic conditions like BBS with such pleiotropic physical phenotypes. In 2024, 4 European Reference Networks (ERNs) came together to devise a new plan for standard of care, diagnosis and overall management of BBS patients in Europe (Dollfus *et al.*, 2024). This included implementing a genetic diagnosis into the diagnostic pathway, as well as promoting genetic counselling for patients and families. The 4 ERNs that contributed are: ERN-EYE (ophthalmology), Endo-ERN (endocrinology), ERKNet (nephrology) and ERN-Ithaca (developmental anomalies). This group has recognised that while genetic testing has progressed and is not as costly as it was, it still remains unavailable in certain cases, and so have described more stringent clinical criteria where a molecular diagnosis was not possible. A ‘High’ and ‘Moderate’ confidence diagnosis can be given depending on what clinical and molecular criteria are met. Where a ‘High’ confidence diagnosis is achieved, the patient may be eligible for future clinical trials for BBS symptoms, receive BBS-specific medications and avail of genetic counselling. Where a ‘Moderate’ confidence diagnosis is achieved, consistent clinical monitoring and regular follow-ups are recommended until either a ‘High’ confidence diagnosis is possible, or a different diagnosis is reached. The same group defined a genetic diagnosis as the presence of biallelic class 4 or 5 variants in one of the previously described BBS-associated genes. If biallelic variants are found in a single gene, the potential presence of triallelism or modifier loci is not taken into account in the initial molecular diagnosis, but is later considered in predictions of disease progression and severity.

Currently in Ireland, the National Genetics and Genomics Office (NGGO) have developed the National Genomic Test Directory to be implemented as part of the national genomics strategy as a way to provide more accessible genetic testing to the Irish population (‘National Genomic Test Directory Rare and Inherited Disease - Version 1’, 2024. Accessed March 2025). Within this Test Directory, a panel for BBS has been described to include 17 genes previously associated with BBS, where either targeted sequencing of this panel or WES will be completed for the patient. While BBS mutations predominantly cause multi-system syndromic disorders such as that observed in the BBS cases described in this Sub-chapter

case study, variants in BBS genes, with the exception of *BBS1*, have been seen in non-syndromic cases of retinal degenerations and RP, however these cases make up the minority of BBS-gene associated disease (Riazuddin *et al.*, 2010; Shevach *et al.*, 2015; Hulleman *et al.*, 2016; DeBenedictis *et al.*, 2020). Therefore, while this remains true in some instances, it is most often the case that BBS occurs as a multi-systemic syndrome, hence it is recommended that BBS patients receive monitoring and diagnoses from multidisciplinary teams taking all aspects of the ciliopathy into account.

BBS is one of the most genetically heterogenous forms of syndromic IRD. Due to the extensive phenotypic overlap between BBS and other ciliopathies and IRD syndromes, a conclusive diagnosis based on physical features alone can be challenging. Therefore, it is of growing importance that genetic testing be adopted into the diagnostic pathway to ensure a definitive clinical diagnosis and optimal disease management plan for each patient. This not only provides the patient with autonomy over their medical decisions, as well as clarity on disease prognosis and prenatal planning, when required. However, it also allows their family members to be better prepared in terms of knowledge of care and further reproductive planning. More globally, it enables the broadening of knowledge regarding the genetic pathogenesis of BBS with the identification of novel gene associations and disease causing variants in known genes. Targeted panel sequencing, as was used in Chapter 3, tends to be very cost-effective and represents an efficient form of preliminary NGS. While this remains true even for BBS, this requires the clinical team to be confident in a BBS diagnosis even without any prior molecular testing. Therefore for syndromes such as BBS, it has more recently been suggested that a comprehensive form of sequencing such as WES or WGS, where available, might be more informative. In summary, as part of the research presented within this Sub-chapter case study, we have identified the first Irish *BBS7* pedigree with two patients both carrying two null *BBS7* mutations *in trans*, which are predicted to result in NMD and therefore have radical effects on the encoded protein. The diversity in clinical phenotypes between these two *BBS7* patients, with regards to both their retinal and extra-ocular phenotypes, highlights the clinical diversity that occurs in syndromes such as BBS, even within a single family. Indeed, we are just at the infancy of elucidating the molecular basis of these syndromic disorders.

4. Variant Classification Across Irish IRD pedigrees: A Family study Collection

4.1 Introduction

The overarching aim of the study, and similar such studies globally, is to provide an overview of the genetic architecture of inherited retinal degenerations (IRDs) and to highlight candidate disease-causing variants. Specifically, as part of the research arm of Target 5000 in TCD, along with the associated clinical teams throughout Ireland, we aim to characterise candidate disease-causing mutations in the Irish IRD patient cohort. These are subsequently validated in an accredited laboratory. As outlined previously in the General Introduction (Section 1.6), this project employs next generation sequencing (NGS) and Sanger validation to genotype as many IRD patients as possible in Ireland (Dockery *et al.*, 2017; Whelan *et al.*, 2020a; KAJ *et al.*, 2021b). More specifically for the families described in the following chapter, target capture sequencing was performed for 254 IRD genes prior to the beginning of this study. This type of NGS was described previously for a cohort of over 680 patients, as part of the Target 5000 study (Whelan *et al.*, 2020a).

Until recently, it has been standard practice in clinical care for individuals with IRDs to receive a purely clinical diagnosis based on disease phenotype alone. As mentioned previously, many genes associated with IRDs can individually cause a multitude of ocular disease phenotypes, and can sometimes cause syndromic disorders which include ocular phenotypes (Duncan *et al.*, 2018c; Ben-Yosef, 2022b). Additionally, multiple genes can lead to the same clinical presentation. As IRDs are a highly clinically and genetically heterogeneous group of diseases, precise diagnosis can be challenging. This heterogeneity makes not only a clinical diagnosis difficult, but also a genetic diagnosis complex. It is important that we do not allow the disease phenotype or presentation of disease to limit our search for the possible genetic causes of an IRD, as if we do so it is likely that we may miss the true genetic cause given the significant heterogeneity outlined above. There are often new genotype-phenotype correlations being described in IRDs, facilitated by comprehensive sequencing methods and large-scale population based studies (X.-F. Huang *et al.*, 2015; Chen *et al.*, 2020; Ellingford, Hufnagel and Arno, 2020; Georgiou *et al.*, 2024; Sahin *et al.*, 2024; Fan *et al.*, 2025). In order to successfully reach our primary objective for the Target 5000 project, a multidisciplinary team approach is needed to gather and interpret various forms of evidence regarding genetic causality on a case-by-case basis, to reach a final informed diagnosis for each patient.

The following chapter of this thesis describes the process of further analysis and validation of the candidate disease-causing variants that have been identified in 13 IRD pedigrees from the Target 5000 patient cohort, outside of those described as part of the macular smMIPs study (see Chapter 3). Family studies, also termed ‘cascade analysis’, are vital in providing the phase of the candidate variants proposed as causative in a pedigree. If candidate variants do not segregate with the disease, or are found to be present in individuals who are determined to be ‘unaffected’ by our clinical teams, then the patient remains unresolved. Therefore, for such patients we must reanalyse NGS data, possibly expanding search parameters or progressing to WGS. Phasing data can provide evidence towards classification of variants, which can then be reported and published in a public database, such as Franklin Genoox, LOVD.nl or ClinVar, in order to help inform the work of other ocular diagnostic laboratories and clinicians internationally (*Franklin*, accessed 2025; Landrum *et al.*, 2014; Harrison *et al.*, 2016; Fokkema *et al.*, 2021).

4.2 Materials and Methods

4.2.1 Patient cohort and clinical assessment

Patients described in this chapter all received a clinical diagnosis from ophthalmologists in either the Royal Victoria Eye and Ear Hospital, or the Mater Misericordiae University Hospital, as part of Target 5000. Blood or saliva samples were obtained from patients and family members, following informed consent, in one of the affiliated hospital sites. Once the patient had consented, a combination of comprehensive tests was carried out. These tests are outlined in the General Materials and Methods section 2.2 and encompass Peripheral vision fields, Colour vision, Full Field electroretinograms, Visual acuity, Fundus colour and autofluorescence photos, and Optical Coherence Tomography (OCT) images (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020b).

4.2.2 Genetic screening

The sequencing methodology carried out for the proband and their family members will be indicated for each case study in the Results section (**Table 4.1**). More details on each type of sequencing are outlined in the Next Generation Sequencing section 2.5 of the General Materials and Methods. The variants called were filtered, with causative variants being identified based on genotype-phenotype correlations, taking into account variants with a minor allele frequency of less than 1% in the Irish in-house and gnomAD databases (Karczewski *et al.*, 2020).

4.2.3 Polymerase Chain Reaction (PCR) and Gel electrophoresis

5x FIREPol Master Mix (Solis Biodyne, Tartu, Estonia) and Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA) were used for PCR reactions, according to the respective manufacturer protocols. All primers for PCRs and Sanger sequencing were designed using Primer3plus software, and were purchased from Sigma-Aldrich (Gillingham, England, UK). An array of percentages of agarose gels (0.8%-4%) were used for the analyses in this study depending on the size and type of product being run, using 1 µg/ml of EtBr (10mg/ml) in each gel. All gels were run at 100-120Volts.

For primer pairs that amplify a fragment larger than the limits indicated by the 5x FIREPol Master Mix protocol (approximately 5kb), two other kits were used to undertake long-range PCRs. These kits are; TaKaRa LA PCR Kit Version 2.1 (TaKaRa Bio, Kusatsu, Shiga, Japan), and GoTaq® Long PCR Master Mix (Promega Corporation, Madison, WI, USA). Both kits were used as per manufacturer protocol.

4.2.4 PCR purification

PCR purification was carried out if Sanger sequencing was not successful upon first attempt, or if unwanted bands were present in the agarose gel images. The band of interest was cut from the gel and a DNA Purification protocol was followed according to the Thermo Scientific GeneJET PCR Purification Kit, eluting into 20µl of nuclease-free water. When gel purification was required and the aforementioned kit was not successful in isolating a high enough concentration of DNA, an alternative was used; NucleoSpin® Gel and PCR Clean-up kit, similarly as per manufacturer's protocol, once again eluting into 20µl of nuclease-free water.

4.2.5 Confirmational Sanger sequencing and phasing analysis

Sanger sequencing was performed on each patient's DNA sample to confirm the presence of the candidate variants identified in the patient. Where family members have consented to participate in the study, Sanger sequencing was performed to determine phasing. Sanger sequencing was carried out through Eurofins Genomics Tubeseq service (Heidelberg, Germany). The final volumes of samples and primers were sent according to Eurofins desired guidelines, with the upper limit of PCR product concentration being sent when possible. When variant calls are confirmed through Sanger sequencing, pedigree trees are created for the available families, in order to make phasing calls for each variant. Phasing must be confirmed in patients in order to give a final research-grade genetic diagnosis.

4.2.6 Variant classification

Variants were classified utilising the phasing analyses undertaken as part of this project, and data from previously published or newly generated *in silico* pathogenicity predictors; MetaLR (Dong *et al.*, 2015), M-CAP (Jagadeesh *et al.*, 2016) and REVEL (Ioannidis *et al.*, 2016). The thresholds for MetaLR and M-CAP are 0.5, 0.025 respectively, with updated recommendations for the REVEL score cutoff being applied (Pejaver *et al.*, 2022). We have also introduced the AlphaFold and AlphaMissense *in silico* prediction tools into our pipeline, using cutoff thresholds recommended by (Nguyen *et al.*, 2024; Zhuo, Aggarwal and Liao, 2024). Various *in silico* tools were used within the Alamut Visual software version 2.13 (Interactive Biosoftware, France; SOPHiA GENETICS, Switzerland) to predict the impact on splicing (Fadaie *et al.*, 2019). These tools included SpliceSiteFinder-like (SSFL) (Zhang, 1998), MaxEntScan (MES) (Yeo and Burge, 2004), NNSPLICE (MG *et al.*, 1997) and GENESplicer (Pertea, Lin and Salzberg, 2001). The SpliceAI online lookup tool was also utilised, through the BROAD Institute web interface

(<https://spliceailookup.broadinstitute.org/#>) to predict splicing effects, with a score of ≥ 0.2 considered as having a potential impact on splicing. The American College of Medical Genetics and Genomics (ACMG) guidelines, (Richards et al., 2015), for classifying pathogenic variants were used to determine likely pathogenicity, with minor alterations applied to account for gene expression patterns in the retina. Some of these adjustments are outlined in (Whelan *et al.*, 2020). As previously described in the Materials and Methods section 3.2.9 of chapter 3, recommendations from the Association for Clinical Genomic Science (ACGS) and ClinGen Sequence Variant Interpretation (SVI) group have been adopted into the classification pipeline (Rehm *et al.*, 2015; Durkie *et al.*, 2024).

4.3 Results

First tier DNA sequencing; panel sequencing and/or WES, was undertaken on many IRD probands recruited to Target 5000 including the 13 Irish IRD probands described herein. Candidate disease-causing variants were identified in 5 IRD genes for these pedigrees (**Table 4.1**). Identification of candidate disease variants and *in silico* analysis of pathogenicity, represent the first of many steps in the pipeline of analysis that is implemented for each proband and pedigree. The *in silico* tools used to predict pathogenicity include REVEL and AlphaMissense, along with a few others as detailed in Materials and Methods section 4.2.6 and **Supplementals 4.4 - 4.6**. As part of this study, 46 additional individuals have been genotyped using confirmational Sanger sequencing to determine whether the candidate disease-causing variants identified in the probands segregate with disease through their respective pedigrees. While informing genetic phase and mode of inheritance, results obtained via cascade studies can be used as a line of evidence to aid in classification/reclassification of variants, based on inheritance information. 13 case studies are detailed below illustrating the importance of family studies when genotyping patients.

The genotype information for all pedigrees is presented in **Table 4.1**. All variants identified as candidate disease-causing variants in these pedigrees have been classified in depth using the ACMG/AMP guidelines, taking into account the ACGS 2024 recommendations, as specified in the Materials and Methods section 4.2.6 (Richards *et al.*, 2015; Durkie *et al.*, 2024). Due to the extensive volume of classification data generated for each of the variants analysed, large and detailed tables have been produced. These results are presented as supplemental tables in the final section of this thesis to maintain clarity and readability within the main text. The evidence applied is indicated in the final column of **Table 4.2**, with detailed notes included in **Supplementals 4.4 – 4.6**. 27 candidate variants were analysed in these pedigrees; 11 pathogenic, 9 likely pathogenic, 4 variants of unknown significance (VUS), and 3 likely benign. When comparing these in-house classifications to those provided by Franklin Genoox, 13 variants differed in classification (**Table 4.2**). Of note, 4 of the candidate variants described are thought to be novel variants, to the best of our knowledge. Natural history and genotype-phenotype correlation studies in IRDs provide information on disease course, prognosis, and may be of value when exploring modifier loci and when deciding on relevant outcome measures for future clinical trials, among other utilities. Therefore, as part of the research described in this chapter of the thesis, detailed retinal phenotypes for each patient are presented.

Table 4.1. Genotype information of candidate variants identified in 13 analysed Irish pedigrees. (Records divided into 2 tables below, 1/2).

Patient barcode	Status	Pedigree	Gene	Allele 1	Allele 2	Clinical Diagnosis	Screening method
1A	Proband	1A	ABCA4	c.161G>A,p.[Cys54Serfs*14,Cys54Tyr]	c.3056C>T,p.Thr1019Met	Pan-Retinal Degeneration	T5K 254 gene panel
2A	Unaffected	1A	ABCA4	+	c.3056C>T,p.Thr1019Met	//	Cascade/Sanger
3A	Unaffected	1A	ABCA4	c.161G>A,p.Cys54Tyr	+	//	Cascade/Sanger
4A	Proband	4A	ABCA4	c.3322C>T,p.Arg1108Cys	c.3329-1G>A,p.?	Cone/Rod dystrophy	T5K 254 gene panel
5A	Unaffected	4A	ABCA4	c.3322C>T,p.Arg1108Cys	+	//	Cascade/Sanger
6A	Unaffected	4A	ABCA4	+	c.3329-1G>A,p.?	//	Cascade/Sanger
7A	Proband	7A	ABCA4	c.5594dupA,p.His1865fs	c.5773A>G,p.Arg1925Gly	Cone/Rod dystrophy	T5K 254 gene panel
8A	Unaffected	7A	ABCA4	c.5594dupA,p.His1865fs	+	//	Cascade/Sanger
9A	Proband	9A	ABCA4	c.4363T>C,p.Cys1455Arg	c.5882G>A,p.Gly1961Glu	Stargardt	whole-ABCA4 smMIPs
10A	Unaffected	9A	ABCA4	+	c.5882G>A,p.Gly1961Glu	//	Cascade/Sanger
11A	Unaffected	9A	ABCA4	c.4363T>C,p.Cys1455Arg	+	//	Cascade/Sanger
12A	Proband	12A	ABCA4	c.3113C>T, p.(Ala1038Val)	c.[4222T>C;4918C>T], p.[Trp1408Arg; p.Arg1640Trp]	Stargardt	whole-ABCA4 smMIPs
13A	Unaffected	12A	ABCA4	c.3113C>T, p.(Ala1038Val)	+	//	Cascade/Sanger
14A	Unaffected	12A	ABCA4	+	c.[4222T>C;4918C>T], p.[Trp1408Arg; p.Arg1640Trp]	//	Cascade/Sanger
15A	Proband	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	Stargardt	T5K 254 gene panel
16A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	T5K 254 gene panel
17A	Unaffected	15A	ABCA4	+	c.[4222T>C; 4918C>T], p.[Trp1408Arg; Arg1640Trp]	//	Cascade/Sanger
18A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	Cascade/Sanger
19A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
20A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	Cascade/Sanger
21A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
22A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
23A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	Cascade/Sanger
24A	Affected	15A	ABCA4	+	c.[4222T>C; 4918C>T], p.[Trp1408Arg; Arg1640Trp]	Stargardt	Cascade/Sanger
25A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
26A	Affected	15A	ABCA4	+	c.[4222T>C; 4918C>T], p.[Trp1408Arg; Arg1640Trp]	Stargardt	Cascade/Sanger
27A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
28A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
29A	Unaffected	15A	ABCA4	c.5603A>T, p.Asn1868Ile	+	//	T5K 254 gene panel/ whole-ABCA4 smMIPs
30A	Unaffected	15A	ABCA4	+	+	//	Cascade/Sanger
31A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	Cascade/Sanger
32A	Unaffected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	+	//	Cascade/Sanger
33A	Affected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	c.[4222T>C; 4918C>T], p.[Trp1408Arg; Arg1640Trp]	Stargardt	T5K 254 gene panel
34A	Affected	15A	ABCA4	c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36])	c.[4222T>C; 4918C>T], p.[Trp1408Arg; Arg1640Trp]	Stargardt	Cascade/Sanger
35A	Proband	35A	ABCA4	c.[5461-10T>C;5603A>T], p.[[Thr1821Aspfs*6,Thr1821Valfs*13];p.Asn1868Ile]	c.[5461-10T>C;5603A>T], p.[[Thr1821Aspfs*6,Thr1821Valfs*13];p.Asn1868Ile]	ABCA4-retinopathy	Cascade/Sanger (Sibling had T5K 254 gene panel - has since left study)
36A	Unaffected	35A	ABCA4	c.[5461-10T>C;5603A>T], p.[[Thr1821Aspfs*6,Thr1821Valfs*13];p.Asn1868Ile]	+	//	Cascade/Sanger

Both the nucleotide and protein changes are indicated for each variant that was found to be present in every patient analysed. Each individual was analysed as part of a pedigree for phasing analysis; the pedigree is indicated in the third column. ('/' = the individual is unaffected, '+' = wildtype allele, 'T5K' = Target 5000).

Table 4.1. (Records continued, 2/2).

Patient barcode	Status	Pedigree	Gene	Allele 1	Allele 2	Clinical Diagnosis	Screening method
37C	Proband	37C	CDHR1	c.[562G>A; 2228G>A],p.[Gly188Ser; Arg743Gln]	c.743delG,p.Gly248fs	Cone-rod dystrophy	T5K 254 gene panel
38C	Unaffected	37C	CDHR1	+	+	//	Cascade/Sanger
39C	Unaffected	37C	CDHR1	+	c.743delG,p.Gly248fs	//	Cascade/Sanger
40G	Proband	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	T5K 254 gene panel
41G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	Cascade/Sanger
42G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	Cascade/Sanger
43G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	Cascade/Sanger
44G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	Cascade/Sanger
45G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	T5K 254 gene panel
46G	Affected	40G	GUCY2D	c.2480A>C,p.Tyr827Ser	+	Dominant Cone-rod dystrophy	T5K 254 gene panel
47P	Proband	47P	PROM1	c.1354dupT,p.Tyr452fs	c.2446G>C,p.Ala816Pro	Rod-Cone/Panretinal	T5K 254 gene panel
48P	Affected	47P	PROM1	c.1354dupT,p.Tyr452fs	c.2446G>C,p.Ala816Pro	Rod-Cone/Panretinal	Cascade/Sanger
49P	Unaffected	47P	PROM1	c.1354dupT,p.Tyr452fs	+	//	Cascade/Sanger
50P	Unaffected	47P	PROM1	+	c.2446G>C,p.Ala816Pro	//	Cascade/Sanger
51P	Unaffected	47P	PROM1	c.1354dupT,p.Tyr452fs	+	//	Cascade/Sanger
52P	Unaffected	47P	PROM1	+	+	//	Cascade/Sanger
53U	Proband	53U	USH2A	c.2276G>T, p.Cys759Phe	c.[2299delG;4714C>T], p.[Glu767Serfs*21;Leu1572Phe]	RP	T5K 254 gene panel
54U	Unaffected	53U	USH2A	+	c.[2299delG;4714C>T], p.[Glu767Serfs*21;Leu1572Phe]	//	T5K 254 gene panel
55U	Affected	53U	USH2A	c.2276G>T, p.Cys759Phe	c.[2299delG;4714C>T], p.[Glu767Serfs*21;Leu1572Phe]	RP	Cascade/Sanger
56U	Unaffected	53U	USH2A	c.2276G>T, p.Cys759Phe	+	//	Cascade/Sanger
57U	Proband	57U	USH2A	c.655delC, p.His219Ilefs*24	c.9391dupG, p.Val3131Glyfs*14	Usher II	T5K 254 gene panel
58U	Unaffected	57U	USH2A	c.655delC, p.His219Ilefs*24	+	//	Cascade/Sanger
59U	Proband	59U	USH2A	c.[2276G>T;6721C>T] p.[Cys759Phe;Pro2241Ser]	c.[2276G>T;6721C>T] p.[Cys759Phe;Pro2241Ser]	RP (simplex)	T5K 254 gene panel

Table 4.2 Candidate variants identified in 13 Irish IRD pedigrees, along with Franklin and In-house ACMG/AMP classifications.

Gene	NM transcript ID	Allele cDNA	Allele genomic	Allele protein	gnomAD-ALL AF	Franklin ACMG/AMP Classification	In-house ACMG/AMP Classification	ACMG/AMP codes applied
ABCA4	NM_000350.3	c.161G>A	1:g.94111579C>T	p.[Cys54Serfs*14,Cys54Tyr]	0.003943% (6)	Pathogenic	Pathogenic	PM3_VeryStrong, PS4_moderate, PP1_Strong, PVS1_moderate, PM2_supporting
ABCA4	NM_000350.3	c.302+68C>T	1:g.94111370G>A	p.?	0.00637% (2)	VUS	Likely benign	PS4_moderate, PM2_supporting, BP7_strong, BP4_moderate, BP2
ABCA4	NM_000350.3	c.3056C>T	1:g.94043470G>A	p.Thr1019Met	0.002628% (4)	Pathogenic	Pathogenic	PM3_VeryStrong, PM5, PS3_moderate, PS4_moderate, PM2_supporting, PP1_strong
ABCA4	NM_000350.3	c.3113C>T	1:g. 94508969G>A	p.Ala1038Val	0.17546% (496)	Likely pathogenic	Likely pathogenic	PM3_strong, PS3_supporting, PM2_supporting, PP1
ABCA4	NM_000350.3	c.3322C>T	1:g.94042767G>A	p.Arg1108Cys	0.01905% (29)	Pathogenic	Pathogenic	PM3_VeryStrong, PM5, PS3_moderate, PS4_moderate, PM2_supporting
ABCA4	NM_000350.3	c.3329-1G>A	1:g.94041403C>T	p.?	0.0006575% (1)	Pathogenic	Likely Pathogenic	PM3_VeryStrong, PVS1_moderate, PS4_supporting, PM2_supporting
ABCA4	NM_000350.3	c.4222T>C	1:g.94496583A>G	p.Trp1408Arg	0.0015913% (4)	Pathogenic	Pathogenic	PM3_VeryStrong, PM5, PS4_moderate, PS3_supporting, PM2_supporting, PP1_moderate
ABCA4	NM_000350.3	c.4363T>C	1:g.94029621A>G	p.Cys1455Arg	0.001314% (2)	Pathogenic	Likely pathogenic	PS4_moderate, PM3_Strong, PP3_moderate, PM2_supporting
ABCA4	NM_000350.3	c.4539+2028C>T	1:g.94027417G>A	p.[=,Arg1514Leufs*36]	0.006371% (2)	Pathogenic	Pathogenic	PM3_Verystrong, PS4_moderate, PVS1_strong, PM2_supporting, PP1_strong
ABCA4	NM_000350.3	c.4918C>T	1:g.94486896G>A	p.Arg1640Trp	0.0031819% (8)	Pathogenic	Pathogenic	PM3_VeryStrong, PM5, PS4_moderate, PS3_supporting, PM2_supporting, PP1_moderate
ABCA4	NM_000350.3	c.5461-10T>C	1: g.94476951A>G	p.[Thr1821Aspfs*6,Thr1821Valfs*13]	0.02197% (62)	Pathogenic	Pathogenic	PM3_VeryStrong, PVS1_moderate, PM2_supporting
ABCA4	NM_000350.3	c.5594dupA	1:g.94476475G>GT	p.His1865Glnfs*47	not in gnomad	Likely Pathogenic	Pathogenic	PVS1, PM3, PM2_supporting
ABCA4	NM_000350.3	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS	VUS	PS4, BS1
ABCA4	NM_000350.3	c.5773A>G	1:g.94008813T>C	p.Arg1925Gly	0.0006582% (1)	Pathogenic	Likely Pathogenic	PP3_strong, PM3_supporting, PS4_moderate, PM2_supporting
ABCA4	NM_000350.3	c.5882G>A	1:g.94008251C>T	p.Gly1961Glu	0.311% (473)	Pathogenic	Likely Pathogenic	PM3_VeryStrong, PM5, PS3_supporting, BS1
CDHR1	NM_033100.4	c.562G>A	10:g.84201843G>A	p.Gly188Ser	0.00682% (19)	Likely pathogenic	VUS (Hot)	PM3_strong, PM2_supporting
CDHR1	NM_033100.4	c.743del	10:g.84203080TG>T	p.Gly248fs	not in gnomad	Likely Pathogenic	Likely pathogenic	PVS1, PM2_supporting
CDHR1	NM_033100.4	c.2228G>A	10:g.84214269G>A	p.Arg743Gln	0.001973% (3)	VUS	Likely Benign	PM2_supporting, BP4_moderate
GUCY2D	NM_000180.4	c.2480A>C	17:g.8014668A>C	p.Tyr827Ser	not in gnomad	Pathogenic	Likely Pathogenic	PP1_strong, PP3_moderate, PM2_supporting
PROM1	NM_006017.3	c.1354dupT	4:g.16006637T>TA	p.Tyr452fs	0.02172% (33)	Pathogenic	Pathogenic	PVS1, PM3, PM2_supporting
PROM1	NM_006017.3	c.2446G>C	4:g.15980465C>G	p.Ala816Pro	0.00329% (5)	VUS	VUS (Warm)	PM3, PP3, PM2_supporting
USH2A	NM_206933.2	c.655delC	1:g.216365081TG>T	p.His219Ilefs*24	not in gnomad	Likely Pathogenic	Pathogenic	PVS1, PM3, PM2_supporting
USH2A	NM_206933.2	c.2276G>T	1:g.216420460C>A	p.Cys759Phe	0.09677% (273)	Pathogenic	Likely pathogenic	PM3_strong, PS4_moderate, PP1_moderate, PP3, BS1_supporting
USH2A	NM_206933.2	c.2299delG	1:g.216420436TC>T	p.Glu767Serfs*21	0.07017% (198)	Pathogenic	Pathogenic	PVS1, PM3_VeryStrong, PS4_moderate, BS1_supporting
USH2A	NM_206933.2	c.4714C>T	1:g.216270469G>A	p.Leu1572Phe	0.07287% (206)	Likely benign	Likely Benign	BP2, BS1_supporting
USH2A	NM_206933.2	c.6721C>T	1:g.216166446G>A	p.Pro2241Ser	not in gnomad	Likely pathogenic	VUS (Cool)	PM5, PM2_supporting, BP2
USH2A	NM_206933.2	c.9391dupG	1:g.215990517A>AC	p.Val3131Glyfs*14	not in gnomad	Pathogenic	Likely pathogenic	PVS1, PM2_supporting

Allele frequency from gnomAD v4.1.0 for each variant is reported, as well as their predicted ACMG/AMP pathogenicity as reported on Franklin Genoox. The in-house classifications are noted, alongside with the criteria that has been applied to achieve these interpretations (further detailed in **Supplementals 4.4 – 4.6**). Novel variants are highlighted in red, and Likely benign variants are highlighted in pale green.

4.3.1 Stargardt and other *ABCA4*-associated ocular dystrophies

The *ABCA4* gene encodes a protein that is a retinal-specific ATP-binding cassette transporter, that is predominantly expressed in the photoreceptor OS and plays a key role in the visual cycle. *ABCA4* works to import retinoid compounds such as N-retinylidene-phosphatidylethanolamine (N-Ret-PE) and 11-cis-retinal from the lumen to the cytoplasmic side of the OS disc membranes (Molday, Zhong and Quazi, 2009a). If *ABCA4* is not functioning correctly, this can result in an accumulation of retinoids in the photoreceptor OS discs, leading to the formation and build-up of A2E and other toxic bisretinoids collectively termed ‘lipofuscin’, in the RPE (Sparrow and Boulton, 2005). As this lipofuscin accumulates, the RPE cells begin to degenerate, ultimately leading to photoreceptor cell death. These deposits are known to cause the characteristic features of Stargardt disease (STGD1), as will be described below. *ABCA4* has also been seen to be expressed to a degree in the RPE, suggesting that *ABCA4* might have an additional role in clearing retinal following the phagocytosis of the OS discs by the RPE (Lenis *et al.*, 2018).

Different combinations of *ABCA4* variants can cause various retinal phenotypes, some of which include; early onset cone/rod dystrophy, pan-retinal degeneration, late onset macular dystrophy (Fundus Flavimaculatus), or classical STGD1. The majority of variants presented in this chapter (~52%) were identified in the *ABCA4* gene using three different forms of preliminary NGS; Target capture sequencing of a panel of 254 IRD genes, WES and whole *ABCA4* sequencing through smMIPs (General Materials and Methods 2.5). Of note, one variant is deemed novel and has not previously been reported in scientific literature/databases, to the best of our knowledge (c.5594dupA, p.His1865Glnfs*47). As can be seen from the genotypes **Table 4.1**, out of the 9 total patients solved with *ABCA4* variants from 7 pedigrees; 6 cases had 2 causative variants, 1 case had 3 variants and 2 cases had 4 variants identified as causative. Phasing analysis has been completed for all of these *ABCA4* patients, thereby confirming the presence of two disease-causing alleles working *in trans* in each patient (**Figure 4.1(a-g)**). Furthermore, we have confirmed that 3 common pathogenic complex alleles are causative of disease in 4 individuals from two different pedigrees (patients 12A, 15A, 33A, 34A). The *ABCA4* variant c.161G>A, p.Cys54Tyr was found to be present in a heterozygous state in the 1A pedigree, where it is seen to contribute to the disease phenotype (**Figure 4.1(a)**). Similarly, the 15A pedigree is seen to carry the *ABCA4* c.4539 + 2028C > T, p.[=, Arg1514Leufs*36] variant, which has been shown to be enriched in the Irish population (**Figure 4.1(g)**). Patient 15A is one of just two homozygotes ever reported worldwide, with the second also being an Irish individual from the Target 5000

study (Whelan *et al.*, 2023). Clinical phenotypes were collated along with multimodal ocular imaging for affected individuals in each *ABCA4* pedigree. A pedigree tree was constructed for each of the 7 *ABCA4* families identified in this study (**Figure 4.1(a-g)**). Along with this, a summary of the clinical information for the 9 affected individuals from these families was constructed and is displayed in **Supplemental 4.1**. Multimodal ocular imaging was completed for the affected individuals as described in the General Materials and Methods section 2.2, and section 4.2.1 of this chapter, by Target 5000 affiliated clinicians, and disease phenotypes are described below.

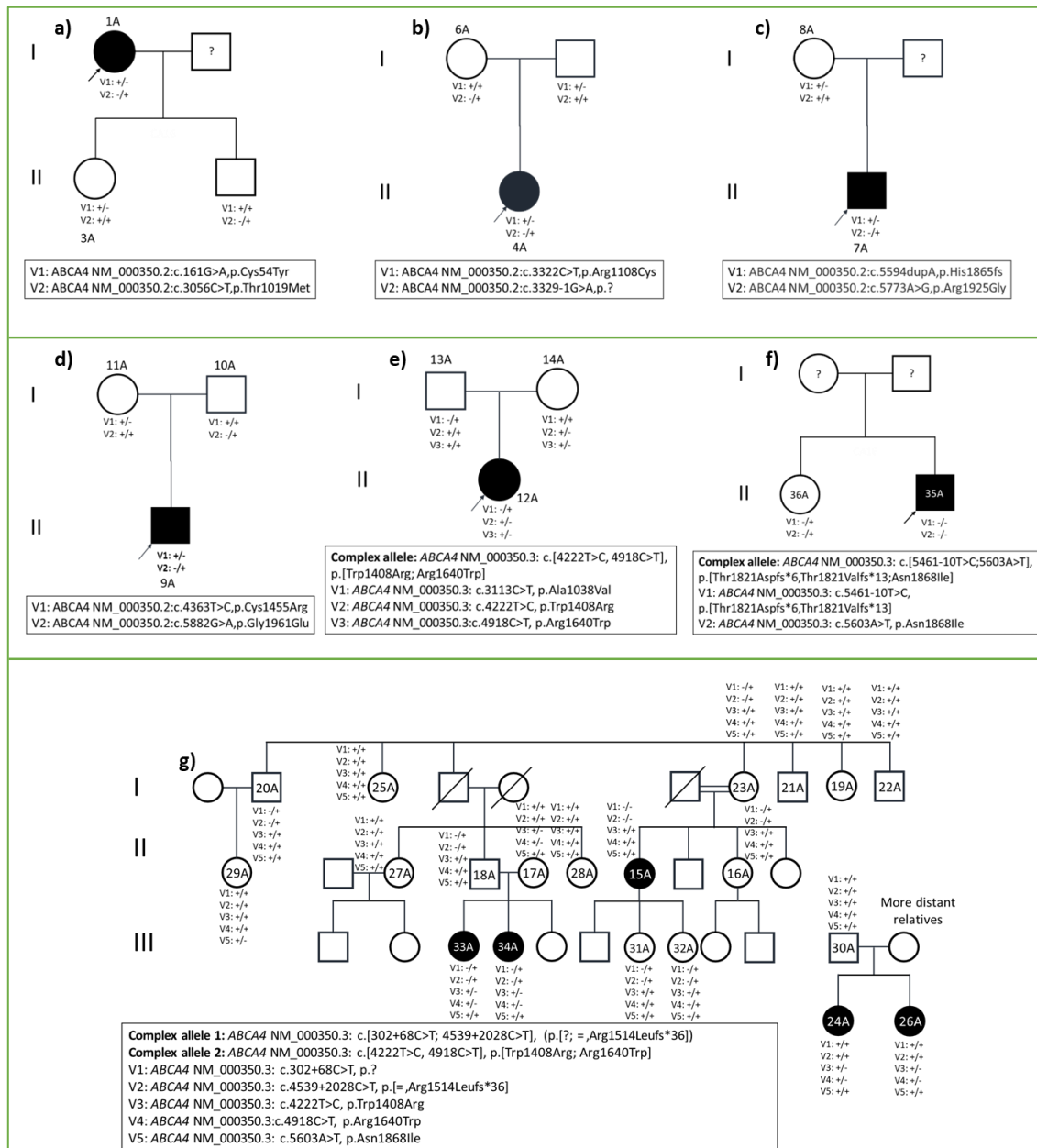


Figure 4.1(a-g). Irish pedigree trees displaying the phasing of candidate variants detected in 6 *ABCA4* families. The segregation patterns of the mutations are shown for each family, with variants indicated under each of the 6 trees. The arrow indicates the probands in each pedigree except for the final large family (g). If two variants create a complex allele, this is indicated under each tree in the corresponding variant annotation boxes (+ = wildtype allele, - = mutant allele).

Proband 1A first presented with ocular symptoms in early childhood. The variants c.161G>A and c.3056C>T were identified in *ABCA4*, and were confirmed to be acting *in trans* within this patient (**Table 4.1, Figure 4.1(a)**). From their colour fundus (**Figure 4.2(a.ii)**), we can observe widespread thinning of the RPE in both eyes. Pigmentary clumping is noted at both maculae, with degenerative changes extending beyond the macula. Fundus autofluorescence (FAF) imaging revealed scalloped edges in both eyes with a petaloid disturbance peripherally (**Figure 4.2(a.iii)**). A marked thinning of whole retina is seen in the optical coherence tomography (OCT), along with photoreceptor debris (**Figure 4.2(a.i)**). Pigmental clumping is seen centrally, aligning with the clumping seen on the fundus imaging. These imaging results, along with the ERG and Goldmann fields summarised in **Supplemental 4.1**, allow for a diagnosis of a pan-retinal degeneration due to the widespread loss of RPE and photoreceptors.

Proband 4A first presented with visual acuity symptoms in their early teens. Variants c.3322C>T and c.3329-1G>A in *ABCA4* were confirmed *in trans* in this patient (**Table 4.1, Figure 4.1(b)**). The colour fundus shows a visible pigmentary disturbance peripherally in the right eye (**Figure 4.2(b.ii)**). There is extensive loss of the RPE centrally, with choroidal vessels just visible. In the FAF, wider RPE disturbance is seen centrally that almost extends out to the periphery, and a dark choroid characteristic of *ABCA4*-related disease due to lipofuscin accumulation (**Figure 4.2(b.iii)**). There are patchy areas of hypofluorescence spreading peripherally, with RPE loss centrally. Marked thinning of the whole retina is observed on OCT in both eyes, with an almost total loss of the photoreceptor layer (**Figure 4.2(b.i)**). The photoreceptor layer is patchy in the left eye, with areas where photoreceptors remain being very thin and sparse. The imaging and ERG results (**Supplemental 4.1**) are indicative of a cone-rod dystrophy.

Proband 7A also presented with a cone-rod dystrophy, with ocular symptoms first occurring in late teens. Variants c.5594dupA and c.5773A>G in *ABCA4* were identified as causative variants, acting *in trans* (**Table 4.2, Figure 4.1(c)**). A light disturbance of colouration at the macula is noted in the colour fundus images, with very few discreet deposits visible (**Figure 4.2(c.ii)**). These deposits align with the areas of hyperfluorescence seen on the FAF (**Figure 4.2(c.iii)**). An area of central hypofluorescence surrounded by a ring of hyperfluorescence in a bullseye pattern is observed in both eyes. The OCT shows that the layers of the retina seem substantially preserved peripherally with debris present centrally (**Figure 4.2(c.i)**). The photoreceptor layer is completely lost subfoveally.

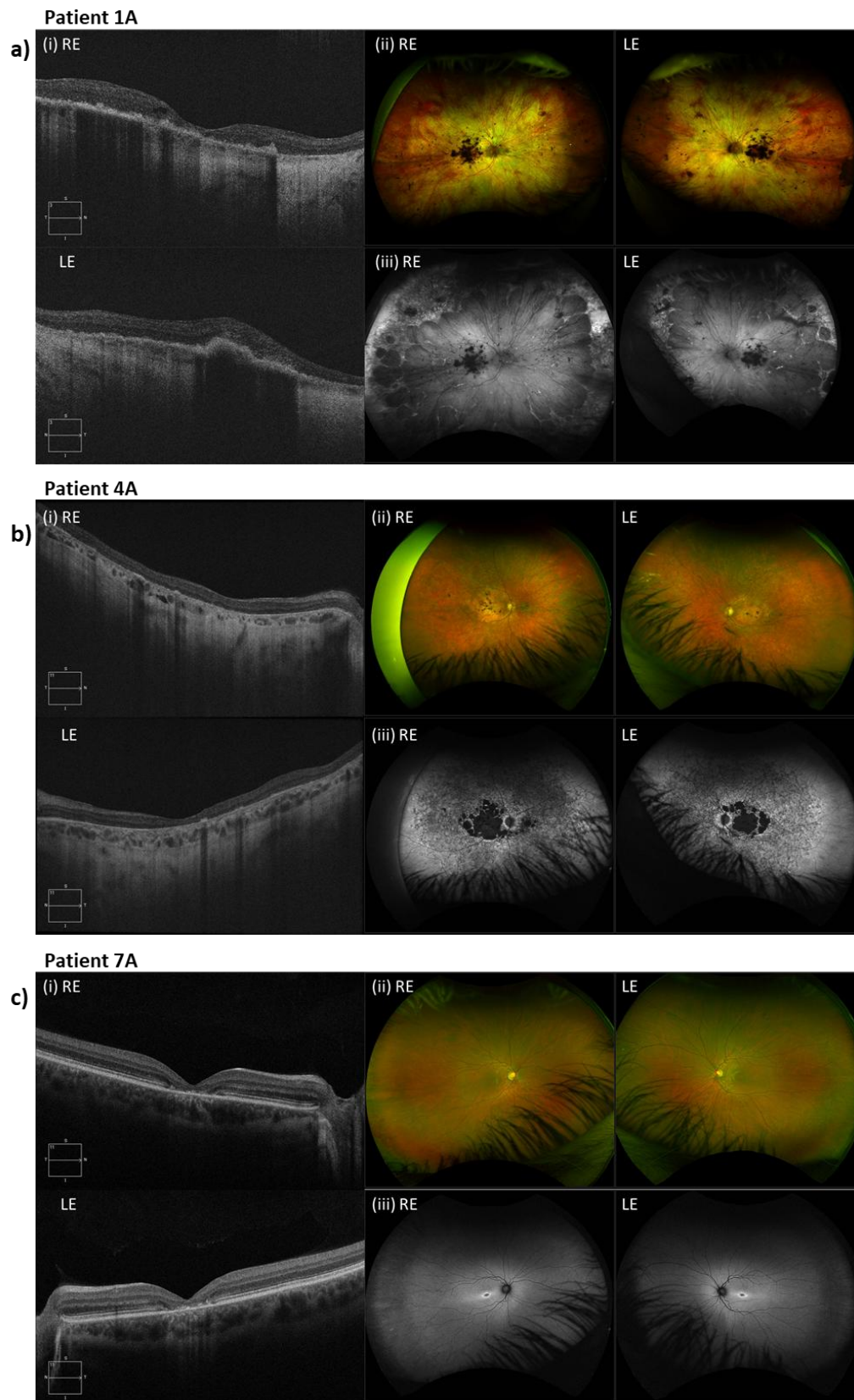


Figure 4.2(a-c). Multimodal imaging for *ABCA4* patients (a) 1A, (b) 4A, (c) 7A, where the images for each patient are annotated as follows; (i) Optical coherence tomography (OCT) images, (ii) Pseudocolour widefield fundus photos, (iii) Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

Patients 9A, 12A, 15A, 33A and 34A presented with classical STGD1 disease, with varying combinations of severe, moderate and mild variants in *ABCA4* (Table 4.1, Supplemental 4.7). The variants identified within *ABCA4* and implicated in disease in patient 9A are c.4363T>C and c.5882G>A (Figure 4.1(d)). As for patient 12A, variant c.3113C>T and the common complex allele c.[4222T>C;4918C>T] were found to be acting *in trans* (Figure 4.1(e)). Probands 9A and 12A first presented with symptoms in adulthood. Colour fundus for patient 9A indicate extensive thinning of the RPE, similar to that seen in patient 4A, so that choroidal blood vessels, usually hidden by the RPE, are visible (Figure 4.3(a.ii)). Their FAF shows a slightly larger area of central hypofluorescence surrounded by a ring of hyperfluorescence seen in both eyes (Figure 4.3(a.iii)). A widespread loss of the photoreceptor layer is seen centrally in OCT images (Figure 4.3(a.i)). While OCT imaging was not available for patient 12A, the colour fundus and FAF are very typical of *ABCA4*-related STGD1. The colour fundus indicates fishtail deposits in both eyes which appear to be extending into the nasal side of the optic disc (Figure 4.3(b.i)). Scattered spotty areas of hyper- and hypo-fluorescence are noted on the FAF in both eyes, aligning with deposits seen in the colour imaging (Figure 4.3(b.ii)).

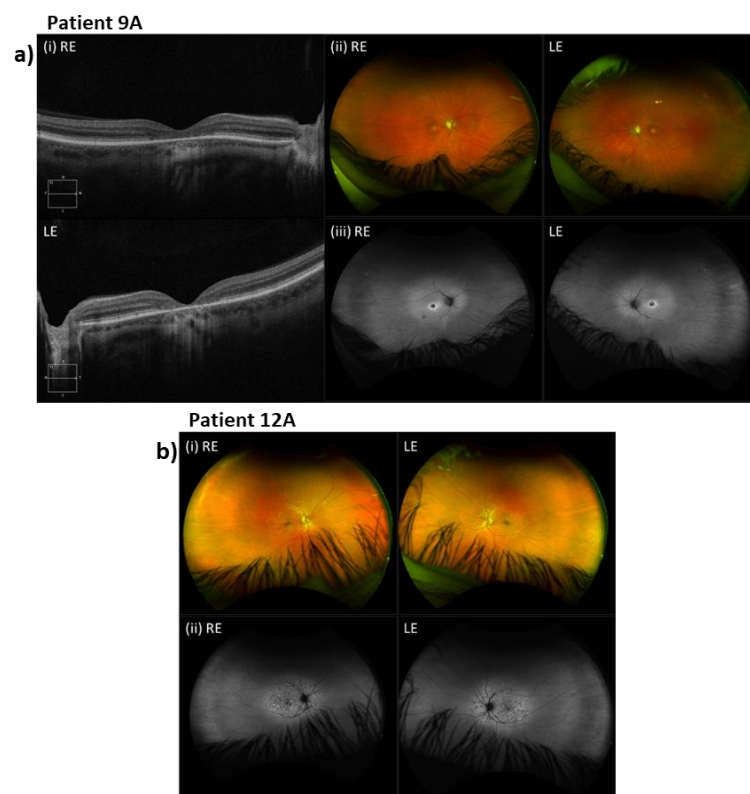


Figure 4.3(a,b). Multimodal imaging for *ABCA4* patients 9A and 12 A. (a) Images for patient 9A (i) Optical coherence tomography (OCT) images, (ii) Pseudocolour widefield fundus photos, (iii) Fundus autofluorescence (FAF). (b) Images for patient 12A (i) Pseudocolour widefield fundus photos, (ii) Fundus autofluorescence (FAF), where the OCT image for this patient was not available. (RE= right eye, LE= left eye).

Interestingly, proband 15A is homozygous for the *ABCA4* c.4539+2028C>T, p.[=,Arg1514Leufs*36] variant that is believed to be enriched in the Irish population ((**Figure 4.1(g)**). Their colour fundus shows a large patch of RPE atrophy centrally in both eyes, with choroidal blood vessels visible in the atrophic regions (**Figure 4.4(a.ii)**). A dark choroid is observed, with a large area of hypofluorescence centrally on the FAF, surrounded by a thin ring of hyperfluorescence in a bullseye pattern (**Figure 4.4(a.iii)**). This area corresponds to the atrophic region seen in the colour imaging. OCT imaging reveals the extensive loss of the photoreceptor layer in a large area centrally, in both eyes (**Figure 4.4(a.i)**). Two other individuals that are affected within this family are siblings 33A and 34A (**Figure 4.1(g)**). While the two patients have the same *ABCA4* genotype (c.[302+68C>T; 4539+2028C>T] and c.[4222T>C, 4918C>T], **Table 4.1**), patient 34A is 5 years younger, partially explaining the markedly less progressed fundus features. The colour fundus for 33A displays fishtail deposits centrally, along with thinning of the RPE centrally in both eyes (**Figure 4.4(b.ii)**). A bullseye pattern is noted on the FAF in both eyes, marked by an area of hypofluorescence centrally with spots of hyperfluorescence seen in a surrounding ring, denoting a thinning RPE (**Figure 4.4(b.iii)**). The OCT trace shows a feathery disturbance in the photoreceptor layer centrally in both eyes, with the photoreceptor layer appearing highly depleted in this area (**Figure 4.4(b.i)**). The colour fundus imaging for 34A was much less informative than the FAF and OCT (**Figure 4.4(c.ii)**). The FAF for patient 34A is similar to that of her sibling but much less progressed. A small central region of hypofluorescence is surrounded by a slight ring of hyperfluorescence (**Figure 4.4(c.iii)**). On the OCT trace, there is a better preservation of layers overall, with less thinning of the retina (**Figure 4.4(c.i)**). But there is loss of photoreceptors subfoveally in both eyes. The images are consistent with the clinical diagnosis of STGD1.

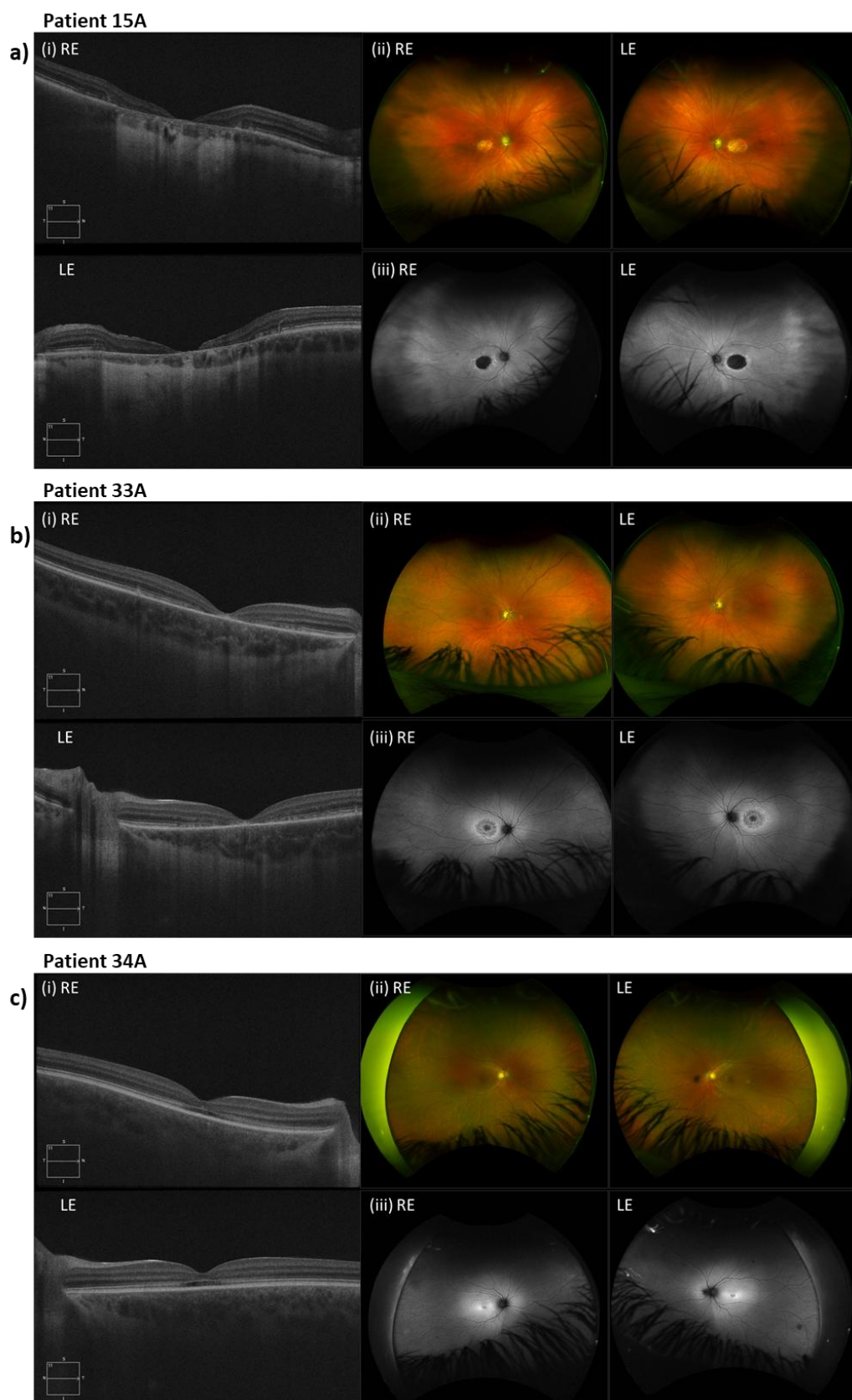


Figure 4.4(a-c). Multimodal imaging for *ABCA4* patients (a) 15A, (b) 33A, (c) 34A, where the images for each patient are annotated as follows; (i) Optical coherence tomography (OCT) images, (ii) Pseudocolour widefield fundus photos, (iii) Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

Proband 35A presented with an *ABCA4*-retinopathy phenotype, unlike the other *ABCA4* patients described within the scope of this study. In this case, Patient 35A was found to carry the complex allele c.[5461-10T>C;5603A>T] homozygously, with second degree consanguinity noted between the parents (**Table 4.1, Figure 4.1(f)**). Gross pigmentary disturbance is evident on the colour fundus, concentrated around the maculae of each eye (**Figure 4.5(i)**). Direct ophthalmoscopy revealed that this disturbance extended out into the far periphery. A loss of autofluorescence involving the whole posterior pole through to the sclera is evident from the FAF for patient 35A, with some retention of autofluorescence noted peripherally (**Figure 4.5(ii)**).

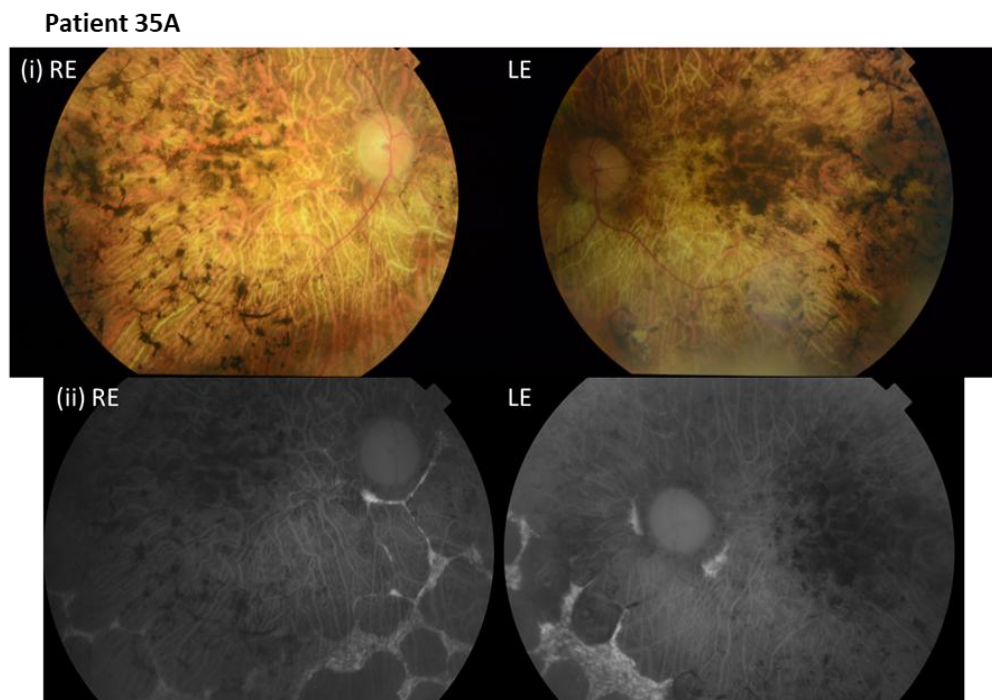


Figure 4.5(i,ii). Multimodal imaging for *ABCA4* patient 35A, where the images are annotated as follows; (i) Pseudocolour widefield fundus photos, (ii) Fundus autofluorescence (FAF). The OCT image for this patient was not available. (RE= right eye, LE= left eye).

4.3.2 Cone-rod dystrophy and pan-retinal degeneration; *CDHR1*, *GUCY2D* and *PROM1* case studies

4.3.2.i *CDHR1*

As the overall focus of this PhD thesis is inherited macular degenerations (iMDs) and cone/cone-rod dystrophies (CCRDs), the next two case studies highlight genes, *CDHR1* and *GUCY2D*, that are frequently implicated in iMDs and CCRDs. Recent targeted NGS studies interrogating macular dystrophy and CCRD cohorts indicate that mutations have been identified in *CDHR1* in approximately 2-4% of cases, and in *GUCY2D* in ~3% (Birtel *et al.*, 2018; Hitti-Malin *et al.*, 2022, 2024). In this section, the clinical features and molecular diagnoses for two families from the Target 5000 cohort with cone-rod dystrophy phenotypes are described.

The cadherin-related family member 1 gene *CDHR1* (MIM * 609502) encodes a structural transmembrane protein called protocadherin. This protein is localised at the base of the rod and cone photoreceptor OS, and has been shown to play a vital role in maintaining photoreceptor structural integrity (Rattner *et al.*, 2001). The knockout *Cdhr1*^{-/-} mouse displays a shortening of photoreceptor outer segments, with progressive photoreceptor cell death (Rattner *et al.*, 2001; Yusuf *et al.*, 2021). Mutations in *CDHR1* have been associated with a multitude of retinopathies including rod-cone dystrophy, cone-rod dystrophy, RP and late-onset macular dystrophy (Malechka *et al.*, 2022; Farag *et al.*, 2023). Hypomorphic alleles in *CDHR1* have most commonly been associated with late onset macular dystrophy, where the atrophy observed can be difficult to distinguish from that seen in dry AMD (Ba-Abbad *et al.*, 2021). The case being presented here is a female patient 37C, presenting with late onset cone-rod dystrophy with significant macular involvement (**Supplemental 4.2**). Targeted sequencing identified 3 variants in *CDHR1*, and cascade analysis was completed to confirm phasing (**Table 4.1, Figure 4.6(a)**). Two of the variants identified; c.562G>A, p.Gly188Ser and c.2228G>A, p.Arg743Gln were found to be *in cis*. These variants have been classified as a ‘Hot’ VUS and likely benign, respectively (**Table 4.2**). The other variant identified; c.743delG,p.Gly248fs, was seen to be *in trans* with the complex allele. The c.743delG variant is a null frameshift variant where the mRNA is predicted to undergo nonsense-mediated decay (NMD) (**Supplemental 4.5**). To the best of our knowledge, this is a novel variant that has not previously been reported in the literature. The frameshift variant was confirmed heterozygously in the proband’s mother, 39C, who is homozygous wildtype for the complex allele (**Figure 4.6(a)**). It is most probable that the father is a heterozygous

carrier of this complex allele, albeit this individual was not available for sequencing. The sibling, 38C, is phenotypically unaffected and carries neither disease allele.

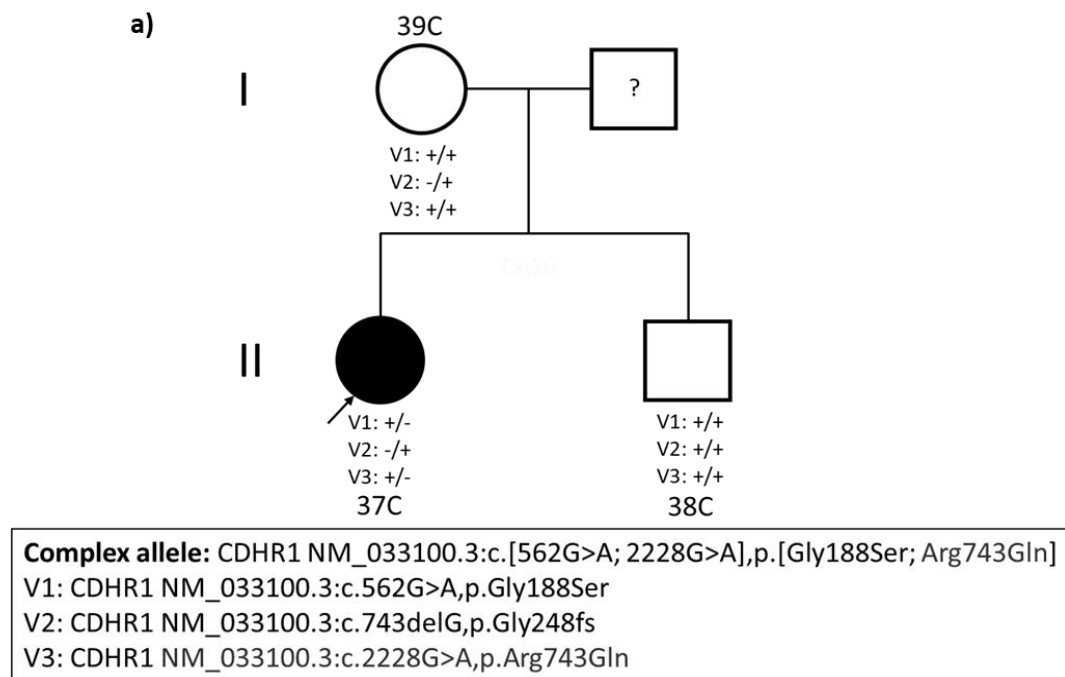
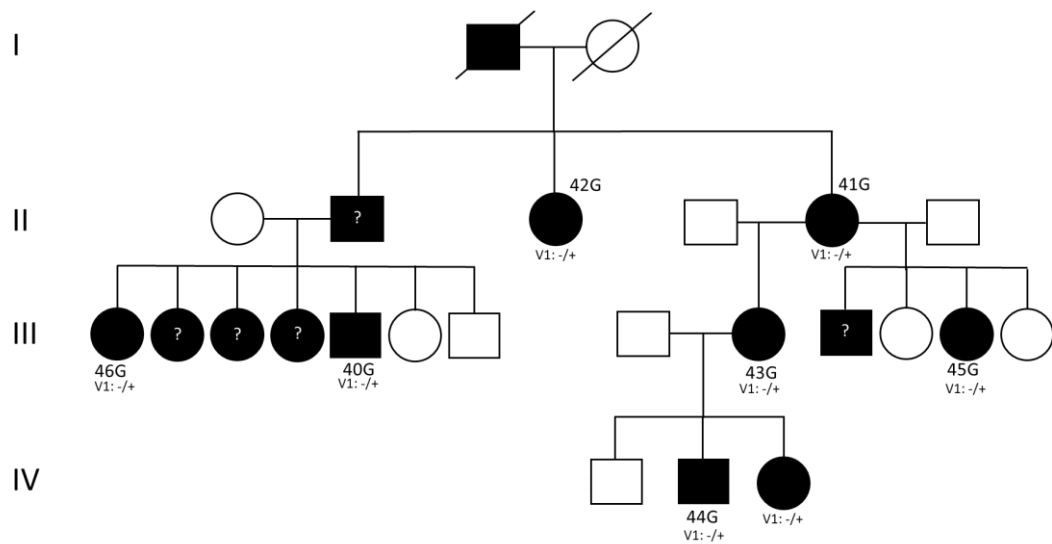


Figure 4.6. (a) Pedigree tree displaying the phasing of candidate variants detected in *CDHR1*. The segregation patterns of the mutations are shown, with variants indicated under the tree. The arrow indicates the proband. The complex allele is indicated the tree in the variant annotation box (+ = wildtype allele, - = mutant allele). **(b) Multimodal imaging for patient 37C (i)** Optical coherence tomography (OCT) image for left eye only, **(ii)** Pseudocolour widefield fundus photos, **(iii)** Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

Patient 37C had adult-onset symptoms, with photophobia and visual acuity decline at age 45. ERG results are consistent with other *CDHR1*-associated CCRD patients, where photopic responses from cones are significantly reduced, while scotopic responses from rods are only slightly subnormal (**Supplemental 4.2**) (Malechka *et al.*, 2022; Sobolewska *et al.*, 2023b). Colour fundus examination shows peripheral spots of thinning, as well as disturbance of pigmentation centrally in both eyes (**Figure 4.6(b.ii)**). Stippling is observed superior to the vascular arcade, bilaterally. FAF reveals a large patchy area of hyper- and hypo-fluorescence centrally (**Figure 4.6(b.iii)**). Peripheral hypofluorescent spots of retinal and RPE thinning align with those seen in colour imaging. The OCT image from the right eye was not informative due to poor image quality and off-centre imaging. In the OCT for the left eye, the photoreceptor layer appears to be preserved peripherally, while centrally only debris remains of the photoreceptor layer (**Figure 4.6(b.i)**).

4.3.2.ii *GUCY2D*

As mentioned, *GUCY2D* is a gene implicated in an array of retinal diseases including autosomal recessive Leber congenital amaurosis (LCA) and autosomal dominant cone/cone-rod dystrophy (CCRD) (Sharon *et al.*, 2018). *GUCY2D* (MIM * 600179) encodes the retinal guanylyl cyclase 1 (RetGC1) enzyme, which is expressed in photoreceptor OS, primarily in cones. RetGC1 works to replenish cytoplasmic cGMP levels during phototransduction to reset the visual system after exposure to light (Snook *et al.*, 2023). The family described herein presents with a dominant cone-rod dystrophy with varying ages of onset seen within the pedigree (**Supplemental 4.2**). All members have been Sanger sequenced for the *GUCY2D* variant c.2480A>C,p.Tyr827Ser (**Table 4.1**). It has been confirmed to be present in all family members available, all of whom are affected (**Figure 4.7**). While this variant is not novel, it has only been reported once before to the best of our knowledge. In this Italian iMD and CCRD study, the variant was also observed to follow an autosomal dominant inheritance pattern in a patient with childhood onset macular dystrophy (Falsini *et al.*, 2022).



V1: GUCY2D NM_000180.3:c.2480A>C,p.Tyr827Ser

Figure 4.7. Pedigree tree displaying the phasing of a dominant acting variant detected in *GUCY2D*. The segregation patterns of the mutations are shown, with the variant indicated under the tree. No proband is indicated here. Question marks indicate family members where genotype is not known. (+ = wildtype allele, - = mutant allele).

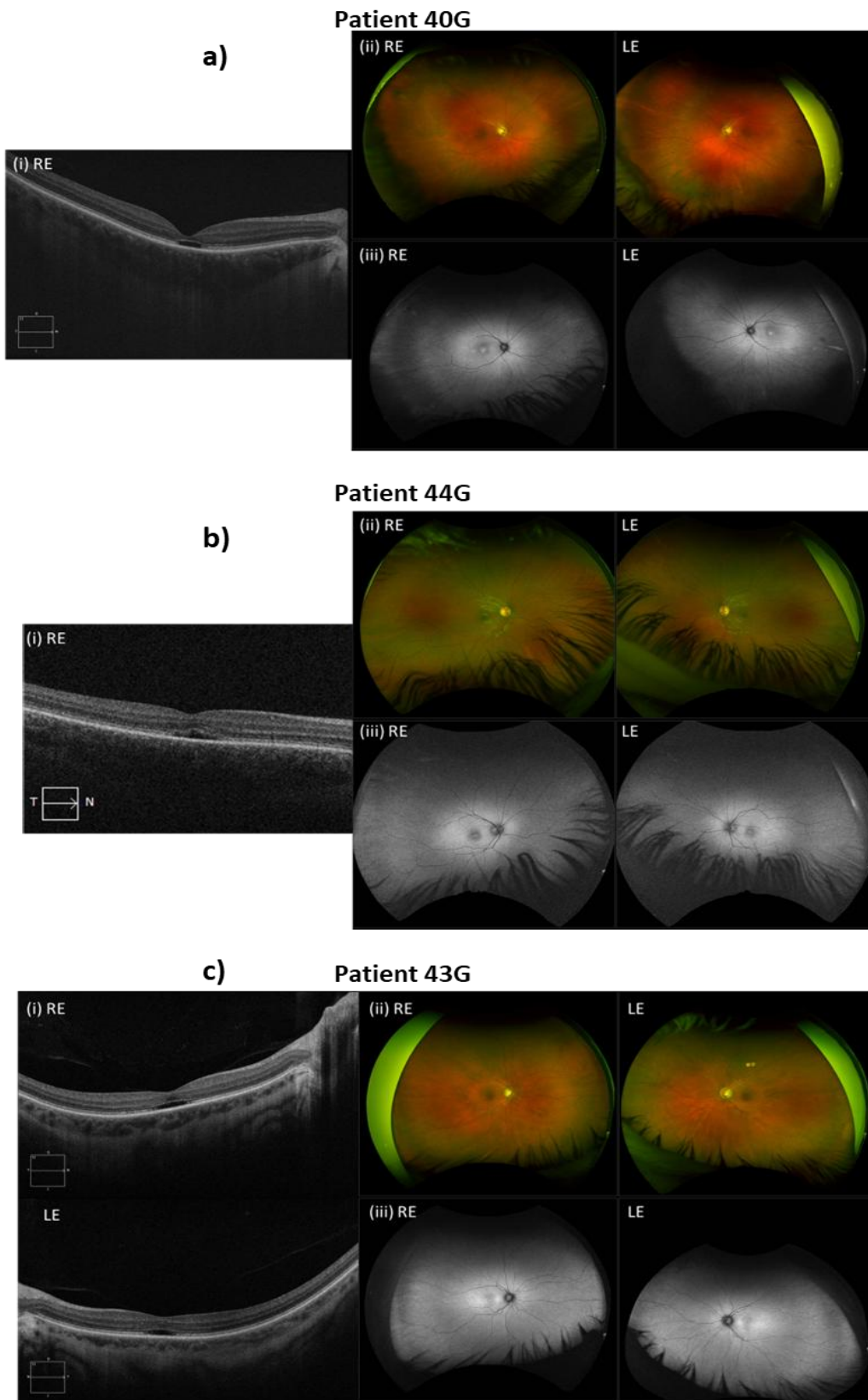


Figure 4.8 (a-c). Multimodal imaging for *GUCY2D* patients (a) 40G, (b) 44G, (c) 43G, where the images for each patient are annotated as follows; (i) Optical coherence tomography (OCT) images, (ii) Pseudocolour widefield fundus photos, (iii) Fundus autofluorescence (FAF). Only the OCT images from the right eyes of patients 40G and 44G were informative. (RE= right eye, LE= left eye).

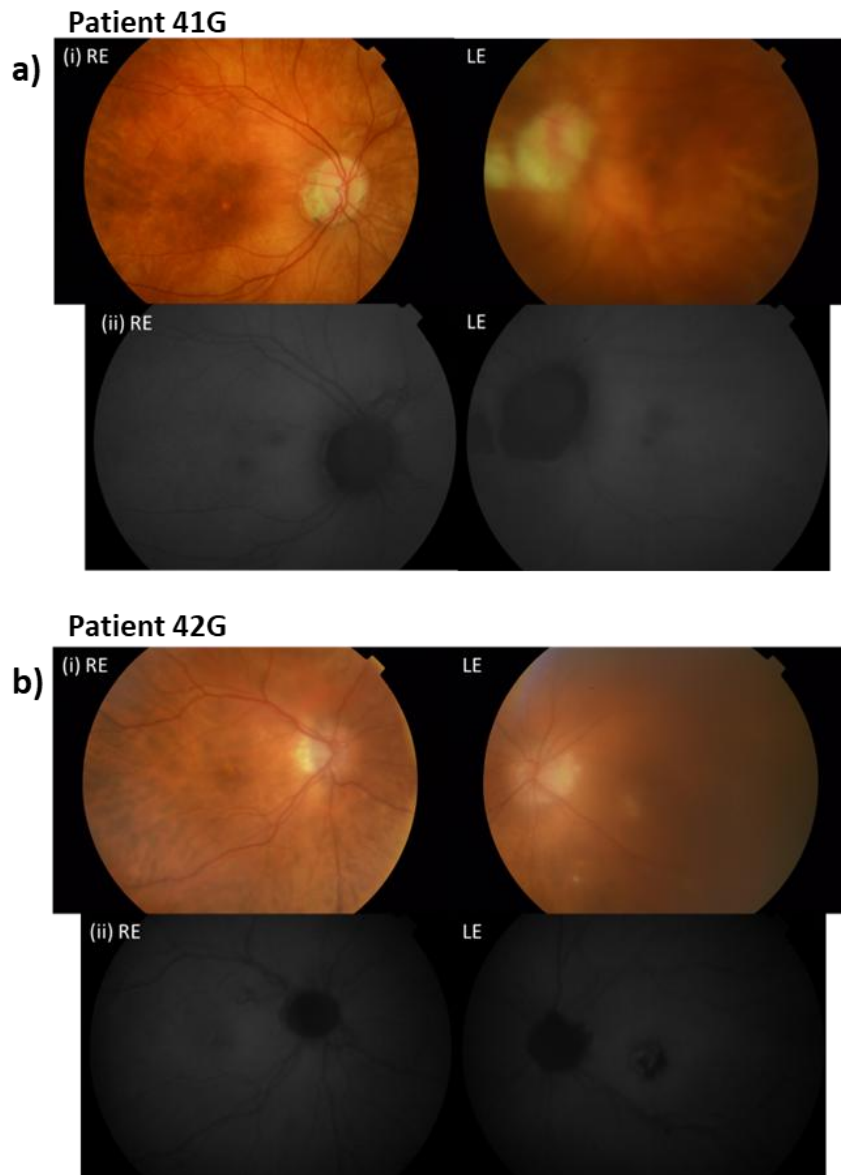


Figure 4.9 (a,b). Multimodal imaging for *GUCY2D* patients (a) 41G, (b) 42G, where the images for each patient are annotated as follows; (i) Pseudocolour widefield fundus photos, (ii) Fundus autofluorescence (FAF). OCT images for these two patients were not available. (RE= right eye, LE= left eye).

Clinical information was available for all patients in this pedigree except for two (45G and 46G). In patients 40G, 43G and 44G, the FAF was more informative than the colour fundus (**Figure 4.8(a-c.iii)**). The FAF for patient 40G shows small spots of hyperfluorescence along the vascular arcade in both eyes (**Figure 4.8(a.iii)**). The disturbance noted in the autofluorescence centrally fits with the area of photoreceptor loss seen subfoveally, which extends out slightly in the OCT of the left eye (**Figure 4.8(a.i)**). The OCT image from the right eye was not informative due to image quality and off-centre imaging. Patient 43G, had only a subtle disturbance noted in the autofluorescence centrally in each eye (**Figure 4.8(c.iii)**). The OCT images reveal a loss of the photoreceptor layer centrally at the fovea,

extending out slightly (**Figure 4.8(c.i)**). Feathery disturbance of the photoreceptor layer can be seen moving out from the fovea, whereas the layers of the retina appear normal peripherally. Similar imaging patterns are observed in 44G, son of 43G. The glistening observed in the colour fundus is characteristic of a young eye. Slight hypofluorescence is seen centrally on the FAF, surrounded by small area of increased fluorescence (**Figure 4.8(b.iii)**). Again, the OCT image from the left eye was not informative due to poor image quality and off-centre imaging. But in the right eye, there is a clear loss of photoreceptors subfoveally (**Figure 4.8(b.i)**). The colour fundus for patients 41G and 42G was informative and was taken using conventional Topcon imaging and it is focused on the macula, hence the different appearance of the images for these patients (**Figure 4.9(a,b.i)**). The colour fundus examination for 41G reveals a loss of the pigment epithelium centrally in the right eye (**Figure 4.9(a.i)**). The patient is a high myope, which could result in the blurring of the imaging seen in the left eye due to the altered shape of the eye. There is a generalised loss of autofluorescence in both eyes on the FAF, with hypofluorescent spots centrally corresponding to the RPE loss that was noted in the colour fundus of the right eye (**Figure 4.9(a.ii)**). The colour fundus for patient 42G displays pigmentary disturbance denoting the loss of RPE in both eyes (**Figure 4.9(b.i)**). These areas of loss correspond to the patches of disturbance to the autofluorescence seen centrally in the FAF, where the autofluorescence is reduced globally (**Figure 4.9(b.ii)**).

4.3.2.iii *PROM1*

The *PROM1* gene (MIM * 604365) encodes the Prominin-1 protein, and has been associated with a spectrum of IRDs. Prominin-1 is a 5-transmembrane domain glycoprotein. While *PROM1* was originally described as a human stem cell surface marker expressed in several stem and progenitor cell systems, it has since been found to have a profound function in the visual system. In the retina it is expressed in the OS discs of photoreceptors and has been shown to be involved in disc morphogenesis and the structural organisation of the outer segments (Yang *et al.*, 2008; Yanardag *et al.*, 2024). In addition, prominin-1 has been shown to play a key role in regulating the autophagy of photoreceptor discs by the RPE (Bhattacharya *et al.*, 2017). Mutations in the *PROM1* gene have been associated with a wide range of retinal phenotypes displaying a broad spectrum of severity and autosomal dominant or recessive inheritance patterns. Such phenotypes include; RP and RP with macular involvement, pan-retinal degeneration, cone-rod dystrophy, rod-cone dystrophy, bull's-eye maculopathy, maculopathy with rod-cone dystrophy and Stargardt-like disease 4 (STGD4) macular dystrophy (Cehajic-Kapetanovic *et al.*, 2019; Del Pozo-Valero *et al.*, 2019b).

Autosomal recessive variants in *PROM1* have been observed to cause RP, cone-rod dystrophy, or panretinal degeneration with early macular involvement. Conversely, autosomal dominant variants have been associated with STGD4-like macular dystrophy or late onset cone-rod dystrophy (Grosspoetzl *et al.*, 2024).

Target capture sequencing of 254 IRD genes of proband 47P revealed two variants in *PROM1*; c.1354dupT,p.Tyr452fs and c.2446G>C,p.Ala816Pro (**Table 4.1**), with confirmational Sanger sequencing undertaken for all family members participating in the study. Phasing analysis was completed for one family with two variants in *PROM1*, segregating in an autosomal recessive pattern (**Figure 4.10(a)**). Two affected individuals, 47P and 48P, are brothers displaying similar ocular symptoms with varying levels of progression. Both patients had received a clinical diagnosis of rod-cone/pan-retinal degeneration, based on the clinical data displayed in **Supplemental 4.2**, and their ocular imaging (**Figure 4.10(b,c)**). For patient 47P, colour fundus imaging revealed the loss of the RPE centrally in both eyes, where these patches seem to align with the dark choroid observed in the FAF (**Figure 4.10(b.ii)**). Pigmentary disturbance is also observed extending out into the mid-periphery bilaterally. In the FAF, a dark choroid patch can be seen centrally in both eyes (**Figure 4.10(b.iii)**). The area of hypofluorescence is surrounded by a ring of hyperfluorescence in both eyes. Disturbance of the autofluorescence is also noted in the mid-periphery. A patchy loss of the photoreceptor layer centrally is present on the OCT imaging, which fits with the thinning observed in both the colour fundus and FAF (**Figure 4.10(b.i)**). The retinal layers have collapsed centrally in the right eye. Similarly, Patient 48P presents with extensive thinning of RPE centrally, observed in the colour fundus photos, with a slightly larger area of loss observed than was seen in 47P (**Figure 4.10(c.i)**). The FAF has a patchy area of hypofluorescence surrounded by hyperfluorescence, corresponding to photoreceptor and RPE loss observed in the colour image (**Figure 4.10(c.ii)**). OCT was not available to us for this patient.

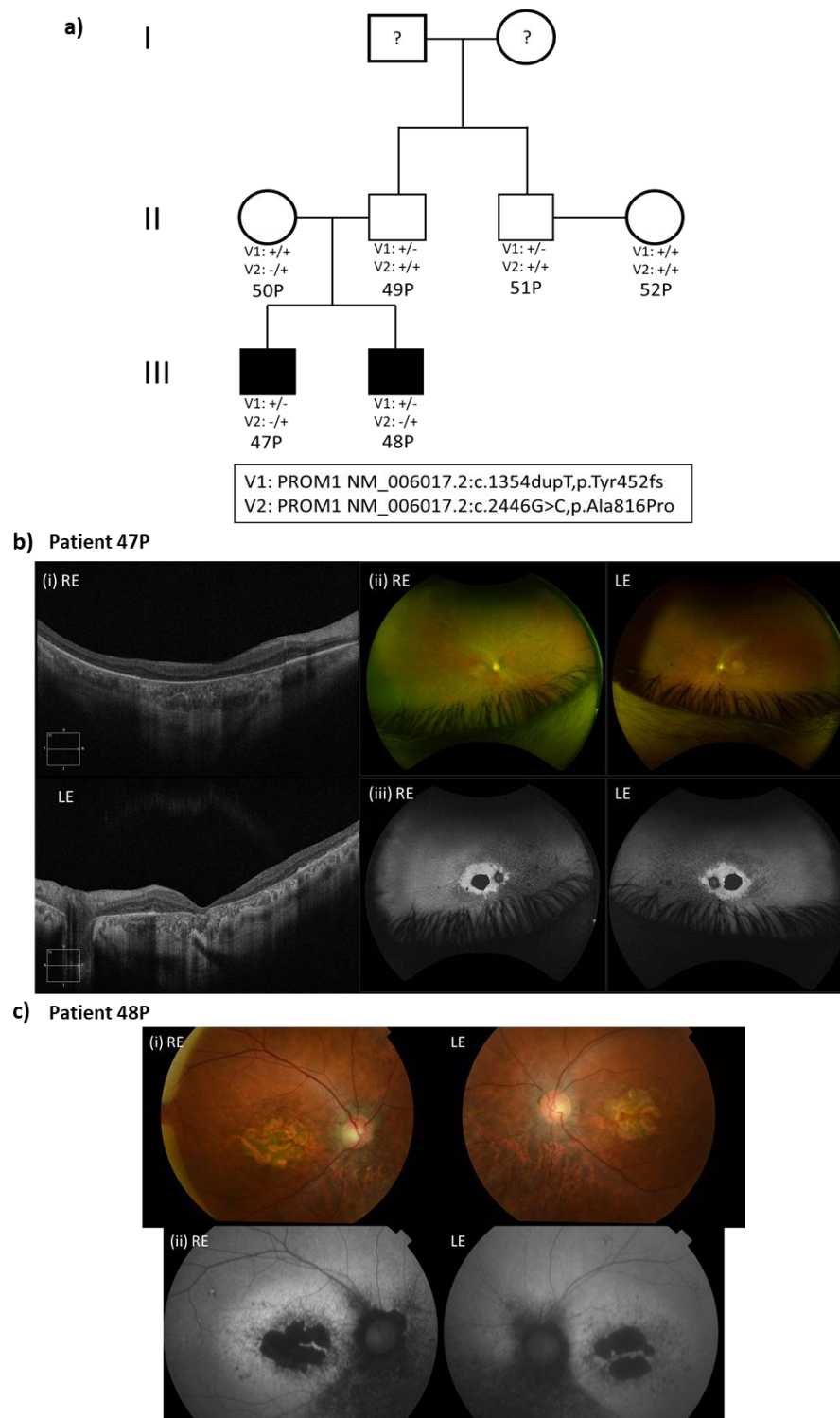


Figure 4.10 (a) Pedigree tree displaying the phasing of candidate variants detected in *PROM1*. The segregation patterns of the mutations are shown, with variants indicated under the tree. The arrow indicates the proband. The complex allele is indicated the tree in the variant annotation box (+ = wildtype allele, - = mutant allele). **(b) Multimodal imaging for patient 47P (i)** Optical coherence tomography (OCT) image for left eye only, **(ii)** Pseudocolour widefield fundus photos, **(iii)** Fundus autofluorescence (FAF). **(c) Multimodal imaging for patient 48P (i)** Pseudocolour widefield fundus photos, **(ii)** Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

4.3.3 Retinitis Pigmentosa and Usher II; *USH2A* case studies

The *USH2A* gene (MIM *608400) encodes a protein called Usherin which is exclusively expressed in photoreceptor cells and the cochlear hair cells during development (Liu *et al.*, 2007). Usherin has been shown to be vital in the development of cochlear cells as well as in maintaining homeostasis of the photoreceptors. Usher Syndrome Type II (Usher II) is the most common form of Usher Syndrome, accounting for over half of the reported cases worldwide. While the *USH2A* gene is the most frequent cause of Usher II (up to 85% of cases (Weston *et al.*, 2000; Koenekoop *et al.*, 2023)), mutations in this gene have also been implicated in non-syndromic autosomal recessive RP (arRP). *USH2A* mutations are seen to account for between 8-23% of non-syndromic arRP cases globally, with variations being seen between populations depending on origin (Hartong, Berson and Dryja, 2006; Eisenberger *et al.*, 2013; Perea-Romero *et al.*, 2021b). In a recent study based in Ireland, carried out on the Target 5000 cohort, it was found that over 69% of Usher II Irish cases were caused by mutations in the *USH2A* gene (Stephenson *et al.*, 2023b). Usher II syndrome that is caused by variants in *USH2A* is characterised by mild to severe sensorineural hearing loss from birth, retinitis pigmentosa (RP) often diagnosed in the second decade of life, and little to no vestibular involvement (Zaw *et al.*, 2022). Hearing loss is commonly the first symptom experienced by Usher's patients. This can often lead to a misdiagnosis of an isolated hearing impairment without genetic testing, until the child begins to display signs of RP in late adolescence. Some of the mutations occurring in *USH2A* can be found only in individuals presenting with non-syndromic RP. Three such *USH2A* non-syndromic RP patients shall be described herein, along with one patient presenting with Usher II. Two of the variants identified in *USH2A*; c.655delC, p.His219Ilefs*24 and c.6721C>T, p.Pro2241Ser, have not been previously reported in the literature to the best of our knowledge, and so it was important to classify them as thoroughly as possible (**Table 4.2, Supplemental 4.6**).

The 53U pedigree has two affected individuals (53U and 55U), presenting with non-syndromic RP, carrying two common *USH2A* variants; c.2276G>T, p.Cys759Phe and c.2299delG, p.Glu767Serfs*21 (**Table 4.1**). The second of the two is found to be inherited as part of a complex allele along with the variant c.4714C>T, p.Leu1572Phe which is classified as 'Likely Benign', in part due to the fact that it has never been observed without the c.2299delG variant. Phasing studies confirmed that these causative variants were *in trans* (**Table 4.1, Figure 4.11(a)**). Full multimodal imaging was not available for patient 53U, except for the colour fundus of which only the image for the left eye was informative, where

bone spicules are present peripherally (**Figure 4.11(b)**). However, full imaging was available for sibling 55U due to a recent appointment. Clinicians have described the imaging as ‘classic RP’ for patient 55U (**Figure 4.11(c.i-iii)**). Boney spicules are evident in the mid-periphery extending into the wider periphery, as is similarly seen in the FAF (**Figure 4.11(c.ii)**). A bullseye pattern is seen in both eyes centrally in the FAF for patient 55U, with a small area of hypofluorescence surrounded by hyperfluorescence (**Figure 4.11(c.iii)**). OCT traces reveal a fluid build-up in the middle layers of the retina, termed ‘cystoid macular oedema’, present in both eyes (**Figure 4.11(c.i)**). It is of note that the photoreceptor layer is well preserved centrally in both eyes for patient 55U.

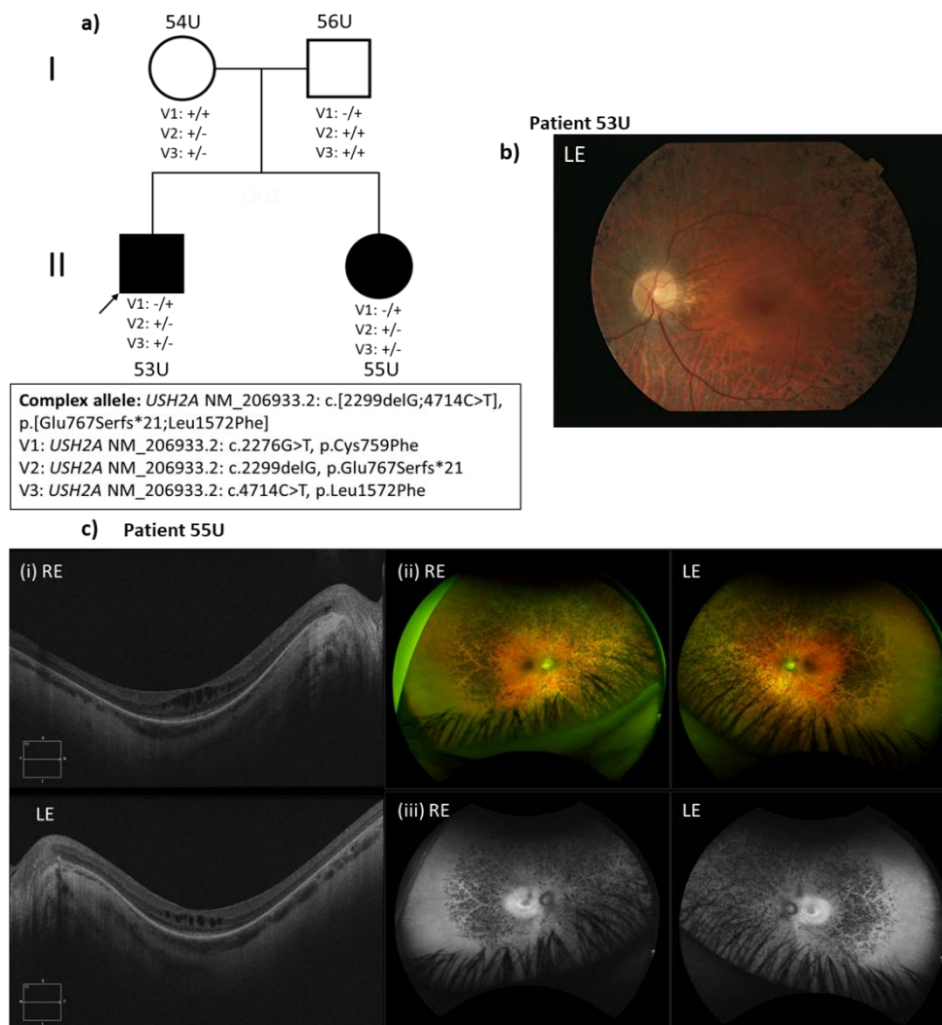


Figure 4.11 (a) Pedigree tree displaying the phasing of candidate variants detected in *USH2A* Irish family. The segregation patterns of the mutations are shown, with variants indicated under the tree. The arrow indicates the proband. The complex allele is indicated the tree in the variant annotation box (+ = wildtype allele, – = mutant allele). **(b) Colour fundus image for patient 53U.** Only the image for the left eye is informative, with other modes of imaging unavailable **(c) Multimodal imaging for patient 55U (i)** Optical coherence tomography (OCT) images, **(ii)** Pseudocolour widefield fundus photos, **(iii)** Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

Similar to patient 55U, the ocular imaging for patient 57U has also been described as characteristic of ‘classic RP.’ However, patient 57U presents with Usher syndrome having experienced hearing loss symptoms at 3 months of age, receiving their Usher syndrome diagnosis then at 9 years of age (**Supplemental 4.3**). The 57U proband is seen to carry two pathogenic null variants; c.655delC, p.His219Ilefs*24 and c.9391dupG, p.Val3131Glyfs*14, with the former being novel (**Figure 4.12, Table 4.1 and 4.2**). While the later (c.9391dupG) has not been previously reported in affected individuals in the literature, it remains well categorised as disease-causing in multiple ClinVar submissions. The colour fundus, reveals heavy bone spicule deposition (**Figure 4.13(a.ii)**). There is a ring of hyperfluorescence centrally in both eyes, with a bulleyes appearance evident in the FAF for patient 57U (**Figure 4.13(a.iii)**). The best-corrected visual acuity (VA) recorded for this patient remains within normal range; 6/7.5 in both eyes (**Supplemental 4.3**). The OCT imaging for patient 57U reflects this as a small narrow area of healthy photoreceptor layer remains centrally in both eyes (**Figure 4.13(a.i)**).

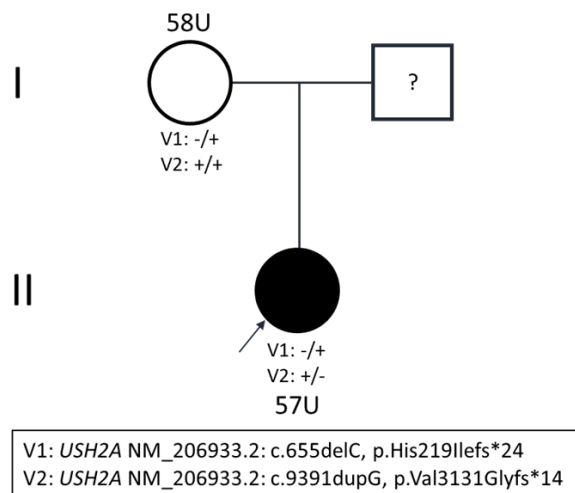


Figure 4.12 Pedigree tree displaying the phasing of candidate variants detected in *USH2A* patient 57U. The segregation patterns of the mutations are shown, with variants indicated under the tree. The arrow indicates the proband 57U. (+ = wildtype allele, – = mutant allele).

Conversely, 59U presents with non-syndromic RP. No family members were available for segregation for patient 59U, but the patient was confirmed to be homozygous for two variants in *USH2A*, that are thought to be acting as a complex allele; c.[2276G>T;6721C>T], p.[Cys759Phe;Pro2241Ser], where variant c.6721C>T is novel and has not been reported in the literature before to the best of our knowledge (**Table 4.1 and 4.2**). Marked thinning of the RPE and retinal layers through to the sclera centrally and surrounding the optic disc is evident in the colour fundus imaging for patient 59U (**Figure 4.13(b.ii)**). This matches with the loss seen in the OCT traces where no visible photoreceptor layer remains in both eyes

(**Figure 4.13(b.i)**). A pan-retinal photoreceptor degeneration diagnosis is backed up by these images. Patchy areas of hyper- and hypo-fluorescence are visible in the FAF in both eyes (**Figure 4.13(b.iii)**). The blur covering each of the images is potentially due to the thickening of the posterior capsule post cataracts surgery, which is noted in the clinical data for this patient (**Supplemental 4.3**).

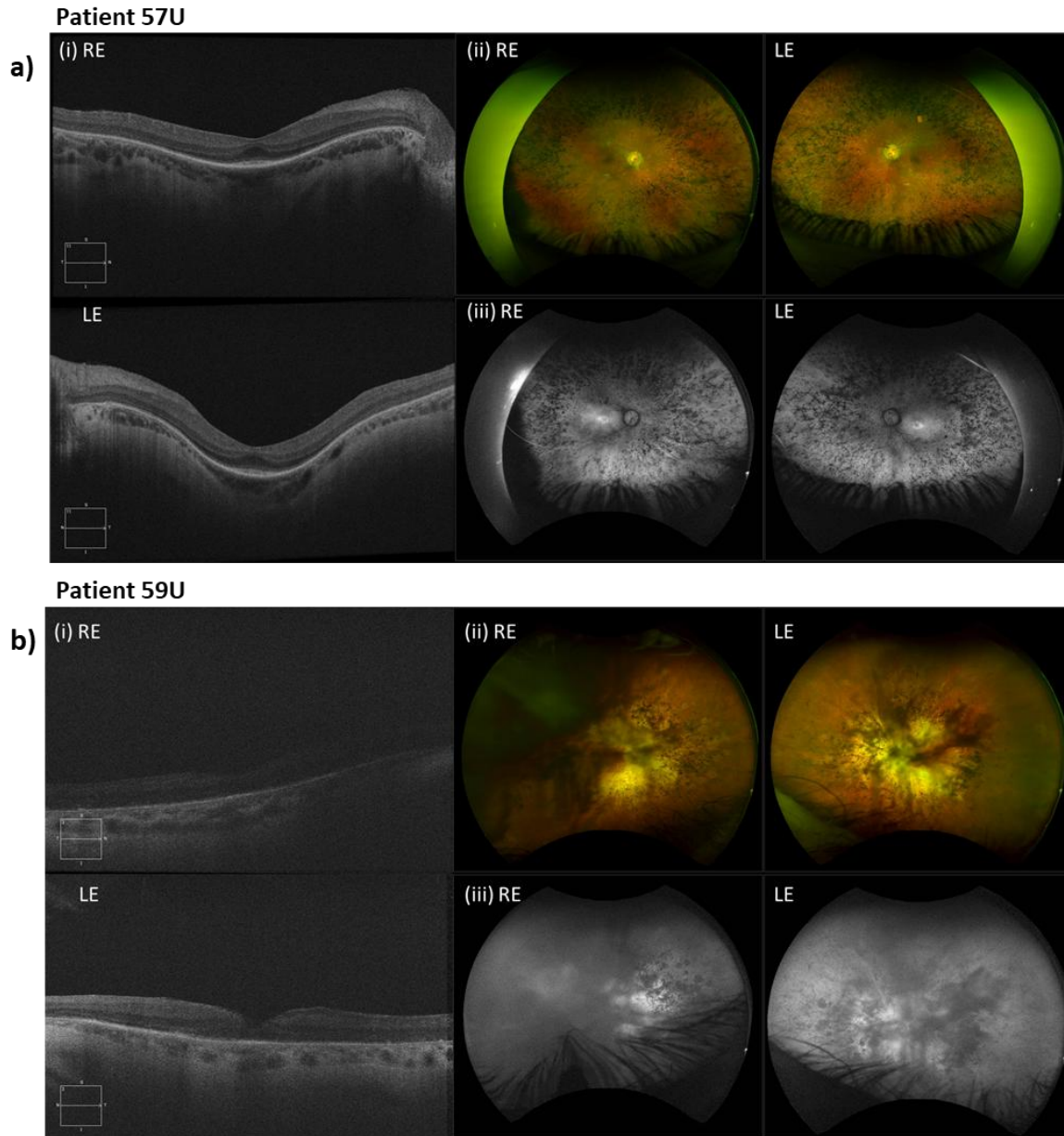


Figure 4.13.(a,b) Multimodal imaging for *USH2A* patients (a) 57U and (b) 59U, where the images for each patient are annotated as follows; (i) Optical coherence tomography (OCT) images, (ii) Pseudocolour widefield fundus photos, (iii) Fundus autofluorescence (FAF). (RE= right eye, LE= left eye).

4.4 Discussion

To date the most popular form of first tier sequencing for projects such as this one, has tended to be targeted NGS of a specific panel of loci, as discussed throughout this doctoral thesis. Given that panel NGS is limited to specific genomic regions, it is possible that the disease-causing variants might not be identified upon first tier sequencing, as these panels tend to focus largely on sequencing the coding regions of a comparatively small number of genes and very specific intronic loci where well-known pathogenic variants have been identified in IRDs. If a candidate variant is not identified after first tier target-capture/panel NGS, a multidisciplinary team approach must guide the next steps for the patient. One consideration would be whether or not the patient might be a good candidate to progress forward for WGS. IRDs are generally great candidates for WGS as a large proportion of them tend to follow a Mendelian pattern; ideally using trios or multiple family members will aid in the interpretation of WGS data. While WGS is a more complete and comprehensive form of sequencing, and is capable of identifying large CNVs and SVs, it too has its pitfalls. In many jurisdictions, it is not practical to run WGS immediately on patients yet, as a first-tier sequencing strategy. WGS opens up the possibility of generating many secondary findings, as well as increasing the workload and, potentially, the capacity for error with respect to highlighting potential candidate variants. When employing WGS, along with generating greater quantities of sequence data for analysis, comes a requirement for significantly greater data storage capacity. Many filtering steps and algorithms are involved in narrowing down candidate variants and so this challenging task takes a considerable amount of time. Hence, while WGS is not recommended as a first tier sequencing strategy for IRD patients, it has been found that using WGS for second tier sequencing, similar to the approach adopted in our Target 5000 study, provides a greater than 40% diagnostic yield for patients who remain unresolved after initial target-capture first tier sequencing (Smedley *et al.*, 2021).

4.4.1 ABCA4

This chapter has provided insights into the retinal phenotypes caused by mutations in *ABCA4*, ranging from classical STGD1 disease to early onset pan-retinal degeneration. Seven *ABCA4* pedigrees are described with different types of mutations, all of which have been previously assigned varying levels of severity. These variants were classified using the ACMG/AMP framework, along with suggested alterations and upgrades recommended by ACGS 2024 and the Cornelis *et al.* cohort (Cornelis *et al.*, 2023; Durkie *et al.*, 2024), using data available through the Target 5000 study, as well as open source databases (**Table 4.2, Supplemental Table 4.4**). STGD1 is often distinguishable from other forms of MD based

on very specific clinical features, some of which can be observed in the fundus imaging. However, this can become more challenging when the disease has progressed to later stages and greater severity. A grading system for patients with *ABCA4*-derived STGD1 has been described by Cremers *et al.* in 2020 (Cremers *et al.*, 2020), with genotype-phenotype studies since then corroborating its validity (Mc Clinton *et al.*, 2023).

Two severe pathogenic *ABCA4* variants were identified to segregate within the 1A pedigree, where patient 1A displayed an early onset CCRD with choroidal involvement which has since progressed into a pan-retinal phenotype (**Table 4.1**). First symptoms began in early childhood, with nyctalopia and visual acuity decline being observed as early as age 8, according to the patient. The disease course appears progressive in this patient, with their most recent ERG having no recordable photoreceptor responses, and visual acuity having decreased significantly to only hand movement recognition remaining in both eyes. *ABCA4* c.161G>A, p.[Cys54Serfs*14,Cys54Tyr] was identified heterozygously in patient 1A. This variant is a non-canonical splice site variant, predicted to lead to the skipping of exon 3 and is classified as having a moderately severe effect on splicing (Fadaie *et al.*, 2019). While this is true, this variant also has a very high REVEL score (0.98), suggesting that the missense variation also contributes to the severity of the variant (**Supplemental 4.4**). Therefore in the most recent re-evaluation of severity of *ABCA4* variants in LOVD.nl, this variant was upgraded to a severe class (Cornelis *et al.*, 2023). The second severe variant confirmed to be acting *in trans* in this patient is c.3056C>T,p.Thr1019Met (**Table 4.2**). This variant was reported to have an functional index (F-index) value of 0.01, where the cutoff used for PS3_moderate ACMG/AMP criteria is an F-index value less than 0.15 (Curtis *et al.*, 2020; Molday *et al.*, 2022a). An F-index close to zero is typically indicative of the absence of significant N-Ret-PE-activated ATPase activity, meaning that the protein is not able to efficiently remove retinoids from the photoreceptor OS, resulting in a severe phenotype, as would be seen with a null variant. This combination of variants has indeed led to a severe and early onset ocular phenotype for this patient, with widespread pan-retinal degeneration, which aligns with the current disease genotype/phenotype model proposed by leading *ABCA4* research groups (Cremers *et al.*, 2020).

The combination, *in trans*, of a moderate and severe allele has been shown to lead to a CCRD, which is seen in patient 4A (**Table 4.1**). This may not always follow the disease model outlined (Cremers *et al.*, 2020), as recent studies have identified a multitude of phenotypes that do not align with this model. This can be due to modifiers at play, such as secondary genes being involved that alter the severity and age of onset as will be discussed

in greater details later in this PhD thesis. Splice variant c.3329-1G>A,p.? has been observed solely in this one patient in the Target 5000 cohort, with only 2 submissions to ClinVar (**Table 4.2, Supplemental Table 4.4**). This variant had a recent PVS1_moderate ACMG/AMP criterion downgrade as it results in an 81-nucleotide elongation which remains in frame. The c.3322C>T variant has been classified to be of moderate severity; this is thought to be due to the profound deleterious nature of this variant observed in prior functional studies (Fakin *et al.*, 2016). While this combination is thought to lead to a more moderate phenotype, it must be noted that patient 4A has more extensively compromised vision than some other patients described here. 4A presents with more severely attenuated and delayed cone ERG responses compared to the other *ABCA4* patients, with ocular imaging demonstrating an almost total loss of the photoreceptor layer, and widespread loss of the RPE centrally in both eyes (**Figure 4.2(b)**).

One novel frameshift variant was identified in patient 7A during the course of this study; c.5594dupA, p.His1865Glnfs*47, and has been classified as pathogenic (**Table 4.2, Supplemental Table 4.4**). Similar null variants that lead to a frameshift in *ABCA4* have been classified as severe, as they result in little to no residual protein activity. This variant is not present in the gnomAD database, supporting its eligibility for the PM2_supporting ACMG/AMP criteria. This criterion can be employed when a variant is absent or at a sufficiently low frequency in control population databases, such as gnomAD. As this variant is located in exon 40 of *ABCA4*, out of a total of 50 exons, nonsense-mediated decay (NMD) is predicted to occur allowing us to apply PVS1 null variant criteria at the original strength. This is the only pedigree we have within the Target 5000 cohort that carries this variant, and we have confirmed that it is acting *in trans* with a second ‘likely pathogenic’ variant in *ABCA4*; c.5773A>G,p.Arg1925Gly (**Tables 4.1 and 4.2**). This missense variant has not previously been assessed for severity, but has been found to be more prevalent in affected individuals compared to unaffected individuals to a degree that is statistically significant (Cornelis *et al.*, 2023). No homozygotes have been recorded in gnomAD, and there have been 3 entries submitted to LOVD.nl with a likely pathogenic classification, as well as 2 submissions to ClinVar. *In silico* tools such as REVEL and Alaphamissense predict a very high likelihood of pathogenicity, where all *in silico* scores and classification logic is outlined in detail in **Supplemental 4.4**. However, the severity of this c.5773A>G variant remains difficult to predict as there are no direct functional assays available that will completely recapitulate its effect at a protein level. Indirect assays may not always correlate with the resulting disease severity or progression experienced by the patient. Large cohort studies that

focus on genotype-phenotype correlations are one of the most informative ways to classify the severity of such variants (X. F. Huang *et al.*, 2015; Zernant *et al.*, 2017, 2018; Lee *et al.*, 2022a; Bianco *et al.*, 2023; Cornelis *et al.*, 2023). Most rare missense variants in *ABCA4* are classified as a VUS, as they are not observed in sufficient numbers to enable genotype-phenotype studies to be carried out. As mentioned above, results from the current array of functional studies often remain inconclusive, as they do not accurately recapitulate what is happening in a patient, despite using multiple *in vitro* studies, and sometimes retinal organoid and/or animal studies. Functional studies in IRD genes, along with working examples resulting from Target 5000, will be discussed further in the next chapter (Chapter 5) and the Discussion section 6 of this PhD thesis.

Patient 7A carries these two aforementioned variants, and presents with a clinical diagnosis of cone-rod dystrophy. The ages of onset of certain ocular phenotypes were provided by the patients themselves and may not be entirely accurate/objective (**Supplemental 4.1**). The patient indicated an age of onset in their late teens. This could be characteristic of multiple variant severity combinations such as; two moderate variants or one severe and one mild fully penetrant variant (Lee *et al.*, 2022a). The ERG for patient 7A reveals severely attenuated cone responses, indicative of a cone-rod dystrophy and aligning with the bilateral central scotoma evident in the Humphrey visual fields test and complete central loss of photoreceptors shown on OCT imagery (**Supplemental 4.1, Figure 4.2(c)**). Discreet deposits are visible upon further inspection of the colour fundus, characteristic of *ABCA4* variation (**Figure 4.2(c)**). This patient presents with a stable disease course, which would not be expected with early onset *ABCA4*-retinopathy where a more progressive and rapid decline is typically observed (Cremers *et al.*, 2020).

The *ABCA4* c.5882G>A variant, present in patient 9A (**Table 4.1**), is seen to be one of the few mild acting variants reported in *ABCA4*. Notably, it has a high allele frequency in the unaffected population, along with reduced penetrance (**Supplemental 4.4**) (Cornelis *et al.*, 2017; Lee *et al.*, 2021b). This variant is also seen to segregate with a milder disease phenotype when *in trans* with a severe variant. Therefore, according to the proposed model (Cremers *et al.*, 2020), the combination of a severe and a mild variant with incomplete penetrance would lead to a later onset STGD1 phenotype, where macular symptoms begin in early to late 30s, progressing in some cases only into extramacular flecks. It is often the case with the c.5882G>A variant that no flecks are observed even though they remain a characteristic of classical STGD1. A recent study by Lee *et al.* 2022 produced a correlation matrix for *ABCA4* variants which placed the most common mild variant c.5882G>A in its

own category (Lee *et al.*, 2022a). They found that the majority of patients where c.5882G>A occurs *in trans* with a severe variant (67%) exhibit a late onset, mild phenotype which presents with foveal atrophy and the periphery remains unaffected. This is consistent with the phenotype observed in our patient 9A, where the variant *in trans* with c.5882G>A in *ABCA4* is the severe and fully penetrant c.4363T>C variant (**Table 4.2, Supplemental 4.4**)(Cornelis *et al.*, 2022). As presented in the results section (**Figure 4.3 (a.i-iii)**), patient 9A presents with a bullseye pattern on their FAF, with central scotoma noted in both eyes on their visual fields while their vision seems spared peripherally. The OCT imaging reveals a loss of photoreceptors centrally, and the most up to date multifocal ERG shows attenuated responses from the foveal and parafoveal segments tested with normal responses peripherally (**Supplemental 4.1**). These results, as well as the lack of extramacular flecks in the fundus imaging suggests that this patient fits with the mild prognostic outcome with late-onset, described by Lee *et al.* (Lee *et al.*, 2022a). The c.5882G>A variant has been associated with small flecks and lesions that remain constricted to the macula and show minimal expansion over time, which is indeed observed here in this patient (Bianco *et al.*, 2023). As well as this, it has been seen to cause a very specific bullseye maculopathy phenotype (Cella *et al.*, 2009). The extensive work that has been completed on this variant, including the intricate genotype-phenotype correlations that have been described, allow us to more accurately infer the prognosis and progression of the disease phenotype, based on their genotype, for this patient. This would not be possible without genetic screening, nor would it be as informative without cascade analysis confirming the phasing of the biallelic variants found. Similarly, it remains vital that the extensive clinical histories continue to be outlined in the scientific literature of patients reported with specific genotype combinations.

Patient 15A is one of only two homozygotes identified globally to date with the c.4539+2028C>T variant in *ABCA4*, with both homozygotes being Irish (**Table 4.1**) (Whelan *et al.*, 2023). This variant was first identified in 2013 (Braun *et al.*, 2013), and has since had functional analyses and genotype-phenotype correlation studies carried out to thoroughly classify this variant as pathogenic (**Supplemental 4.4**) (Albert *et al.*, 2018; Whelan *et al.*, 2023). The functional study was carried out on photoreceptor precursor cells (PPCs) derived from fibroblasts obtained from individuals with STGD1, showing that the c.4539+2028C>T variant results in the inclusion of a pseudo-exon which is 345 nucleotides long (Albert *et al.*, 2018). Semi-quantification of *ABCA4* transcripts showed that ~15% of *ABCA4* transcripts carrying the variant were aberrant. The c.4539+2028C>T variant is seen to occur in *cis* with the c.302+68C>T variant (Schulz *et al.*, 2017; Stéphanie Cornelis *et al.*, 2023). This same

study by *Albert et al.* confirmed that the c.302+68C>T variant does not result in any differences in the mRNA and is predicted not to be pathogenic alone as it does not result in any splice site defects or aberrant recruitment of splice enhancers. Thus far, studies have not been able to definitively confirm whether the c.302+68C>T variant has an additional effect on the severity of this complex allele. This variant is currently categorised as likely benign and remains difficult to classify as it has never been observed alone and always occurs as a part of a complex allele with c.4539+2028C>T (see **Supplemental 4.4** for detailed in-house classification). This is not the case for the c.4539+2028C>T variant, as it has been reported alone in patients and has been well categorised. This variant is thought to be enriched in the Irish population, with only 15 other cases reported globally outside of Ireland to date. (Whelan *et al.*, 2023). Patient 15A was described in Whelan *et al.*, 2023, as referenced above, with the full extended family being sequenced as part of the studies presented in this PhD thesis (**Table 4.1, Figure 4.1(g)**).

The pedigree expanded on here represents one of the only 2 reported homozygous cases of the c.4539+2028C>T deep-intronic variant reported, with the second case also from our Irish Target 5000 cohort. Comparably, we believe a second *ABCA4* variant to also be enriched in the Irish population: *ABCA4* c.161G>A, p.[Cys54Serfs*14,Cys54Tyr], present within the 1A pedigree. This work is currently underway in collaboration with the Alikmets Lab group, where to the best of our knowledge, this variant is most often identified within patients of Irish descent (data in infancy). More than 30 patients carrying this variant in a biallelic manner have been identified within the Irish Target 5000 cohort, 4 of whom were analysed as part of the iMD-smMIPs study (Chapter 3). As evidenced in the iMD study reported on in Chapter 3 of this doctoral thesis, *ABCA4* is the gene most commonly implicated in macular dystrophy cases in Ireland. Within the scope of that study, another novel splice-altering variant has been identified within *ABCA4*: c.5584+5del, that has not been previously reported within any other IRD population globally. Notably, the presence of such deep intronic variants as is c.4539+2028C>T and other non-coding splice-altering variants highlights a feature of the Target 5000 cohort. In the context of a historically and geographically impacted population as is the Irish population, the enrichment of non-coding *ABCA4* variants might reflect the persistence of older alleles that are rare in other more genetically diverse populations. However, as *ABCA4* is highly allelically heterogeneous, further study would be beneficial to determine whether such variants represent broader population-level trends within Ireland, especially as identification of such non-coding

variants represents an expanding area of research in IRDs, particularly *ABCA4*-related disorders.

Within this large pedigree, two sisters are affected; 33A and 34A. They both carry two common disease-causing complex alleles; c.[302+68C>T; 4539+2028C>T], (p.[?; =,Arg1514Leufs*36]) and c.[4222T>C, 4918C>T], p.[Trp1408Arg; Arg1640Trp] (**Table 4.1**). It is evident from their ocular imaging that they have a similar phenotype but at different stages of progression (**Figure 4.4 (b,c)**). As mentioned in the results section, there is a 5-year age difference between the siblings, with 33A being older and displaying more advanced disease. The allele c.[4222T>C, 4918C>T] is very common in *ABCA4* disease cohorts, where it has been classified as likely pathogenic. However, it remains challenging to infer its level of severity. The c.[302+68C>T; 4539+2028C>T] allele has been previously classified as mild to moderate severity, based on level of remaining functional *ABCA4* protein as well as the detailed clinical phenotyping of the only two known homozygotes (Cornelis *et al.*, 2022; Whelan *et al.*, 2023). As both of these siblings appear to have ‘classical’ STGD1 symptoms with first symptoms appearing in late childhood/second decade of life, this suggests that the c.[4222T>C, 4918C>T] allele may be acting as a moderate to severe variant (**Table 4.3**). No definitive conclusions can be drawn from these two patients alone. It also must be understood that the assignment of severity is not a straightforward task and requires constant updating as more patients come to light harbouring these variants. Deep phenotyping and genotype-phenotype correlation studies on a large scale are essential, as well as functional studies and animal/organoid models providing insights into the effect(s) of the mutant protein. There are two other affected individuals in this pedigree; 24A and 26A (**Table 4.1**). These two patients only have one complex allele confirmed through segregation analysis (c.[4222T>C, 4918C>T]), where the two pathogenic variants identified have been confirmed to be acting *in cis* (**Figure 4.1(g)**). It would be of merit to perform whole-*ABCA4* sequencing on these two unresolved patients, as their clinical presentation would suggest that they have a second variant in *ABCA4*.

The c.5461-10T>C is the third most common disease-causing variant found in *ABCA4* in patients with STGD1 (Runhart *et al.*, 2021; Cornelis *et al.*, 2022) Patient 35A, homozygous for this variant, presents with an interesting phenotype as they have received a clinical diagnosis of RP/*ABCA4*-retinopathy (**Table 4.1, Table 4.2**). It has been reported that biallelic severe variants found in *ABCA4* may cause early onset STGD1, where later stages of disease can begin to resemble RP due to the widespread atrophy seen in the ocular imaging. Recently, studies have explored the rescue of the c.5461-10T>C variant using antisense

oligonucleotides (AONs) (Kaltak, de Bruijn, *et al.*, 2023), as this variant has been shown to lead to depleted levels of ABCA4 protein when analysed in patient fibroblasts or patient derived PPCs, utilising minigene splicing assays (Sangermano *et al.*, 2016; Aukrust *et al.*, 2017). The c.5461-10T>C variant leads to the skipping of exon 39 or exons 39 and 40, allowing PVS1 null variant criteria to be applied (**Table 4.2, Supplemental Table 4.4**). This variant is seen to occur commonly as part of a complex allele with the hypomorphic variant c.5603A>T (Aukrust *et al.*, 2017). Homozygotes with this complex allele have been reported previously, but not many have shown the degree of severity in disease progression as seen in patient 35A. As there is no access to more recent tests for patient 35A, it is difficult to comment on the current status of vision. Previously, homozygous patients for the c.5461-10T>C variant have had symptom onset before ten years of age, followed by a rapid decline in vision often leading to legal blindness within the third decade of life (Sangermano *et al.*, 2016). This is consistent with that observed in patient 35A, where similar symptoms and rate of disease progression is seen. Second degree consanguinity is noted between parents, explaining the homozygous presence of the complex allele c.[5461-10T>C;5603A>T] in the patient.

4.4.2 *CDHR1*

Target capture NGS completed prior to these PhD studies identified three variants in *CDHR1* that we believe cause disease in the 37C pedigree (**Table 4.1 and 4.2**). The work reported herein allowed better classification of these variants in order to elucidate pathogenesis of disease in this family and in others who harbour these disease-causing variants (see **Supplemental 4.5** for detailed in-house classifications). Very little is known about the *CDHR1* gene in the context of IRDs, with only a few genotype-phenotype studies focusing on a subset of the most common variants identified. One such variant is c.562G>A, p.Gly188Ser, which was previously identified in three patients described by Ba-Abbad and colleagues (Ba-Abbad *et al.*, 2021). This was the first study to identify this variant, and classified the variant as deleterious based on Polyphen and SIFT data, as well as its conservation across multiple mammalian species. We were only able to classify this variant as a VUS based on its low allele frequency in the gnomAD database (v.2.1; 0.00005256, v.4; 0.0001242) (see **Supplemental 4.5** for *in silico* tool scores). As well as this, we observed its presence *in trans* with a likely pathogenic variant in this 37C pedigree (**Table 4.1**). To allow us to utilise the PM3 allelic/phasing criteria, we also included the 2 pedigrees described by Ba-Abbad *et al.*, 1 patient with confirmed phasing described by Farag *et al.*, and 1 patient with phasing assumed from the Hitti-Malen *et al.* large cohort study (Ba-Abbad *et al.*, 2021;

Farag *et al.*, 2023; Hitti-Malin *et al.*, 2024). This allowed the application of PM3 with a modified level of strength, PM3_Strong (**Supplemental 4.5**) ('SVI Recommendation for in trans Criterion (PM3)-Version 1.0', 2019). In patient 37C, the c.562G>A variant was found *in cis* with a second *CDHR1* variant; c.2228G>A, p.Arg743Gln. This variant has not been reported in the literature before, to the best of our knowledge, but there is one submission to ClinVar where it has been classified as a VUS. Despite this, we have not been able to award it any classification above likely benign (**Table 4.2, Supplemental 4.5**). Therefore, it is uncertain whether this variant is contributing to disease, and if it is indeed contributing, we cannot speculate to what degree without more knowledge regarding its potential effect on the protein. The second allele identified in patient 37C is a frameshift variant c.743del, p.Gly248fs. Loss of function (LoF) is a known mechanism of disease associated with *CDHR1*, with 95 LoF variants reported in ClinVar in *CDHR1* to date. This c.743del frameshift variant is a novel variant, to the best of our knowledge, and is absent from the gnomAD database. We have applied PVS1 null variant criteria without modifying the strength as this variant occurs in the Cadherin-3 domain of *CDHR1*, and will likely undergo NMD as it is in exon 8/13 (**Table 4.2, see Supplemental 4.5** for full in-house classification).

CDHR1 mutations have been associated with a broad range of phenotypes (Stingl *et al.*, 2017; Issa *et al.*, 2019b; Cusumano *et al.*, 2023; Farag *et al.*, 2024), including a late-onset macular dystrophy with predominant cone involvement (Ba-Abbad *et al.*, 2021). Clear correlations have been difficult to establish due to the small individual cohort sizes per study, and the lack of diversity of variants reported, i.e. certain variants such as the common synonymous c.783G>A have been studied in greater detail than other variants (Issa *et al.*, 2019b). Common across all phenotypes observed is the involvement of the macula in atrophy, even in patients with rod-cone dystrophies and RP (Stingl *et al.*, 2017). The Stingl *et al.*, 2017 study also noted that colour vision discrepancies and photophobia were observed in all patients analysed in their cohort, which is consistent with our patient 37C (**Supplemental 4.2**). Patient 37C presented with late onset CCRD in adulthood, with first symptoms noted at age 45 with visual acuity decrease. As outlined in the results section, their imaging reveals mottled and patchy hypo- and hyper-fluorescence centrally in both eyes on the FAF, as well as peripheral spots of complete retinal thinning which are mirrored in the colour fundus (**Figure 4.6 (b.i-iii)**). Consistent with the ERG findings, the photoreceptor layer appears preserved peripherally in the OCT, with only debris remaining of the photoreceptor layer centrally. While the ERG shows only a slight subnormal rod response in both eyes, the cones are significantly delayed in response time and reduced in

amplitude (**Supplemental 4.2**). The *CDHR1* c.562G>A variant has been identified as a hypomorphic allele, and has previously been studied in a patient with RP (Farag et al., 2024). While this remains true, hypomorphic alleles occurring in a biallelic fashion with truncating or null-like variants such as the novel *CDHR1* c.743del, have been associated with late-onset MD or CCRD, which can often be misclassified clinically as AMD (Yusuf, MacLaren and Charbel Issa, 2020). It is hypothesised that this hypomorphic allele c.562G>A in *CDHR1* might allow for some cone function to be retained leading to an RP or rod-cone phenotype. Conversely, when it is seen *in trans* with a null variant as in patient 37C, this preservation of cones is not seen. Similar spectra of phenotypes have been observed in patients with variants in genes whose encoded proteins are also localised to the base of the photoreceptor OS, such as *PRPH2* and *PROM1* (Collison *et al.*, 2019; Ba-Abbad *et al.*, 2021).

4.4.3 *GUCY2D*

An extensive pedigree tree was available for the following family, allowing us to follow the segregation of a dominant acting *GUCY2D* variant in each affected individual analysed (**Figure 4.7**). While this variant was confirmed to be present in each affected family member described, we were unable to obtain DNA samples for any unaffected family members from the 40G pedigree. As previously mentioned, *GUCY2D* is found to be one of the most commonly implicated genes in autosomal dominant progressive CCRDs (Gill *et al.*, 2019). While *GUCY2D* is associated with a considerably smaller range of phenotypic variability, it has been implicated in CCRDs, RP and LCA (Rodilla *et al.*, 2023). The *GUCY2D* c.2480A>C, p.Tyr827Ser missense variant has been identified in the 40G pedigree, and subsequently classified as likely pathogenic (**Table 4.2**, see **Supplemental 4.4** for full in-house classification). This variant is not present in the gnomAD database, and to the best of our knowledge, has only been reported once in previous literature (Falsini *et al.*, 2022) where it was identified heterozygously in a patient with iMD and concluded to be acting in an autosomal dominant manner. Due to the extensive family available to us, we have been able to apply the PP1 segregation criteria at a modified strength, upgrading it to ‘Strong’, based on the number of informative meioses and affected individuals in concurrence with the most up to date recommendations from ClinGen and ACGS (Jarvik and Browning, 2016; Biesecker *et al.*, 2024). This is the only family in Target 5000 where this variant has been identified and implicated in disease. Given that the only other report of the variant was in a single IRD patient, our study involving an extensive dominant pedigree provides a valuable opportunity to examine in much greater depth the retinal phenotype associated with the *GUCY2D* c.2480A>C, p.Tyr827Ser mutation.

Within this family, five individuals (40G, 41G, 42G, 43G, 44G) were identified to be affected with a dominant CCRD phenotype with age of onset ranging from very early childhood to adulthood (4th decade of life) (**Figure 4.7**). Clinically, similar cases such as this have previously been described in association with *GUCY2D*-linked CCRD, albeit different *GUCY2D* mutations (Wu *et al.*, 2024). In pedigree 40G, all affected individuals present with a progressive disease course bar one, 41G, who began to experience photophobia, visual acuity decrease and colour anomalies very early in life (**Supplemental 4.2**). As evident in the results described within this thesis Chapter, the FAF for each individual reveals a disturbance to the autofluorescence centrally, which often correlates with loss of the photoreceptor layer as noted in OCT images, where available (patients 40G, 43G and 44G, **Figure 4.8(a-c)**). The ERG findings show reduced and delayed cone responses in all patients denoting a generalised cone dysfunction, with patients 40G, 41G and 43G displaying mild to severely affected rod responses (**Supplemental 4.2**). This is a frequent characteristic of CCRDs, as scotopic responses decline over time as rod cells slowly degenerate.

4.4.4 *PROM1*

Two brothers (47P and 48P) presenting with a rod/cone pan-retinal degeneration, experienced progressive decline in vision since their early 30s. Two variants in *PROM1* were shown to segregate with disease in this family; c.1354dupT,p.Tyr452fs and c.2446G>C,p.Ala816Pro (**Table 4.1**). The pathogenic c.1354dupT,p.Tyr452fs variant has been well categorised before and has been reported in individuals with varying phenotypes such as CCRD, iMD, maculopathy with rod-cone dystrophy, Stargardt-like disease 4 and RP, and often seen to be inherited in an autosomal recessive manner (Cehajic-Kapetanovic *et al.*, 2019; Panneman *et al.*, 2023; Hitti-Malin *et al.*, 2024; Schließleder *et al.*, 2024). Due to its extensive reporting history, we can apply the PM3 criteria here based on the presence of homozygotes in the literature (see **Supplemental 4.4** for full in-house classification). This variant is a null frameshift variant and is predicted to undergo NMD, permitting the application of PVS1 null variant criterion at its full strength. The *PROM1* c.2446G>C,p.Ala816Pro variant has only been reported once before to the best of our knowledge, in a landscape RP/LCA paper by Panneman *et al.* (Panneman *et al.*, 2023), where it was found in a compound heterozygous state in two unrelated individuals presenting with an rod-cone/RP phenotype. As this is the only pedigree in Target 5000 carrying this variant, we were not able to award a higher strength based on the PM3 allelic/phasing ACMG criteria and therefore we applied it unmodified (**Supplemental 4.4**). We were only able to classify this variant as a warm VUS due to its low frequency in gnomAD (v2.1.1 - 0.00002984 and

v.4 - 0.00005461), and *in silico* predictions which allowed us to apply PP3 computation evidence at supporting level (see **Supplemental 4.4** for *in silico* tool scores). Identification and reporting of this variant in additional affected families may aid in better classification of this variant in the future, further underpinning the importance of such family cascade studies in improving our understanding of IRDs and the pathogenesis of associated genes such as *PROM1*. The exact genotype-phenotype correlation of *PROM1* variants, while widely reported on, remains largely elusive and poorly understood in part due to the clinical heterogeneity observed.

There are both autosomal dominant and recessive forms of *PROM1*-associated retinal degenerations, where the dominant form has been linked to a late onset MD or CCRD phenotype which tends to be milder in severity. Biallelic recessive variants in *PROM1* have been associated with more widespread degenerations such as RP, CCRD, rod/cone dystrophy or pan-retinal degenerations with early macular involvement (Del Pozo-Valero *et al.*, 2019b). The two patients in our study presented with a pan-retinal degeneration with extensive macular involvement. Both brothers, 47P and 48P, presented with early night vision symptoms, which suggests a dysfunction of the rods. This is further emphasised by the ERG results, where there were no recordable rod responses seen in patient 47P, and a gross reduction in rod amplitudes was also noted in patient 48P (**Supplemental 4.2**). The cone and 30Hz flicker were significantly delayed in response time as well as amplitude in both patients. Of note in their retinal imaging, both 47P and 48P displayed extensive thinning centrally of the RPE as revealed in the colour and FAF images, more so in 48P (**Figure 4.10(b,c)**). Both siblings exhibited a severe decline in visual acuity, coupled with diffuse (47P and 48P) and protan (47P) colour discrimination anomalies, indicating a profound impact on cone photoreceptors (**Supplemental 4.2**). It is of note that the four carrier family members; 49P, 50P, 51P and 52P, developed no associated IRD symptoms, but siblings 49P and 51P have been diagnosed with glaucoma, where we have been unable to find any evidence implicating *PROM1* variations in glaucoma. As visual field loss is common to both glaucoma and RP, this raises a suspicion that a retinopathy, rather than glaucoma, accounted for the field loss in these 2 individuals. An early genetic diagnosis of a recessive *PROM1*-associated retinal disease would allow for better understanding of the disease prognosis for patients. Additional longitudinal studies, as were cited above, can expand the landscape of knowledge regarding disease progression and therapeutic approaches by adding to the repertoire of patients reported thus far, alongside detailed phenotyping making it possible to expand on genotype-phenotype correlations.

4.4.5 *USH2A*

With the publication of the ACMG/AMP framework for variant interpretation, the intention of these guidelines was for them to be applicable universally for all Mendelian disorders (Richards *et al.*, 2015). As they became a more mainstream set of guidelines used by research and clinical teams around the world, it became clear that some of the evidence utilised for variant classification required gene and disease specific alterations in order for them to be informative. This has led to some discrepancy between different teams worldwide and how they approach variant classification (further discussed in Section 6. General Discussion). In an effort to provide recommendations for classifying variants specific to a particular disease and to evaluate the evidence associating a particular disease with a genetic cause, ClinGen have put together Variant Curation (VCEPs) and Gene Curation (GCEPs) panels of experts. One set of VCEP guidelines is available for genetic hearing loss (HL-EP), wherein recommendations have been outlined for the classification of variants in *USH2A* (Hearing Loss VCEP, 2022). These guidelines, while specific to sensorineural hearing loss variation, are potentially outdated as they do not take into account the recent ClinGen ACGS recommended updates that were released in 2024 (Durkie *et al.*, 2024). For the variants identified in this study, the Version 2 rules set out in the HL-EP 2022 guidelines were utilised, along with the application of the updated ACGS recommendations where appropriate.

Four affected patients were analysed as part of three pedigrees with *USH2A* variants (**Table 4.1**). The allele frequency cutoffs for PM2_supporting and BS1 criteria set out by the HL-EP were utilised here. A BS1_supporting criterion was applied to three of the *USH2A* variants identified due to their high frequency in the control gnomAD database, using an allele frequency threshold of ≥ 0.0007 (**Table 4.2**, see **Supplemental 4.5** for full in-house classifications) (Hearing Loss VCEP, 2022). The remaining three variants surpassed the threshold for the PM2_supporting evidence (AF < 0.0007). It is of note that variants c.2299delG and c.2276G>T, found in this study, are two of the most commonly implicated variants in *USH2A*-associated disease (Rivolta *et al.*, 2000; Dreyer *et al.*, 2001; Baux *et al.*, 2014; Calzetti *et al.*, 2018). It is for this reason, that even though they appear at a high frequency in the general population, HL-EP recommends the application of PS4 case-control criterion as they are observed in a biallelic manner in affected individuals statistically significantly more frequently than in unaffected carriers. We have applied PS4 for both of these variant classifications at a lower strength (moderate), to be stringent. It is also worth noting that without the PS4_moderate criteria, these two variants would still achieve a

pathogenic and likely pathogenic classification, respectively (**Table 4.2**, see **Supplemental 4.5** for full in-house classifications). Benign BP2 allelic/phasing criterion was applied to variants c.4714C>T (53U, 55U) and c.6721C>T (59U) as they have been detected *in cis* with common pathogenic and likely pathogenic variants, respectively (**Table 4.1**).

Patient 59U did not have any family members available to participate in this study, but was confirmed homozygous for the c.[2276G>T;6721C>T] p.[Cys759Phe;Pro2241Ser] *USH2A* complex allele, where c.6721C>T is a novel variant not previously reported in the literature, to the best of our knowledge (**Table 4.1 and 4.2**). A second novel variant in *USH2A*; c.655delC, p.His219Ilefs*24 was identified in patient 57U, acting *in trans* with the null allele c.9391dupG, p.Val3131Glyfs*14 (**Table 4.1**). These two variants, along with the common variant c.2299delG, have been designated PVS1 null variant evidence as they create a premature stop codon that is believed to lead to NMD of the resulting mRNA and no viable protein (**Table 4.2**, see **Supplemental 4.5** for full in-house classifications). Deleterious variants in *USH2A* have been associated with Usher II and a more severe RP phenotype than is observed in patients with non-syndromic RP due to *USH2A* variants (Pierrache *et al.*, 2016). Patient 57U is the only patient of the 4 described within this Chapter who presents with Usher II syndrome, while the remaining three present with non-syndromic RP (**Supplemental 4.3**). As mentioned previously, clear genotype-phenotype correlations have proven difficult to achieve for *USH2A* mutations. Typically, it is observed that null or null-like variants such as nonsense, frameshift or splicing variants, occurring in a biallelic fashion or *in trans* with a missense variant lead to an Usher II phenotype. In contrast, two hypomorphic missense variants typically result in non-syndromic RP (Pierrache *et al.*, 2016; Stephenson *et al.*, 2023b). Previous genotype-phenotype studies have suggested that the common *USH2A* variant c.2276G>T is mainly associated with a non-syndromic phenotype restricted to the eye. One such study suggested that retinal-specific variants such as this one, can cause non-syndromic RP with hearing being preserved (Lenassi *et al.*, 2015). The Lenassi *et al.*, study categorised 5 additional variants; c.2802 T>G, c.10073 G>A, c.11156 G>A, c.12295-3 T>A, and c.12575 G>A in *USH2A*, all associated with a retinal-specific disease phenotype. Of note, we have 3 patients in the current study presenting with the c.2276G>T variant in a biallelic fashion (**Table 4.1**). Interestingly, all three patients appear to support this categorisation with each exhibiting an ocular restricted phenotype. The eventual hearing abnormalities reported in patient 59U in his mid-60s are believed to be associated with age (**Supplemental 4.3**). The two most common mutations in *USH2A*, c.2299delG and c.2276G > T are both found within exon 13. Recent studies have suggested

the potential use of AONs to skip exon 13 of *USH2A*, as a therapeutic approach, showing great promise in cellular models animal models as proof of concept (Dulla *et al.*, 2021). AONs and their use as a potential therapeutic strategy for IRDs will be discussed in greater detail in Chapter 5 and throughout the General Discussion Section. 6 of this doctoral thesis.

The age of onset of RP symptoms can be highly variable in individuals with variants in *USH2A*, depending on the type of variation present. Usually, symptoms appear during adolescence or early adulthood in the second and third decades of life (Koenekoop *et al.*, 2023). Patient 57U with Usher II displays very early onset of ocular dystrophy, with night vision problems noted as early as 4 years of age (**Supplemental 4.3**). The remaining three patients with RP all begin to develop symptoms in their mid-teens to mid-20s. Symptom onset was reported by the patients themselves; therefore, this is subject to recall bias and may be misleading. Patients 53U, 55U and 57U present with almost normal VA, with patient 59U presenting with a severe VA decrease in their 20s; only hand movements were registered during the most recent test (**Supplemental 4.3**). It is important to note the presence of a cystoid macular oedema on the OCT trace in both eyes for patient 55U (**Figure 4.11(c.i)**). This is a feature observed in up to 50% of RP patients (Stephenson *et al.*, 2023b). Patient 59U presented with the most severe ocular phenotype of the *USH2A* patients described here, with no recordable photoreceptor responses from their ERG and visual fields within 5 degrees in both eyes (**Supplemental 4.3**). As discussed in the results section, there is no visible photoreceptor layer remaining in either OCT trace for patient 59U, with a marked thinning of the RPE and retinal layers through to the sclera centrally noted in the colour fundus (**Figure 4.13(b)**). It is possible that the complex allele identified in this patient, when present homozygously, results in a more severe disease prognosis. As for patient 57U who received an Usher syndrome diagnosis as a young child, while their fundus and OCT traces display classical RP imaging, the ERG and multifocal ERG show no recordable responses from photoreceptors, and only very small islands of peripheral vision remain in their visual fields (**Figure 4.13(a)**). While *USH2A* is a major contributor to RP and Usher syndrome globally, its relative contribution to disease in Ireland is quite high in comparison to other populations, as briefly stated within the results section of this thesis chapter. We have described 2 novel variants in *USH2A* within this chapter, but as evidenced by the above discussion, we see the reoccurrence of the same common disease variant often within the Irish population, even within this smaller selection of pedigrees. This might suggest that a proportion of *USH2A* variants contribute disproportionately to the disease burden within the Irish population. However this remains speculation, as the prevalence and genotype-

phenotype models of Usher syndrome in Ireland are not yet widely reported on, save from a recent study completed as part of Target 5000 in 2023 (Stephenson *et al.*, 2023b).

4.4.6 Variant Classification Guidelines and additional Recommendations

The focus of the study described in this Chapter of the PhD thesis was to undertake a detailed analysis of the genetic basis of inherited retinal degenerations in 13 pedigrees from the Target 5000 cohort. The study involved undertaking cascade studies, collating evidence regarding the pathogenic or benign nature of the identified genetic variants in retinal degeneration genes, largely using a diverse array of *in silico* tools, and categorising these variants according to the latest available international guidelines. The study illuminates the value of such work enabling pathogenic classifications for many variants and therefore providing patients with a genetic diagnosis. Indeed, for example, the classification of 13 variants was updated by undertaking these additional studies. Furthermore, the study highlights the significant work involved in the clinical and genetic evaluation of IRD pedigrees. While this study is funded by research grants, ultimately to accomplish such tasks optimally for large numbers of patients, many additional supports are required in the health services in Ireland and in many other jurisdictions.

Studies such as described in this thesis Chapter are only possible due to the great strides that have been made in NGS technologies and genetic testing, resulting in an exponential increase in the identification of variants in sequenced patients associated with IRDs. This accelerated rate of variant identification has overtaken the rate at which classification guidelines and functional assays are evolving, with over 40% of variants identified in ClinVar being listed as VUS (Finnegan *et al.*, 2025). Some of the challenges posed by the current standard of interpretation guidelines are discussed throughout this PhD thesis and within the General Discussion (Section 6). When classifying variants, population frequency, open access databases such as ClinVar and LOVD.nl, and *in silico* prediction tools are all useful in elucidating the pathogenicity of a mutation. Many such *in silico* tools are beginning to employ machine learning techniques and artificial intelligence (AI), in an effort to improve genetic interpretation and identification on a large scale. Two such tools employed by us in routine variant interpretation is AlphaMissense and AlphaFold from Google DeepMind, when analysing coding missense variants (Nguyen *et al.*, 2024; Varadi *et al.*, 2024; Zhuo, Aggarwal and Liao, 2024). Often it is the case that functional studies, segregation/cascade analysis, deep phenotyping and allelic/*de novo* information is required to upgrade the classification of a variant. While these data and findings inform our understanding of the impact of the variant on the encoded protein, genotype-phenotype

correlations and on their segregation with disease, they require a substantial amount of time and resources that are not always readily available. Brnich *et al.* have published recommendations, as part of the SVI Working Group, on how to apply PS3/BS3 criteria which apply to functional evidences (Brnich *et al.*, 2020). As the original ACMG/AMP guidelines did not specify in detail how to evaluate the validity and application of a functional assay, these recommendations have provided structure and detailed advice on applying this PS3 functional evidence and on the assessment of functional assays relating to the gene in question. As IRDs are a largely heterogenous group of disorders, this means that various functional assays must be utilised to assess the numerous genes and pathways involved. Assays should be designed and carried out on a gene-by-gene basis in a way that takes into consideration the interpretation and classification of the variants themselves being analysed, as well as the gene. ClinGen's VCEPs will greatly aid in accomplishing this feat, as the panels of experts work to provide guidance on appropriate assays based on the underlying mechanism of disease in specific genes or disease groups. As described for the *USH2A* case reports, ClinGen's VCEPs will illuminate some of the gene-specific issues that are encountered when classifying variants. They will undoubtedly prove helpful in proposing functional assays that can be used to apply PS3 at varying strength levels, as they have done within the Hearing Loss VCEP among others (Hearing Loss VCEP, 2022).

It is of note that the PM3 allelic/phasing classification criterion was applied to 20 of the variants interrogated as part of this group of IRD case studies, where 17 of these variants were awarded a Likely pathogenic, or Pathogenic classification (**Table 4.2, Supplementals 4.4 – 4.6**). The PM3 rule is utilised for recessive disorders when a variant is found *in trans* with a pathogenic or likely pathogenic variant in a proband (Richards *et al.*, 2015; 'SVI Recommendation for in trans Criterion (PM3)-Version 1.0', 2019; Durkie *et al.*, 2024). In 15/17 cases, if PM3 had not been utilised to varying degrees of strength, the variant being classified would have received a lower classification by one or two classes, depending on the case. 7/15 of these variants would have remained as a VUS had phasing information not been available, with the remaining 8/15 being downgraded from Pathogenic to Likely pathogenic. This alone highlights the value in phasing and cascade studies, and how allelic data adds to the evidence of the pathogenicity of a variant in a given gene. Sometimes, even in cohorts like Target 5000, we do not have access to family members who have given consent to participate in the study, as was observed in Chapter 3. For such cases, the emergence of targeted long-read sequencing has been shown to provide haplotyping data, allowing the phase of variants to be identified without parental or familial testing (Gupta *et*

al., 2023). This method has proven helpful in recessive cases where a second pathogenic or likely pathogenic variant has been identified, and also in the identification of potential *cis*-modifier loci. VUS are not clinically actionable, posing a problem not only for clinicians, but most importantly, for patients as this impedes their future access to a potential therapy, clinical trial(s) or effective care pathways. A recent review on variant reclassification suggests that in some cancer cases, patients and clinicians have still opted to pursue surgeries or treatment based on a VUS result (Walsh *et al.*, 2024). While there are significant costs and supports needed to do this, VUS require the most attention and it has been suggested that these variants be reclassified on a yearly basis as knowledge evolves and open access genomic databases advance.

As part of the research arm of Target 5000, through close collaborations with clinical and scientific teams nationally and internationally, we have the opportunity to seek further expertise when needed. Having a greater understanding of IRDs is not only beneficial for the patients in our Target 5000 cohort, but is also valuable for ocular researchers and clinicians in providing more comprehensive care to patients globally. As outlined above, categorisation of some candidate IRD causing variants in addition to *in silico* analyses and cascade/phasing analyses will require additional wet laboratory-based functional studies as per the research findings presented in Chapter 5 of this PhD thesis. It is clear that to undertake such work optimally in the future on a population-wide basis, many additional government supports will be required.

5. In Vitro Functional Assays Investigating Aberrant Splicing in IRDs

5.1 Introduction

The focus of the studies described within this PhD thesis Chapter is the functional analysis of known and novel candidate disease causing mutations in IRD genes. Specifically, candidate mutations that may alter splicing and thereby may be causative of retinal disease have been explored, through the use of *in vitro* midgene splice assays. The first variant studied using such assays is c.1307G>A in the *RPGR* gene, which is implicated in disease in a family with X-linked retinitis pigmentosa (XLRP). Additionally, the disease pathogenicity and impact on splicing of a novel variant, *ABCA4* c.5584+5del identified in Chapter 3 as part of the iMD-smMIPs study, has also been described.

XLRP is a particularly severe form of RP, that predominantly affects males, as it is caused by aberrations to genes located on the X-chromosome (Parmeggiani *et al.*, 2011). XLRP in males is most commonly characterised by an early onset of vision loss and nyctalopia, with steady and rapid progression. This typically progresses to peripheral vision impairment and eventually leads to central vision loss. Affected males often are declared legally blind by their early 40s (Sandberg *et al.*, 2007). Mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene (OMIM: *312610) are the most commonly implicated in XLRP, with the *RPGR* gene accounting for approximately 70% of XLRP cases worldwide (Sharon *et al.*, 2003; Branham *et al.*, 2012). *RPGR* has been implicated in X-linked cone-rod dystrophies, and various forms of XLRP, also termed RP3. As stated before, these IRDs predominantly affect males, with milder symptoms occasionally being apparent in some carrier females (Comander *et al.*, 2015). *RPGR*'s primary function is in ciliogenesis and in maintaining the viability of photoreceptor cells in the retina. The gene is expressed in the outer region of both rod and cone photoreceptor cells and *RPGR* protein has been found to localise to the connecting cilia (Yang *et al.*, 2021; Gocuk *et al.*, 2025). To date, approximately 700 pathogenic/likely pathogenic variants have been reported in *RPGR* on ClinVar (Landrum *et al.*, 2014; 'RPGR, gene', accessed June 2025).

Thus far within the scope of this thesis, the ATP-binding cassette transporter, alpha 4 subunit (*ABCA4*) gene (OMIM: *601691) has been discussed at great length due to its profound involvement in iMDs worldwide (Chapters 3 and 4). *ABCA4* encodes a transmembrane protein that localises to the discs of the photoreceptor outer segments. The *ABCA4* protein's

primary role is in retinoid transport and recycling, preventing the toxic buildup of bisretinoids that accumulate during the visual cycle (Molday, Rabin and Molday, 2000; Molday, Zhong and Quazi, 2009b). To date, more than 1300 pathogenic/likely pathogenic variants have been submitted to ClinVar, with recent studies contributing to the LOVD.nl curated database categorising 1,248 pathogenic/likely pathogenic *ABCA4* variants, including details regarding disease severity (Stéphanie Cornelis *et al.*, 2023). Biallelic variants in the *ABCA4* gene lead to a spectrum of retinopathies including Stargardt disease (STGD1) and Fundus Flavimaculatus, CCRDs, and RP in some cases. This phenotypic heterogeneity is believed to be dependent on the combination of variants present in *ABCA4* and their relative severity (Cremers *et al.*, 2020; Cornelis *et al.*, 2022a; Lee *et al.*, 2022a), as previously discussed (Chapters 3 and 4). The *ABCA4* gene exhibits a high incidence rate of disease-causing non-canonical splice site (NCSS) variants and deep-intronic variants (DIVs), with many studies focused on elucidating variant impact(s) on splicing through *in silico* assessments and *in vitro* functional assays (Khan *et al.*, 2020; Corradi *et al.*, 2023; Cornelis *et al.*, 2023). It is hypothesised by research groups in RadboudUMC studying *ABCA4*-associated retinopathies, that all recessive *ABCA4*-associated cases retain no more than 40% *ABCA4* protein activity expressed from both alleles combined (Sangermano *et al.*, 2018a; Cremers *et al.*, 2020), where the level of residual protein influences the severity of the allele and resulting disease phenotype.

Along with the extra context that the noncoding genome provides, also comes the substantial gap in knowledge on how to interpret noncoding variation and its impact on cellular processes such as gene expression and splicing. Splicing, as previously outlined in the general introduction, is a complex process that can be perturbed by variants in the protein coding, intronic and regulatory regions of genes. Splicing occurs during the maturation of pre-mRNA into mature mRNA ((Lee and Rio, 2015; Qian *et al.*, 2021). It is a very meticulous process that relies heavily on the recognition of specific splice site motifs, to allow for precise excision of introns from the pre-mRNA. These motifs can be mutated, leading to exon skipping or aberrant intronic inclusion, among other defects. Mutations in the coding regions of a gene can also lead to the creation of inappropriate splice site motifs within exons. Variants that are found more than 100bps into an intron are known as deep-intronic variants, and are thought to make up part of the missing heritability seen in genetically unresolved IRD cases. In recent years, continued applications of NGS technologies has allowed for the identification of deep intronic variants in IRD-associated genes (Sangermano *et al.*, 2019; Qian *et al.*, 2021b; Corradi *et al.*, 2023; Whelan *et al.*, 2023). This means that the splice

machinery may recognise newly produced ‘cryptic’ splice sites, rather than the canonical sites (CSS) where splicing should naturally occur, resulting in a possible mature mRNA defect. In fact, recent predictions state that between 9-30% of causative variants in Mendelian disease contribute to impairing the splicing process (Stenson *et al.*, 2020; Fernández-Suárez *et al.*, 2025). It is notable that the branchpoint is an important motif that allows for the coordination of the correct splicing pattern. There is a lot still unknown regarding splicing branchpoints present in humans. These regions are highly conserved and located close to the 3’ splice site (acceptor) in the pre-mRNA (**Figure 5.1**). This consensus sequence always appears as the short ‘yUnAy’ sequence, where the y is a pyrimidine and the n is any nucleotide (Gao *et al.*, 2008). The U and A nucleotides are more conserved than the pyrimidines, with the adenosine nucleotide representing the branchpoint lariat point of formation. The 5’ splice site (donor) is linked with the branchpoint that is next to the 3’ end of the intron-to-be spliced out of the mRNA. This link forms the lariat structure, which is recognised by the spliceosome and is subsequently removed to join together the two surrounding exons (Will and Lührmann, 2011) (**Figure 5.1**). Mutations that destroy the branchpoint have been closely associated with disease causation, with these mutations typically leading to exon skipping (Padgett, 2012). However, due to the high levels of degeneracy of the sequences that surround the branchpoint in introns, not many variants in these sequences have as yet been reported as being involved in causing disease pathologies. The *RPGR* c.1307G>A,p.? variant to be discussed in this chapter, is predicted to lead to the production of a cryptic branchpoint site within the exon located downstream of a naturally occurring cryptic acceptor site leading to pathology in an expansive Irish pedigree.

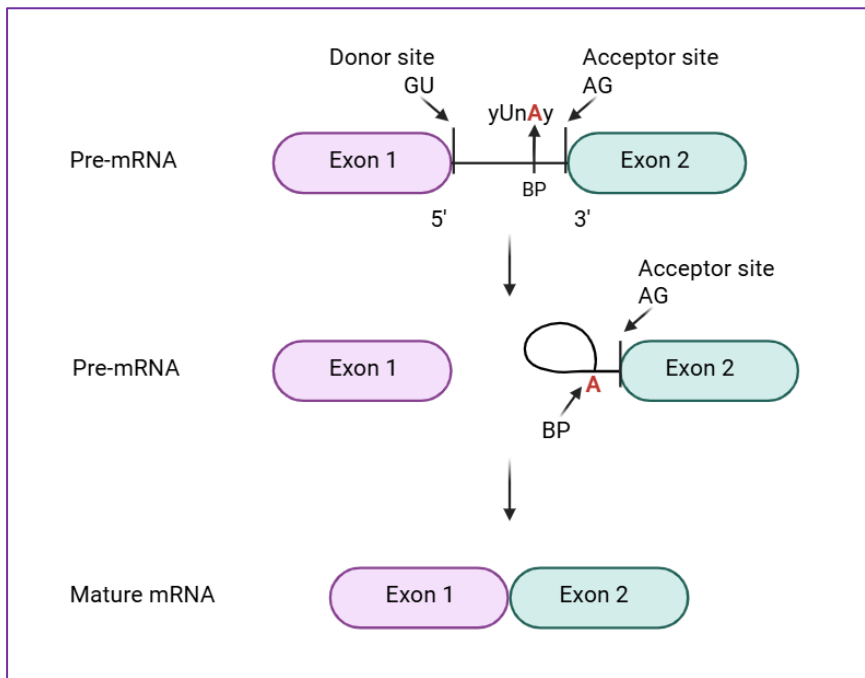


Figure 5.1. Pre-mRNA splicing schematic indicating the location of the branchpoint within an intron. The branchpoint is typically located in the intron, between 20-50bp upstream of the acceptor site. The ‘yUnAy’ motif is indicated here for clarity. The GU sequence motif marks the 5' splice site (donor) and the AG sequence marks the 3' splice site (acceptor). Created using BioRender, (BP=branchpoint). (Will and Lührmann, 2011; Pan *et al.*, 2015).

As part of this thesis, a large body of work is focused on the characterisation of genetic mutations causative of the disease pathology in Irish IRD patients. Variant classification or re-classification represents an area of high priority, as this will ultimately allow us to provide preliminary research-grade genetic diagnoses for patients, requiring validation by a clinically accredited laboratory. It can often be the case, as encountered in previous chapters of this thesis, that variants appear to have good evidence to support their pathogenicity but remain classified as a variant of unknown significance (VUS). To further interrogate this with respect to the potential effects of such variants on splicing, we evaluated whether results from *in silico* tools such as Alamut Visual Plus software (SOPHiA GENETICS, Switzerland) and SpliceAI (de Sainte Agathe *et al.*, 2023a) support the hypothesis that these variants can indeed have an effect on splicing. Variants with a Splice AI score of ≥ 0.2 are considered as having a potential impact on splicing, warranting further investigation. Most recent recommendations by the ClinGen SVI group have supported this cutoff threshold, indicating that a SpliceAI score of ≥ 0.2 is sufficient evidence to apply the PP3 criteria at a supporting level (Walker *et al.*, 2023). If the scores suggest pathogenicity due to splicing disruption, then typically we utilised a functional *in vitro* splice assay known as the midigene assay, as

previously described (Sangermano *et al.*, 2018a, 2019). Variants that have been predicted to have an impact on splicing, whether they are located within the coding or non-coding region of an IRD gene, often require further functional analysis in order to assess the validity of the *in silico* splicing predictions, categorise the effect on splicing and ultimately describe the pathogenicity of the variant. Routinely for splice-altering variants identified in IRD genes, Mini- or Midigene *in vitro* splice assays are carried out to analyse the impact a variant may have on pre-mRNA splicing, with resulting wildtype and experimental constructs transfected in HEK293 cells (Sangermano *et al.*, 2018a, 2019). Within this study, we have utilised a Midigene assay as opposed to smaller minigene assays, where only a single exon is analysed. Midigene splice assays include multiple exons and larger flanking intronic sequences within the wildtype and experimental constructs, as opposed to minigenes which usually only contain one exon and very little intronic context. This allows for Midigenes to provide greater genomic context and for more complex or multifaceted splicing defects to be identified, such as multiple exon skipping and pseudoexon inclusion. Similarly, Midigenes may be used instead of patient RNA, as many IRD genes like *ABCA4*, *USH2A* and *RPE65* are often poorly expressed in most readily available patient-derived tissues such as fibroblasts (Weber *et al.*, 2025). Previous studies have shown that Midigene assays can be utilised to investigate the effect of variants on splicing in IRD-associated genes with great success, proving them to be an efficient and cost-effective initial screening process to define the consequences of a predicted splice-altering variant in HEK293 cells. The outcomes of this functional assay could allow us to apply PVS1_RNA criteria at varying strengths, facilitating the reclassification of splice-altering variants and improving molecular diagnosis in IRDs.

In this doctoral thesis chapter, to demonstrate the importance of functional studies and to assess the effect(s) of coding and noncoding variation on mRNA and splicing, two *in vitro* midigene splice assays of candidate disease variants; *RPGR* c.1307G>A and *ABCA4* c.5584+5del have been outlined in further detail. To progress a candidate variant from a VUS to a pathogenic variant, it is important to understand the functional effect(s) of such variants. Splice assays such as midigene assays enable us to do so for a category of variant that operates at the level of perturbed splicing. Interestingly, to the best of our knowledge, *RPGR* c.1307G>A is the first pathogenic variant reported that leads to creation of a branchpoint within an exon associated with an IRD. The midigene protocol is detailed within the next section (Materials and Methods section 5.2.6).

5.2 Materials and Methods

5.2.1 HEK293 cell culture

5.2.1.i Growth conditions

HEK293 cells (ATCC: accession number CRL-1573) were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% Sodium pyruvate, routinely in T75 flasks at 37°C, 5% carbon dioxide.

5.2.1.ii Sub-culturing

Cells were passaged biweekly with splits of 1:10 or 1:20 using standard procedures, in a total volume of 20mls DMEM media in T75 flasks. Cells were detached with 0.25% Trypsin-EDTA at 37°C for approximately 2 minutes.

5.2.1.iii Freezing and Thawing

To freeze the HEK293 cells, cells were detached with Trypsin-EDTA as above, and Trypsin-EDTA was subsequently deactivated with 4X of supplemented DMEM. The cells were pelleted by centrifugation at 300gs for 5 minutes. This cell pellet was resuspended in freezing medium (90% FBS and 10% DMSO; 1ml per $\sim 10^6$ cells). The cell suspension was rapidly aliquoted into cryotubes and frozen using the Nalgene® Mr. Frosty freezing container at -70°C. This allows for a slow rate of cooling (approximately 1°C per minute) of the cells to decrease the likelihood of ice crystals forming that would damage the cells. After 24 hours, the frozen cells were transferred into regular storage boxes and kept in either liquid nitrogen storage or at -70°C.

To thaw the HEK293 cells for use, cells were defrosted quickly and transferred immediately into 10ml of prewarmed DMEM media (supplemented with 10% FBS, 1% L-Glutamine and 1% Sodium pyruvate). The cells were then pelleted, as before, by centrifugation at 300gs for 5 minutes. Cell pellets were resuspended in 20mls of DMEM media and transferred into a T75 and maintained at 37°C, 5% carbon dioxide.

5.2.1.iv Cell counting

Following detachment of cells with Trypsin-EDTA, cells were suspended in 10mls of DMEM media (supplemented with 10% FBS, 1% L-Glutamine and 1% Sodium pyruvate) and were counted using a haemocytometer, according to protocol indicated by manufacturer. Briefly, the cells in four large grid squares were counted, averaged together and then this figure multiplied by 10^4 to give the number of cells per ml in the cell suspension (Absher, 1973; Camacho-Fernández *et al.*, 2018).

5.2.2 Preparation of Competent cells; XL1-Blue

For all instances of Gateway cloning used for the midigene splice assays, the commercial DH10B™ competent *E. coli* cells (Thermo Fisher Scientific Inc, Waltham, MA, USA) were used. XL1-Blue competent cells (Bullock, 1987; Achatz and Skerra, 2024) were only used for the transformation of the destination vector utilised within this study (see section 5.2.3 Transformation below).

The XL1-Blue cells were streaked onto a Tetracycline (0.025g/l) Agar plate and incubated overnight at 37°C. A single colony was picked from the overnight plate and used to inoculate 5ml of LB. This was grown at 37°C overnight shaking at ~220rpm. After ~16-18 hours, 2mls of this overnight culture was added into 200mls LB in a conical flask and incubated at 37°C with agitation for ~4 hours, until bacteria reached the exponential growth phase. The LB culture was placed on ice for 15 minutes and subsequently centrifuged at 6000g for 5 mins at 4°C. This cell pellet was resuspended in 100ml of cold calcium chloride (50mM CaCl₂), and placed on ice for a further 30 minutes before being centrifuged as above. The resulting cell pellet was resuspended in a mixture of 17ml of cold 50mM CaCl₂ and 3ml of glycerol for freezing. 1ml aliquots were immediately distributed into Nalgene freezing tubes and snap frozen in liquid nitrogen to be stored at -70°C.

The transformation efficiency of the XL1-Blue competent cells was tested by transforming a serial dilution of DNA plasmid with an ampicillin resistance gene ranging from 100ng-1pg. Observation of bacterial colonies on an LB agar plate with ampicillin at 1pg confirmed that the XL1-Blue cells were sufficiently competent for subsequent ligations.

5.2.3 Transformation

Plasmid DNA (between 10-150ng) was transformed into 100 µl of competent XL1-blue *E. coli* cells or DH10B™ competent *E. coli* by heat shock at 42°C for 50 seconds, followed by 2 minutes on ice. 1 ml of prewarmed media was added and cells were incubated (37°C), shaking at 100rpm for 1 hour. 200 µl of cells was then plated onto agar plates supplemented with appropriate antibiotic and incubated upside down overnight at 37°C. Individual colonies were picked the next day and inoculated in LB medium supplemented with an appropriate antibiotic for overnight growth. The resulting cultures were maxiprep using the HiSpeed® Plasmid Maxi Kit (Qiagen Ltd., Germany) or miniprep using GeneJET Plasmid Miniprep Kit (Thermo Scientific™, Lithuania), according to the manufacturer's protocols (see section 5.2.4 Preparation of plasmid DNA below).

5.2.4 Preparation of plasmid DNA

5.2.4.i Maxiprep

A single colony was picked from an overnight transformation plate, and used to inoculate 5mls of LB broth containing the appropriate antibiotic (Ampicillin 0.5g/l, Kanamycin 0.5g/l). This was incubated at 37°C for around 8 hours with agitation. After the incubation period, 2ml of this starter culture was used to inoculate a 200ml flask of LB with antibiotic selection, and was left to grow at 37°C overnight at 220rpm. The next day, the cells were harvested from the flask through centrifugation at 6000gs for 15 minutes at 4°C. Plasmid DNA was extracted using a HiSpeed® Plasmid Maxi Kit (Qiagen Ltd., Germany) following the manufacturer's protocol. Plasmid DNA was eluted into 1ml of nuclease-free water. DNA concentration was determined in ng/μl using a NanoDrop ND1000 spectrophotometer.

5.2.4.ii Miniprep

A single colony was picked from an overnight transformation plate and was used to inoculate 3ml of LB containing the appropriate antibiotic (Ampicillin 0.5g/l, Kanamycin 0.5g/l). These tubes are incubated overnight at 37°C in an orbital shaker set at 220rpm. Each bacterial culture was centrifuged in a microcentrifuge at 12,000rpm for 5 mins to pellet the cells. The plasmid DNA was extracted using a GeneJET Plasmid Miniprep Kit (Thermo Scientific™, Lithuania) following the manufacturer's protocol. Plasmid DNA was eluted into 50μl of nuclease-free water, after a 5-minute incubation at room temperature. DNA concentration was determined in ng/μl using a NanoDrop ND1000 spectrophotometer.

5.2.5 Restriction digests

The SnapGene Viewer software was utilised to choose restriction enzymes (New England Biolabs, NEB). Restriction enzyme digestions of DNA were set up as shown in the table below, and were performed for 2 hours at 37°C. 2μl of digested DNA was subsequently separated on a 1% agarose gel (supplemented with 1μg/ml of EtBr) to visualise DNA under UV light. Digested DNA was sized with a 1Kb Plus DNA ladder (Invitrogen™). For the *RPGR* plasmids, enzyme EcoRV-HF was utilised, as it digested the plasmid in two locations (**Figure 5.7**). For the *ABCA4* plasmids, enzymes MluI-HF and NaeI were used (**Figure 5.12**). Restriction digests were used as a diagnostic tool to confirm successful cloning of DNA fragments into plasmids.

Restriction enzyme digestion mix:

1 μ l	rCutsmart Buffer
0.5 μ l	NEB Restriction enzyme
X μ l	Miniprepplasmid (max. 500ng)
X μ l	Nuclease-free water

Make up to 10 μ l final volume

5.2.6 Midigene splice assay

5.2.6.i Variant and wild type midigene cloning

A midigene assay is a form of functional assay designed to test the potential effect a variant might have on the splicing of pre-mRNA. These midigene assays were designed and carried out in accordance with previous reports (Sangermano *et al.*, 2018a; Khan *et al.*, 2020) and a simplified workflow schematic is shown in **Figure 5.2** below. The sequence of genomic DNA containing the variant of interest was amplified using either the TaKaRa LA PCR Kit Version 2.1 (TaKaRa Bio, Kusatsu, Shiga, Japan) or GoTaq® Long PCR Master Mix (Promega Corporation, Madison, WI, USA), from patient DNA using primers that were designed with 5' attB1 and attB2 tags (tags highlighted in red in Supplemental 5.1). This enables Gateway cloning to be carried out. The wildtype and mutated inserts were introduced separately into pDonor221 (pDONR) using an Invitrogen™ Gateway BP Clonase II enzyme kit (ThermoFisher, Waltham, MA, USA), as per the manufacturer's protocol, and subsequently transformed into DH10B™ competent *E. coli* (Thermo Fisher Scientific Inc, Waltham, MA, USA) (**Figure 5.2**). Following the transformation, resulting bacterial colonies were picked and plasmid DNA isolated using a miniprep kit as described in Materials and Methods 5.2.4.ii, creating the 'entry clones.' 'Entry clones' were subject to digestion using appropriate restriction enzymes to confirm the insert size and orientation (see 5.2.5 Restriction digests above). The insert sequence was confirmed by Sanger sequencing (Eurofins Genomics Tubeseq service, Heidelberg, Germany) (see 4.2.5 Confirmational Sanger sequencing, in Chapter 4). 150ng of each 'entry clone' was then cloned into the pCI-NEO-RHO Gateway-adapted vector (Sangermano *et al.*, 2016), using an Invitrogen™ Gateway LR Clonase II enzyme kit (ThermoFisher, Waltham, MA, USA) as per the manufacturer's protocol. Resulting colonies were picked and plasmid DNA miniprepplasmid as per Materials and Methods 5.4.2.ii. The resultant wildtype and mutant expression clones

were sent for Full plasmid sequencing (Eurofins Genomics Full plasmid service, Heidelberg, Germany), to validate the contents of the construct, the orientation of the insert, and verify no SNPs had been introduced during recombination.

5.2.6.ii Variant and wild-type plasmid transfection

Variant and wild type expression plasmids were transfected into HEK293 cells. Cells were seeded at a density of 2×10^5 cells per well, in six-well plates. These plates were incubated at 37°C until approximately 70-80% confluence was reached. Cells were transfected with 0.5µg of expression plasmid suspended in 25µl of Opti-MEM™ Reduced Serum Medium (Gibco™) mixed with 0.5µl of Lipofectamine™ 2000 suspended in 25µl of Opti-MEM™, as per Lipofectamine™ 2000 Transfection Reagent manufacturer's protocol. Transfections were performed in duplicate for each experiment.

5.2.6.iii mRNA analysis

Variant and wild type mRNA was isolated from HEK293 cells 24-hours post transfection using an RNeasy RNA isolation kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. However, an amendment was made to the protocol, where an on-column DNase digestion step was performed for 2 hours at 37°C, to ensure that all of the transfected plasmid DNA was digested and removed. RNA concentration was determined with a NanoDrop ND1000 spectrophotometer. cDNA was subsequently synthesised from the RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. RT-PCRs were performed on resulting cDNA using primers that bind within the pCI-NEO-RHO backbone. These were performed with 5x FIREPol Master Mix (Solis Biodyne, Tartu, Estonia). All primer sequences are provided in Supplemental 5.1. The amplified DNA fragments were separated and sized by agarose gel electrophoresis (2% gels). Amplification products were excised from gels and purified using the NucleoSpin® Gel and PCR Clean-up kit, as per manufacturer's protocol, eluting into 20µl of nuclease-free water. Purified amplification products were Sanger sequenced to identify the resulting sequence after splicing. *Beta Actin* and exon 5 of *RHO* were also amplified as expression and transfection controls, respectively (see controls primers in Supplemental 5.1 and *Beta Actin* control gel in Supplemental 5.5). The cDNA, RNA and protein notation of all variants were obtained using the MANE transcript listed for each gene.

5.2.6.iv Protein Modelling

The AlphaFold Server online interface, developed by Google DeepMind was used to produce the protein fold models predicted to occur based on the transcripts resulting from

the mutant midigenes (Varadi *et al.*, 2024). UniProt was used to retrieve wildtype protein sequences for RPGR and ABCA4, and aid in generating HGVS nomenclature of the protein consequences for each mutant transcript (Bateman *et al.*, 2022). ChimeraX (version 1.10) was used to analyse the protein models, in order to visualise and highlight the residues of interest. No hydrogen bond changes or inter-residue clashes were noted. InterPro online interface was used to identify the protein domains lost/impacted in each transcript.

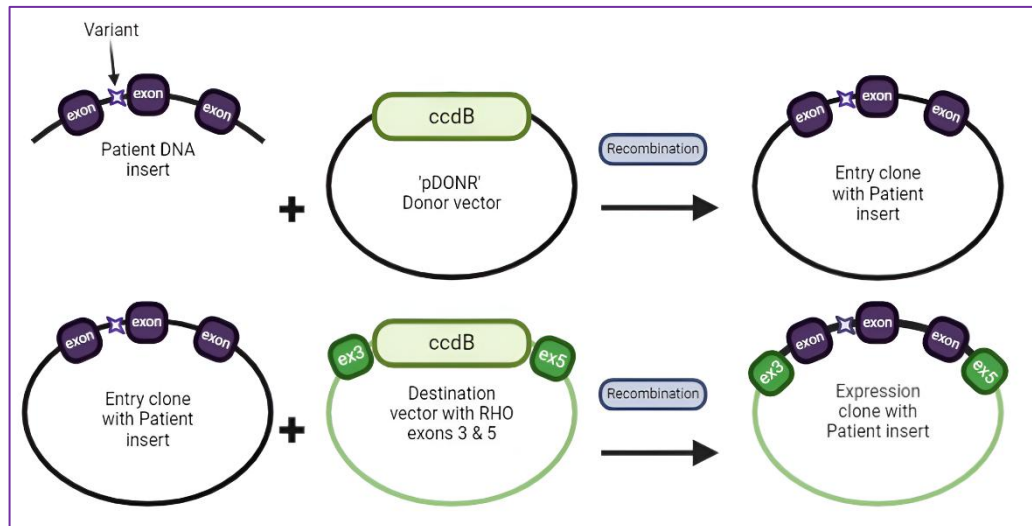


Figure 5.2. Schematic representation of Gateway cloning used for midgene functional splice assay. Top row depicts recombination event that occurs during BP reaction, between DNA insert of interest and pDONR donor vector to create an entry clone. Bottom row depicts recombination event that occurs subsequently as part of the LR reaction, between the entry clone and the pCI-NEO-RHO destination vector to create the final expression clone. (ccdB= death gene). Created using Biorender.

5.3 Results

Within this thesis chapter we have described the *in vitro* splice assays undertaken on two variants identified in Irish IRD patients through two different forms of target capture sequencing. The VUS in *RPGR*, c.1307G>A, p.[Gly436Asp, p?], was initially identified in an extensive Irish pedigree through targeted sequencing of the coding regions of 254 IRD genes, undertaken using the Target5000 panel (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020a). The second variant, *ABCA4* c.5584+5del, p.? is a novel splice site variant and was identified in a single STGD1 pedigree as part of the iMD-smMIPs study described in Chapter 3 of this thesis. Both variants exhibit intriguing *in silico* predictions of splice defects and therefore were selected for functional studies to further analyse their potential pathogenicity and impact on splicing. RT-PCR analysis was carried out on *RHO* exon 5 for each construct, as a transfection efficiency control. For details of *in silico* SpliceAI prediction and RT-PCR methodologies see Materials and Methods section 5.2 of this Chapter. While not described herein, additional work was carried out on midigene assays on *PROM1*, *CHM* and *CACNA1F* throughout the course of this doctorate, which are listed as manuscripts within the Thesis Outcomes section of this thesis.

5.3.1 *RPGR* c.1307G>A, p.[Gly436Asp, p?]

8 affected males are noted in the *RPGR* pedigree, with 5/8 participating in the Target 5000 study. These 5 affected male family members including the proband 4.5, are hemizygous for the *RPGR* c.1307G>A, p.Gly436Asp variant (**Figure 5.3**). The variant has been confirmed to only be present in affected family members, as well as causing a mild phenotype in patient 2.5 who is a carrier female. Patient 3.5 is also a carrier female but no symptoms have been recorded. The other three females in the pedigree tree that are marked in black and white have not been genotyped but are annotated as obligate carriers. Individual 10.5 is confirmed homozygous wildtype for this variant and is the only female we have genotyped who is not a carrier.

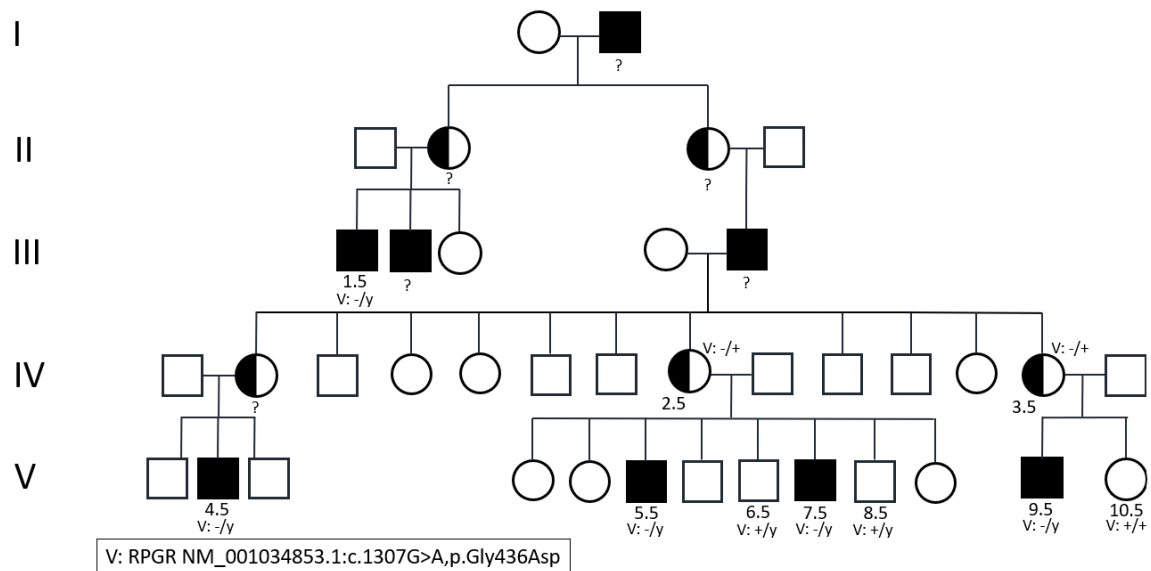


Figure 5.3. An Irish pedigree tree in which the variant was first found, displaying phasing of the *RPGR* branchpoint variant. The segregation pattern of the candidate *RPGR* splice-altering mutation is presented. Note female family members denoted by a black and white circle indicate a female carrier phenotype. (+ = wildtype allele, - = mutant allele, Y = Y chromosome, ? = not genotyped as samples were unavailable).

Multimodal imaging for unaffected family members 6.5 and 10.5 is shown in **Figure 5.4**, where both individuals exhibit healthy colour fundus images and preserved retinal layers evident in the OCT. Patient 2.5 is also represented in **Figure 5.4**. The female carrier's colour fundus revealed subtle pigmentary disturbances in the peripheral retina. Whereas, the OCT for Patient 2.5 reveals subtle thinning of the outer nuclear layer with a well-maintained ellipsoid zone throughout the macula. These features are characteristic of a mildly symptomatic X-linked female carrier. Patient 1.5 is in the 3rd generation of this pedigree; in the same generation as the grandparents of proband 4.5. As is noted in the clinical data (**Supplemental 5.2**), Patient 1.5 has a visual acuity that has been reduced to only being able to observe hand movements, with no recordable ERG or visual fields to the largest target. Extensive bone spicule deposits in a 360-degree pattern were noted in the colour fundus of patient 1.5 by clinicians, but dense cataract in both eyes prevented extensive imaging. A complete outline of the clinical phenotype for the remaining 4 affected male family members is also displayed in **Supplemental 5.2**.

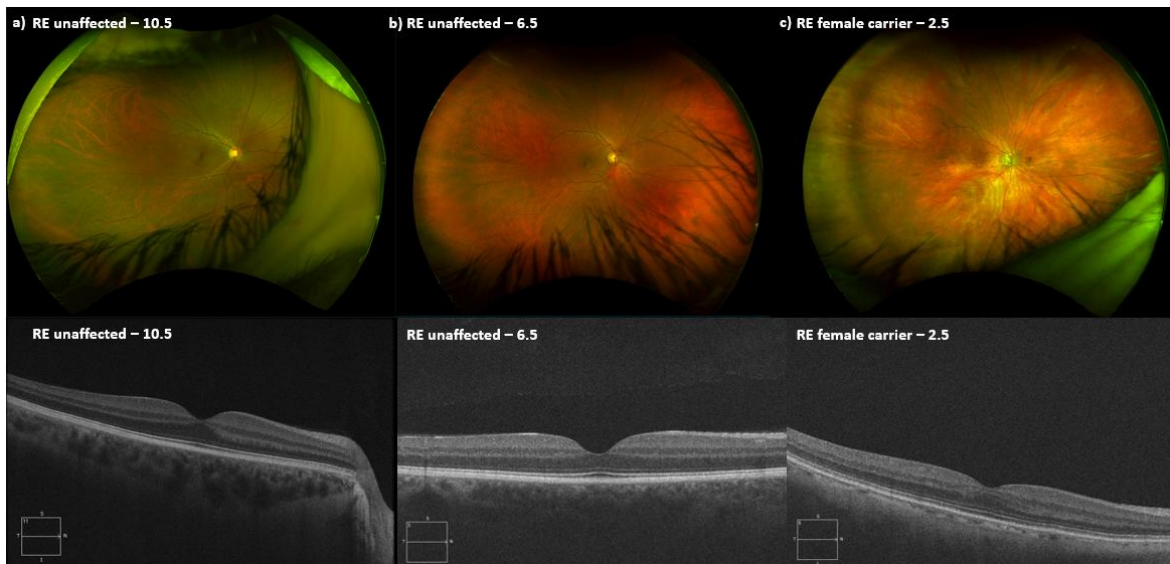


Figure 5.4(a-c). Multimodal imaging from unaffected or female-carrier family members in the *RPGR* family. The top row images are Pseudocolour widefield fundus photos for each family member, with the bottom row displaying Optical coherence tomography traces. (a) Unaffected patient 10.5 (b) Unaffected patient 6.5 (c) Female carrier 2.5. (RE = right eye).

Patients 1.5, 4.5, 7.5 and 5.5 share a similar clinical phenotype (**Supplemental 5.2**). As patient 9.5 is younger than the other affected males, he appears to have a less progressed ocular phenotype, with clinicians confirming his clinical imaging was similar to that of their relatives when they were his age (**Figure 5.6**). The proband 4.5 displays extensive peripheral pigment migration and some patchy RPE atrophy along with attenuated retinal vessels and a pale optic disc when compared to the unaffected individuals. Bone spicule deposits are noted to extend out 360 degrees, as is evident from the peripheral fundus image (**Figure 5.5(a)**). The OCT for patient 4.5 reveals outer retinal atrophy with loss of the ellipsoid zone and a breakdown of retinal cell layers (**Figure 5.5(b)**). Patients 7.5 and 5.5 are first cousins of proband 4.5. Patient 7.5 has asteroid hyalosis in right eye, evident from the fundus imaging (**Figure 5.5(c,d)**). These are calcium deposits in the vitreous humour of the eye that often have no effect on vision, but here they have obscured the image quality of the colour fundus and autofluorescence. Focusing on the fundus imaging of the left eye for patient 7.5, a pale disc along with very narrow retinal vessels are evident (**Figure 5.5(c)**). A similar appearance is noted in the colour fundus for patient 5.5 (**Figure 5.5(f)**). Lots of bone spicule intraretinal deposits are noted in a 360-degree pattern, similar to patients 4.5 and 5.5. The FAF for patient 7.5 displays patchy areas of hypo-fluorescence in the mid to far periphery, with an evident bullseye appearance indicating an area of photoreceptor loss centrally (**Figure 5.5(d)**). Extensive photoreceptor cell loss with few inner segment remnants

scattered across the posterior pole are noted in the OCT of patient 7.5, where loss appears worse in the right eye than in the left (**Figure 5.5(e)**). Patient 5.5 exhibits patchy thinning of the RPE as denoted by the brighter central area on the colour fundus in both eyes (**Figure 5.5(f)**). A total loss of the RPE is evident in the FAF of the left eye for patient 5.5, with a bullseye pattern of fluorescence present in both eyes (**Figure 5.5(g)**). The OCT for patient 5.5 exhibits a significant loss of the photoreceptor layer, extending out into the mid-periphery (**Figure 5.5(h)**). Some of the inner segments of photoreceptors are preserved in the periphery of the left eye, but mostly the photoreceptor layer is gone.

All ocular imaging for patient 9.5 is characteristic of early stage XLRP (**Figure 5.6**). Bone spicule deposits in the mid-periphery are evident in the colour fundus imaging of patient 9.5 in both eyes (**Figure 5.6(a)**). Fewer bone spicules are noted in patient 9.5 than are seen in his relatives with a more progressed form of XLRP. Similarly, a small bullseye pattern is noted in the FAF of patient 9.5, representing small areas of photoreceptor loss centrally (**Figure 5.6(b)**). The OCT traces for patient 9.5 show a better preservation of the photoreceptor layer centrally, meaning it is possible that this patient currently retains good vision in a tight visual field centrally (**Figure 5.6(c)**). This makes patient 9.5 a good potential candidate for possible gene therapies, as will be discussed in the next section. No other systemic features believed to be associated with *RPGR* have been noted in any of the family members.

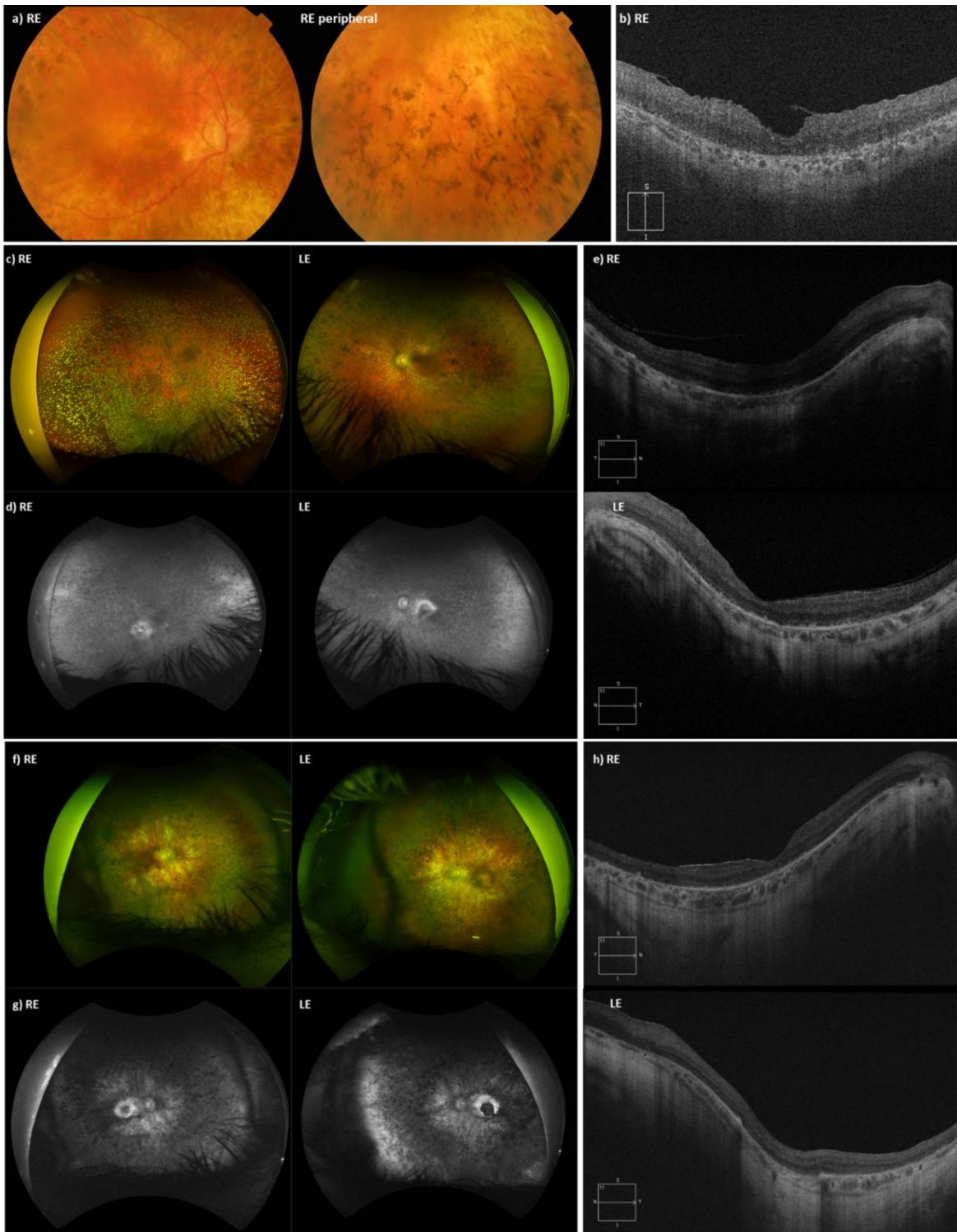


Figure 5.5(a-h). Multimodal imaging from patients 4.5, 7.5 and 5.5 in the *RPGR* family. (a, c, f) Pseudocolour widefield fundus photos for patients 4.5, 7.5 and 5.5 respectively. (b, e, h) Optical coherence tomography traces for patients 4.5, 7.5 and 5.5 respectively. (d, g) Fundus autofluorescence photos for patients 7.5 and 5.5 respectively. (RE = right eye, LE = left eye).

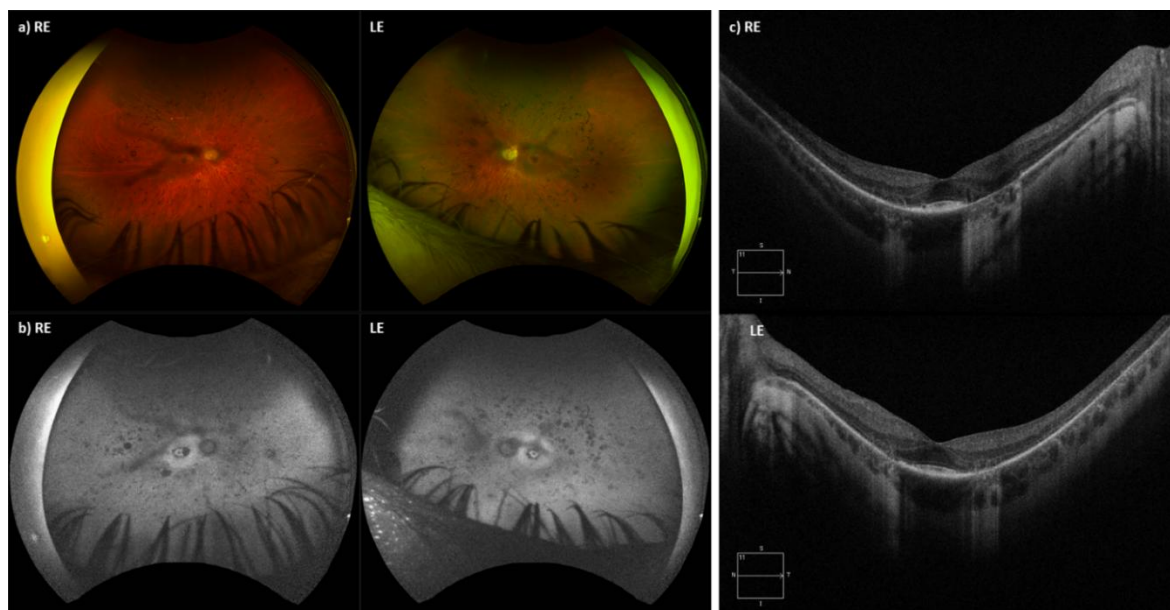


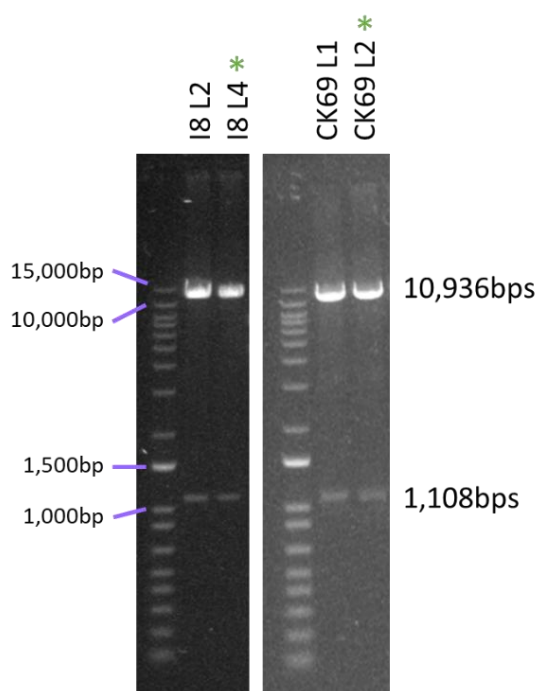
Figure 5.6(a-c). Multimodal imaging from patient 9.5 in the *RPGR* family. (a) Pseudocolour widefield fundus photos for patients 9.5 (b) Fundus autofluorescence photos for patient 9.5. (c) Optical coherence tomography traces for patients 9.5 (RE = right eye, LE = left eye).

The *RPGR* c.1307G>A variant was first identified as a missense variant in a study carried out in 2000 (Guevara-Fujita et al., 2000). The variant exhibits very poor missense scores, far below the threshold of pathogenicity, and therefore when first identified in our patients it was not deemed to be a likely disease-causing candidate variant. The *in silico* scores such as REVEL, Alphamissense, MetaLR, and MCAP are listed in **Table 5.1**, along with the full variant classification post-midigene assay. While this candidate disease causing variant has also been reported in databases such as ClinVar and LOVD.nl, it is not present in gnomAD and no functional studies have been carried out previously. It is currently classified as a VUS on Franklin Genoox and Varsome. Variant c.1307G>A in *RPGR* has a Splice AI score of 0.41, predicting an acceptor gain 30 bases downstream from the variant, as well as a 0.48 score indicating acceptor loss at its canonical acceptor site 61 bases upstream. Its SpliceAI scores and predicted effect on splicing are outlined in the Alamut schematic depicted in **Figure 5.8(a,b)**. Interestingly as shown in the schematic **Figure 5.8(a)**, c.1307G>A alters the Branch Point score from zero in the wild-type trace, to 86.1 in the mutant as a result of the creation of the adenine nucleotide within a pre-existing sequence motif that matches that of a canonical branchpoint.

As described in the Materials and Methods section of this chapter, the method of cloning used for this midigene study is Invitrogen ‘Gateway’ cloning, and employs multiple recombination reactions rather than more traditional restriction enzyme methods of cloning (Katzen, 2007). ‘Entry clones’ are generated during the first (BP) step of Gateway cloning,

where BP clonase enzyme is used to catalyse the recombination of our midigene mutant or wildtype insert and the pDONR vector. To facilitate this reaction, primers are designed for a long-range PCR to include 5' attB sites, so that the DNA fragment of interest from the patient contains these tags when amplified (see **Supplemental 5.1** for primers). Recombination then occurs between the attB sites on the fragment, and the attP sites located on the pDONR vector, allowing for the creation of entry clones. Once transformed into competent cells, bacterial colonies were picked and grown and plasmid clones isolated by miniprep (see 5.2.4.ii Miniprep section). Both the mutant and wildtype entry clones were subject to diagnostic restriction enzyme digestion by various restriction enzymes to validate plasmid and insert size and to confirm the orientation of the insert (see 5.2.5 Restriction digests section). The resulting digested DNA fragments were separated by gel electrophoreses and sized using a 1kb DNA ladder as depicted in **Figure 5.7** below, showing successful insertion of both wildtype (CK69 L1, CK69L2) and mutant (I8 L2, I8 L4) fragments into the backbone. Sanger sequencing of each plasmid over the insert confirmed the presence of the *RPGR* c.1307G>A variant in mutant constructs and its absence in wildtype constructs.

Figure 5.7. Gel images of diagnostic restriction enzyme digests performed on *RPGR* wildtype



and mutant plasmids generated from BP step in Gateway cloning, for midigene splice assay. Plasmids were digested with EcoRV-HF (Materials and Methods 5.2.5). Digestion of the plasmid resulted in two fragments of 10,936bp and 1,108bp, as was expected from a correct recombination reaction to insert the fragments into the pDONR vector. 1kb Plus DNA ladder was used here, with band sizes noted to the left of the image. Green asterisks indicate which plasmids were chosen to progress into the next cloning phase as described below.

After this validation step, we progressed on to the second (LR) step, to produce ‘expression clones.’ LR clonase enzyme was used to catalyse the recombination between the attL sites on the pDONR vector flanking our DNA insert, and the attR sites located in the pCI-NEO-RHO Gateway-adapted destination vector (Sangermano *et al.*, 2016). Once transformed into competent cells, and resulting bacterial colonies had been picked, grown and minipreped, the resulting wildtype and mutant expression clones were sent for Full plasmid sequencing to validate the contents of the construct, the orientation of the insert, and verify no SNPs had been introduced during recombination. Only plasmids I8 L4 (mutant) and CK69 L2 (wildtype) were progressed on through the LR step, as indicated by a green asterisk in **Figure 5.7**. As whole plasmid nanopore sequencing (Eurofins Genomics) may be error prone leading to potential sequencing artefacts, especially in repetitive regions of sequence, each plasmid sequence was aligned to a reference sequence of the expression plasmid including the respective inserts and the ‘erroneous’ SNPs were analysed to see if any might have a potential impact on splicing and hence the experiment (**Supplemental 5.3**). It was confirmed that no potential SNPs were likely to impact the midigene assay, and the plasmid DNAs were progressed on to transfection into HEK293 cells as described in the Materials and Methods section of this chapter. Each construct was re-transfected, with experimental duplicates for completeness and to show replicability.

Whole RNA was extracted from HEK293 cells, containing either the wildtype or mutant midigene constructs, and cDNA was generated. RT-PCRs were performed to analyse the resulting splicing profile, with all primers utilised listed in **Supplemental 5.1**. From the wildtype *RPGR* midigene construct, a fragment of ~700 base pairs (bp) containing the wildtype sequence along with approximately 200 bp of flanking *RHO* exon 3 and 5 was identified (**Figure 5.8(d,e)**). Sanger sequencing of this purified PCR fragment confirmed the presence of exons 10-13 in their entirety (**Figure 5.8(f)**). In comparison to the wildtype, the mutant midigene did not express any residual wildtype transcript. We observed a ~610 bp fragment which corresponds to the predicted 90bp deletion that is occurring in exon 11 due to the c.1307G>A variant’s predicted creation of a branchpoint motif within an exon. This partial exon deletion results in the creation of a premature stop codon 30 amino acids away, meaning the protein consequence is p.(Glu416Serfs*30) using standard HGVS nomenclature. Additionally, a ~410 bp fragment was detected in the mutant midigene, corresponding with the 90bp deletion from exon 11 as well as the skipping of exon 10. This deletion also results in premature stop codon creation (p.(Val354Serfs*30)). Both of these transcripts produced by the *RPGR* variant are predicted to undergo nonsense mediated decay

(NMD). While absence of the transcript due to NMD is likely, and therefore also the protein, nevertheless, we investigated the potential impact of the mutation on protein domains (**Figure 5.9**). This was achieved through the use of the Alphafold protein modelling interface, as described (see 5.6.4 Protein modelling). While this *RPGR* midigene assay has demonstrated the aberrant splicing that occurs due to the c.1307G>A variant, we have also observed the skipping of exon 10 in the mutant mRNA. It remains to be confirmed whether this is a natural exon skipping event due to the existence of many splicing isoforms, or whether it is simply an artefact created due to the lack of retina-specific splicing factors in HEK293 cells.

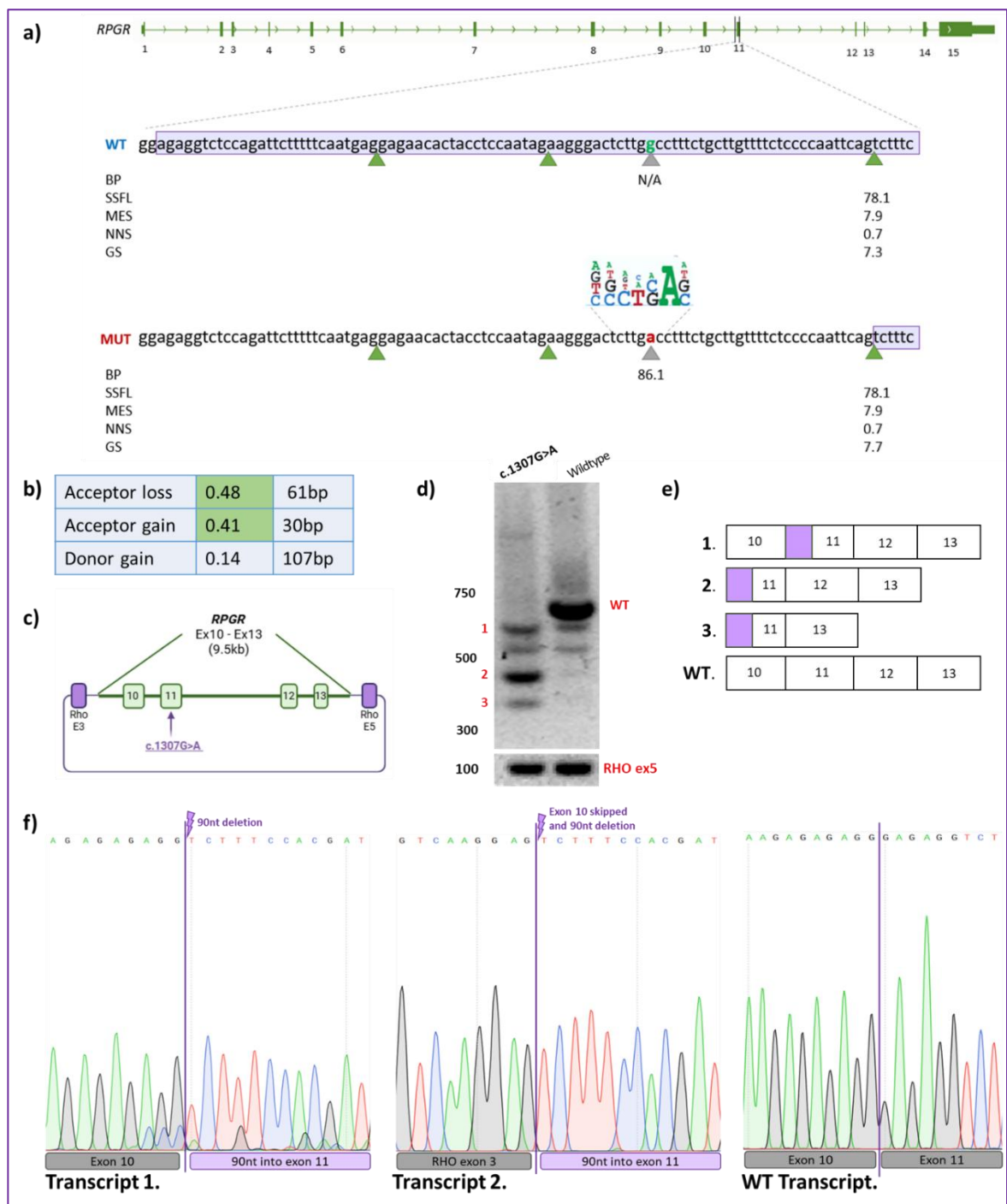


Figure 5.8. Results from functional characterisation of the *c.1307G>A*, p.Gly436Asp variant in *RPGR*. (a) A representation of the *RPGR* gene, focused on exon 11, and the Alamut™ Visual *in silico* splice prediction for the *c.1307G>A*, p.Gly436Asp variant. The top row shows the wildtype (WT) prediction, and the bottom is the mutated (MUT) prediction. This schematic shows the predicted splice sites within this region. The purple box overlaid on the WT and MUT sequences indicates the predicted location of the exon in the wildtype and predicted mutant (for clarity, there is a 90base pair depicted by the absence of this box in the mutant). The grey arrow indicates the creation of the branchpoint within exon 11, and the green arrows show the location of the predicted cryptic splice acceptor sites in both wildtype and mutated sequences. A conserved branchpoint motif is marked above the mutant sequence at the branchpoint. The red 'a' denotes the

c.1307G>A,p.Gly436Asp variant in the mutant sequence. The SpliceSiteFinder-like (SSFL, range 0-100), MaxEntScan (MES, range 0-12), and GeneSplicer (GS, range 0-24) scores for the splice acceptor site, and Branch Points (BP, range 0-100) scores for the branchpoint are indicated below each sequence. **(b) *in silico* SpliceAI delta prediction scores.** Acceptor gain is predicted 30bp downstream of the variant, with acceptor loss predicted 61 bp upstream relative to the variant. **(c)** Depiction of the *pCI-NEO-RHO* vector containing exons 10 to 13 of *RPGR*, being flanked by *RHO* exons 3 and 5. The wildtype and mutant midigenes were transfected into HEK293 cells. **(d,e)** Agarose gel image of the RT-PCR products from the mutant and wildtype midigene constructs, illustrating the WT band in the wildtype construct and multiple resulting bands from the mutant midigene construct along with the *RHO* exon 5 control below each lane. Here, the purple boxes indicate the 90bp deletion present in all mutant transcripts **(f)** Sanger sequence analysis of the gel purified RT-PCR fragments. Transcript 1 from the mutant midigene depicts a 90bp deletion at the start of exon 11. Transcript 2 also expressed from the mutant midigene contains this same deletion along with exon 10 skipping. And the WT transcript was confirmed from the wildtype midigene.

Utilising the publicly available online server (Varadi *et al.*, 2024), an AlphaFold protein model was generated for the wildtype RPGR-ORF15 protein and the 2 predicted outcomes; p.(Glu416Serfs*30) and p.(Val354Serfs*30) (**Figure 5.9**). As both of the resulting mutant transcripts result in the creation of a premature stop codon, it is evident from the protein models that a significant portion of the protein is lost. The green highlighted residue which is also marked by a green arrow is residue 416, while the pink residue denoted by the pink arrow is residue 354. The model for the p.(Glu416Serfs*30) transcript shows the protein ending abruptly after the green 416 residue. This change results in loss of the final 706 amino acid residues; in turn this also means that the entire C-terminal tail encoded by ORF-15 is lost. Furthermore, it is also evident from the model for the p.(Val354Serfs*30) transcript that the protein is once again truncated. In this instance, 768 amino acid residues are lost resulting in the preservation of folding of the RCC1-like domain, typically ending at residue 350, followed by a very short and unstructured tail where the C-terminal tail is once again lost. However, as mentioned above, NMD will likely degrade such transcripts containing premature termination codons.

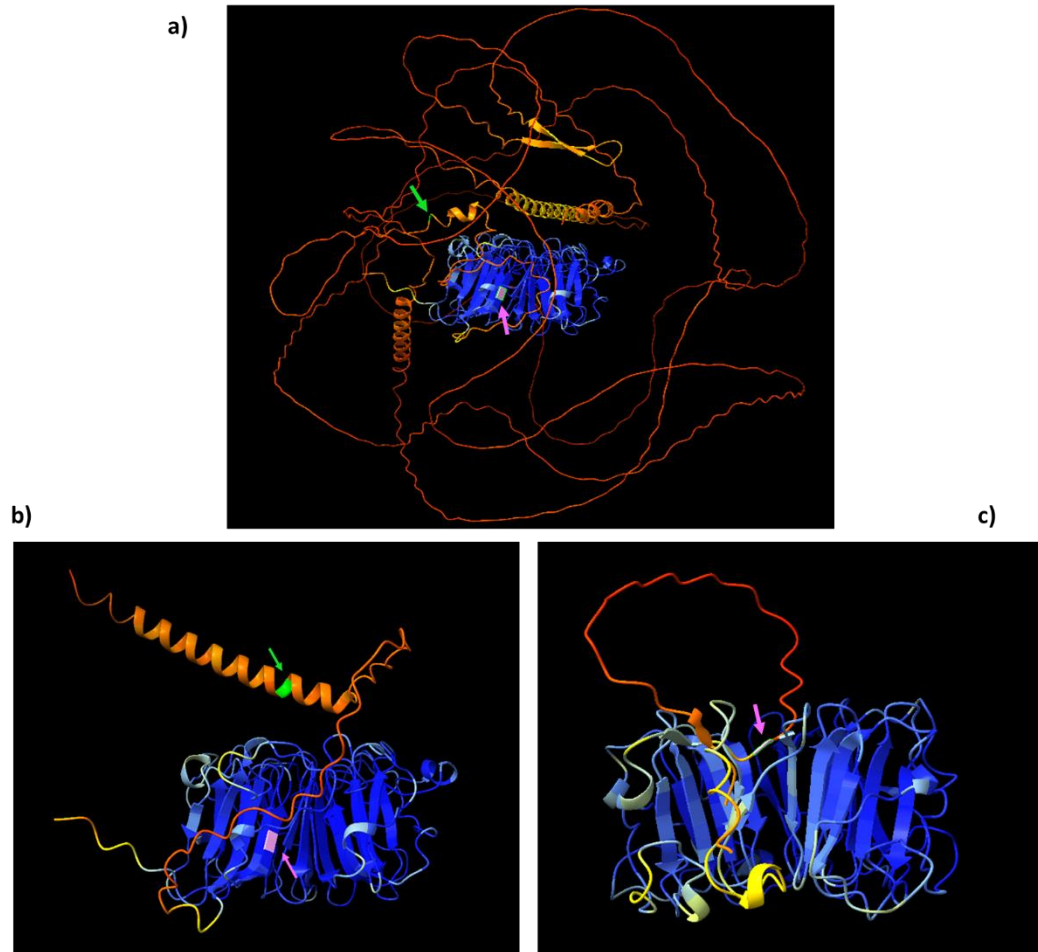


Figure 5.9. AlphaFold protein folding predictions for (a) wildtype compared to the amino acid sequence produced as a result of the *RPGR* c.1307G>A variant; (b) p.(Glu416Serfs*30) and (c) p.(Val354Serfs*30). The green residue also indicated by the green arrow is residue 416. The pink residue indicated by the pink arrow is residue 354. Their position is indicated in each protein prediction image.

5.3.2 *ABCA4* c.5584+5del, p.?

As outlined in Chapter 3, covering the iMD-smMIPs study, the *ABCA4* c.5584+5del variant has been identified in one individual (patient 11.5) and is thought to be acting *in trans* with the complex allele c.[2588G>C;5603A>T] as indicated in **Figure 5.10**. This variant has since been confirmed to be present in this patient's affected sister (patient 12.5), through familial testing by a clinically accredited lab. To the best of our knowledge, this is a novel variant and therefore has not been identified in any other IRD patients in the literature thus far. Both patient 11.5 and their sister, patient 12.5, present with classical STGD1 (**Figure 5.11**). Both colour fundus and FAF imaging are displayed in **Figure 5.11(a,b)** for patients 11.5 and 12.5. Observing the FAF of both siblings, the dark choroid is less obvious in sister 12.5 as disease progression is less advanced by a few years. Expansive subretinal fishtail deposits are noted at both maculae extending nasally in the colour fundus of patient 11.5. This is mirrored in the disturbance observed in the FAF. These deposits are slightly denser in the right eye than in the left. Likewise, these classical STGD1 fishtail deposits are noted in the colour fundus of sister 12.5 also to a slightly lesser degree. Similarly, preservation of photoreceptors centrally is seen in the OCT for patient 12.5, whereas the photoreceptor cell layer in both eyes of patient 11.5 is lost extensively centrally (**Figure 5.11(c,d)**). It is evident from the OCT of patient 11.5 that the disturbance of photoreceptors is more widespread in the left eye, extending toward the mid-periphery, than in the right.

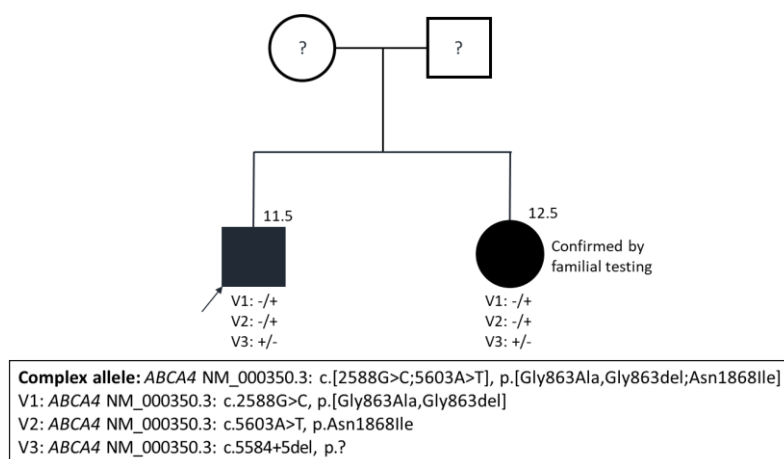


Figure 5.10.
pedigree tree

An Irish
in which the

ABCA4 variant was first found, displaying phasing of the novel c.5584+5del variant and a common complex allele. This shows the segregation pattern of the candidate *ABCA4* splice-altering mutation that we have analysed. The variant in patient 12.5 was confirmed by Sanger sequencing through a clinically accredited lab as requested by clinicians. The black arrow indicates the proband. (+ = wildtype allele, - = mutant allele, Y = Y chromosome, ? = not genotyped as samples were unavailable).

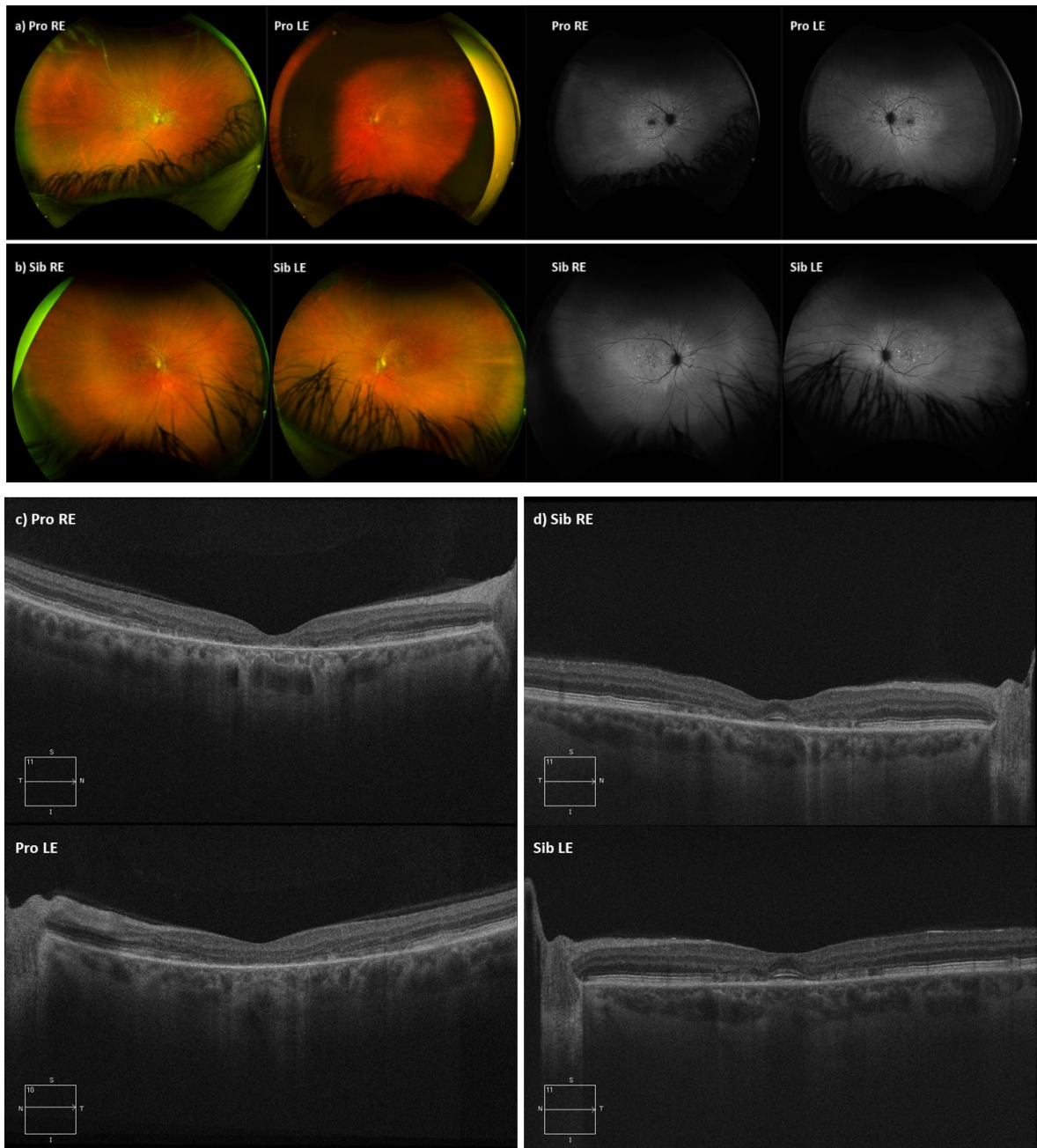


Figure 5.11.(a-d) Multimodal imaging from patients 11.5 and 12.5 respectively. Patient 11.5 is indicated as ‘Pro’ for proband, and patient 12.5 is indicated by ‘Sib’ for sibling. **(a)** Pseudocolour widefield fundus and Fundus autofluorescence photos for patients 11.5 (Pro). **(b)** Pseudocolour widefield fundus and Fundus autofluorescence photos for patients 12.5 (Sib). **(c vertically)** Optical coherence tomography traces for patient 11.5 (Pro). **(d vertically)** Optical coherence tomography traces for patient 12.5 (Sib). (RE = right eye, LE = left eye).

The c.5584+5del single base deletion is located 5 bases into intron 39 at the canonical donor site. This candidate variant has not previously been reported to ClinVar or LOVD.nl, and it does not appear in the gnomAD database. Similarly to the *RPGR* variant described above, it is currently classified as a VUS on Franklin Genoox and Varsome. As it is located within the 5' splice site motif (donor) it is predicted to impact splicing, with high SpliceAI scores indicating a donor loss (score 0.88) 6bp upstream and a donor gain (score 0.27) predicted 1bp downstream (**Figure 5.13(b)**). Additional *in silico* tools embedded into Alamut visual such as SpliceSiteFinder-like, MaxEntScan and GeneSplicer support the total abolishment of the canonical donor site due to this variant, as indicated in the schematic in **Figure 5.13(a)**.

As described previously for the *RPGR* midigene and in the Materials and Methods section 5.2.6, 'entry clones' are generated first during the BP step of Gateway cloning, where both the mutant and wildtype entry clones were subject to diagnostic restriction enzyme digestion to validate insert size and orientation, as well as overall plasmid size. The resulting digested DNA, was separated on an agarose gel by electrophoresis and is depicted in **Figure 5.12** below, showing successful insertion of both wildtype (CH43 B2) and mutant (CH43A5) fragments into the plasmid backbone. Sanger sequencing of each plasmid over the insert confirmed the presence of the *ABCA4* c.5584+5del variant in mutant constructs and its absence in the wildtype construct. Of note, the red asterisk in **Figure 5.12** marks a DNA band that we believe to be the linearised construct, where the restriction enzyme digestion reaction was incomplete and only cut once. This DNA band confirms the total size of the plasmid is correct (9406bp).

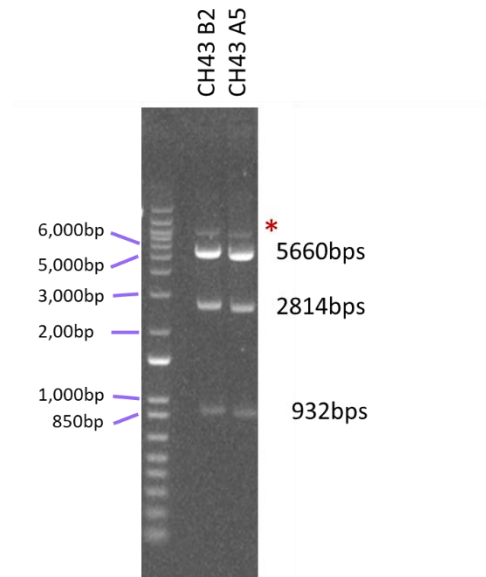


Figure 5.12. Gel images of diagnostic restriction enzyme digests performed on *ABCA4* wildtype and mutant plasmids generated from BP step in Gateway cloning, for midigene splice assay. Plasmid DNA was digested with MluI-HF and NaeI (Materials and Methods 5.2.5). Digestion of the plasmids resulted in three fragments, 5,660bp, 2814bp and 932bp, as was expected from a correct recombination reaction to insert the fragments into the pDONR vector. The red asterisk marking the band at approximately 9000bp denotes residual linearised plasmid where digestion was incomplete. 1kb Plus DNA ladder was used here, with band sizes noted to the left of the image.

Similarly to the *RPGR* midigene assay, after the restriction enzyme digest validation step, the second LR step was undertaken to produce expression clones. Once transformed into competent cells, resulting colonies were picked, grown and plasmid DNA isolated. Resultant wildtype and mutant expression plasmid clones were sent for Full plasmid sequencing to validate the contents of the construct, the orientation of the insert, and verify no SNPs had been introduced during cloning (Eurofins Genomics). As before, any SNPs noted in each plasmid when they were aligned to a reference sequence were analysed for their potential impact on the assay (**Supplemental 5.4**). None of the SNPs identified appeared to have a predicted impact on the midigene assay, and the plasmid DNAs were progressed on to transfection into HEK293 cells as described in the Materials and Methods section 5.2.6.iii. Due to the errors often noted in nanopore sequencing of whole plasmids, we believe many of these SNPs to be sequencing artefacts. Each construct was re-transfected, with experimental duplicates for completeness and demonstrated that the results are replicable.

The midigene *in vitro* splice assay indeed confirmed that the *ABCA4* c.5584+5del variant resulted in multifaceted aberrant splicing (**Figure 5.13(d,e)**). Cells transfected with the wildtype construct expressed a fragment of ~750bp, with Sanger sequencing confirming this

to be the full wildtype sequence of exons 38-41. This wildtype band is also observed in the mutant midigene lane, but to a much lesser degree, indicating that a small amount of residual wildtype transcript is still expressed in the presence of the c.5584+5del variant. Three additional mRNA fragments were expressed from the mutant midigene construct of varying sizes, 1020bp, 590bp and 450bp (**Figure 5.13(d)**). The largest fragment of ~1020bp corresponds to the retention of intron 39 between exons 39 and 40, which is predicted to occur due to the loss of the canonical donor site. We have also confirmed that the second fragment at ~590bp contains a partial deletion of exon 39, where only the first 11bp are retained. This is believed to occur due to the loss of the canonical donor site, as a cryptic donor site has been identified where the 11bp retention from exon 39 ends, indicated in **Figure 5.13(f)**. The final ~450bp DNA fragment corresponds to the deletion of exons 39 and 40. This same band was also expressed to a similar degree by the wildtype midigene and therefore is not believed to be occurring as a result of the variant of interest. Resulting protein consequences of the *ABCA4* c.5584+5del are believed to be as follows: p.(Glu1863Glyfs*33) and p.(Phe1825*) respectively (**Figure 5.13f**). Both of the aberrant transcripts produced by the *ABCA4* variant are predicted to undergo NMD, with very little wildtype transcript evident from the agarose gel image. Moving forward, it would be beneficial for severity classification reasons to quantify the amount of residual wildtype transcript.

Once again, AlphaFold protein models were generated for the wildtype transcript of *ABCA4* along with the two resulting aberrant transcripts; p.(Glu1863Glyfs*33) and p.(Phe1825*) (**Figure 5.14**). Similarly to the *RPGR* midigene results, both transcripts are predicted to undergo NMD due to the resulting RNA truncation but for completeness they have been modelled below to observe the potential impact on protein domains. This truncation is evident from both models as significant portions of the protein are lost. The green highlighted residue which is also marked by a green arrow is residue 1863, while the pink residue denoted by the pink arrow is residue 1825. As a result of p.(Glu1863Glyfs*33), 377 amino acid residues are predicted to be lost leading to the loss of the C-terminal tail along with a partial loss of the nucleotide-binding domain 2 (NBD2) which occurs between amino acids 1650 and 1900. The p.(Phe1825*) resulting transcript creates a truncated protein where 448 amino acids are deleted. Therefore, much like the previous transcript, NBD2 and the C-terminal tail after residue 1825 are lost due to this truncation as is evidenced by the tail highlighted in pink (**Figure 5.14(c)**). It is also likely that extracellular helices as well as a transmembrane domain are disrupted by this truncation. Previously variants impacting the

above-mentioned domains were shown to lead to reduced/lost ATPase activity and a reduction in ABCA4 protein expression due to misfolding (Molday *et al.*, 2022b).

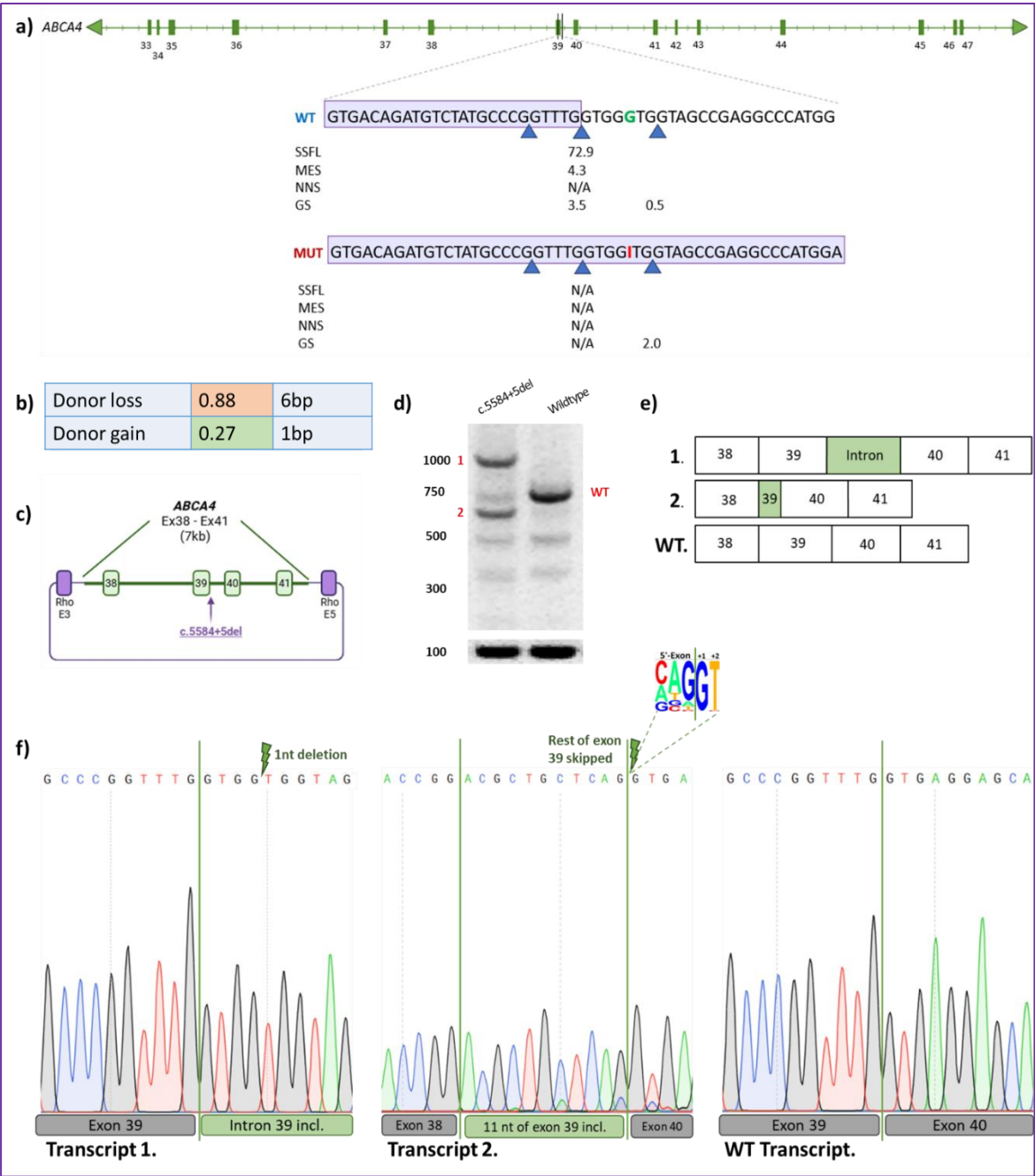


Figure 5.13. Results from functional characterisation of the c.5584+5del, p.? variant in *ABCA4*. (a) A representation of the *ABCA4* gene, focused on exon 39, and the Alamut™ Visual *in silico* splice prediction for the c.5584+5del, p.? variant. The top row shows the wildtype (WT) prediction, and the bottom is the mutated (MUT) prediction. This schematic shows the predicted splice sites within this region. The purple box indicates the predicted location of the exon in the wildtype and mutated sequence. The blue arrows show the location of the predicted cryptic splice donor sites in both wildtype and mutated sequences. The red 'I' denotes the location of the single base deletion due to the c.5584+5del, p.? variant in the mutant sequence, with the above green 'G' being the wildtype base. The SpliceSiteFinder-like (SSFL, range 0-100), MaxEntScan (MES, range

0-12), and GeneSplicer (GS, range 0-24) scores for the splice donor sites are indicated below each sequence. **(b) *In silico* SpliceAI delta prediction scores.** Donor gain is predicted 1bp downstream of the variant, with donor loss predicted 6bp upstream relative to the variant. **(c)** Depiction of the *pCI-NEO-RHO* vector containing exons 38 to 41 of *ABCA4*, being flanked by *RHO* exons 3 and 5. The wildtype and mutant versions of this construct were transfected into HEK293 cells. **(d,e)** Agarose gel image of the RT-PCR products from the mutant and wildtype midigene constructs, illustrating the WT band expressed from the wildtype construct and multiple resulting bands expressed from the mutant midigene construct along with the *RHO* exon 5 control below each lane. **(f)** Sanger sequence analysis of the gel purified RT-PCR fragments. Transcript 1 from the mutant midigene depicts the inclusion of intron 39. Transcript 2, also expressed from the mutant midigene displays the retention of only 11bps of exon 39 due to the strengthening of a cryptic donor site, with the rest of the exon deleted. And the WT transcript was confirmed from the wildtype midigene.

Both the *RPGR* and *ABCA4* variants have been reclassified due to the RNA splicing analyses that have been completed as part of this study (**Table 5.1**). Vitally, the *RPGR* c.1307G>A variant has been upgraded from a VUS to pathogenic. The *ABCA4* c.5584+5del has been upgraded from a cool VUS to a hot VUS, where additional family members carrying this variant or long-read sequencing for phasing analysis would enable cascade studies and potentially facilitate further upgrades when the variant classification is routinely revisited in the future. Both of these splice-altering variants have been upgraded due to the ability to apply the PVS1 and PVS1_Strong criteria following RNA analysis, further underpinning the importance of functional studies in genetic diagnoses (Walker *et al.*, 2023).

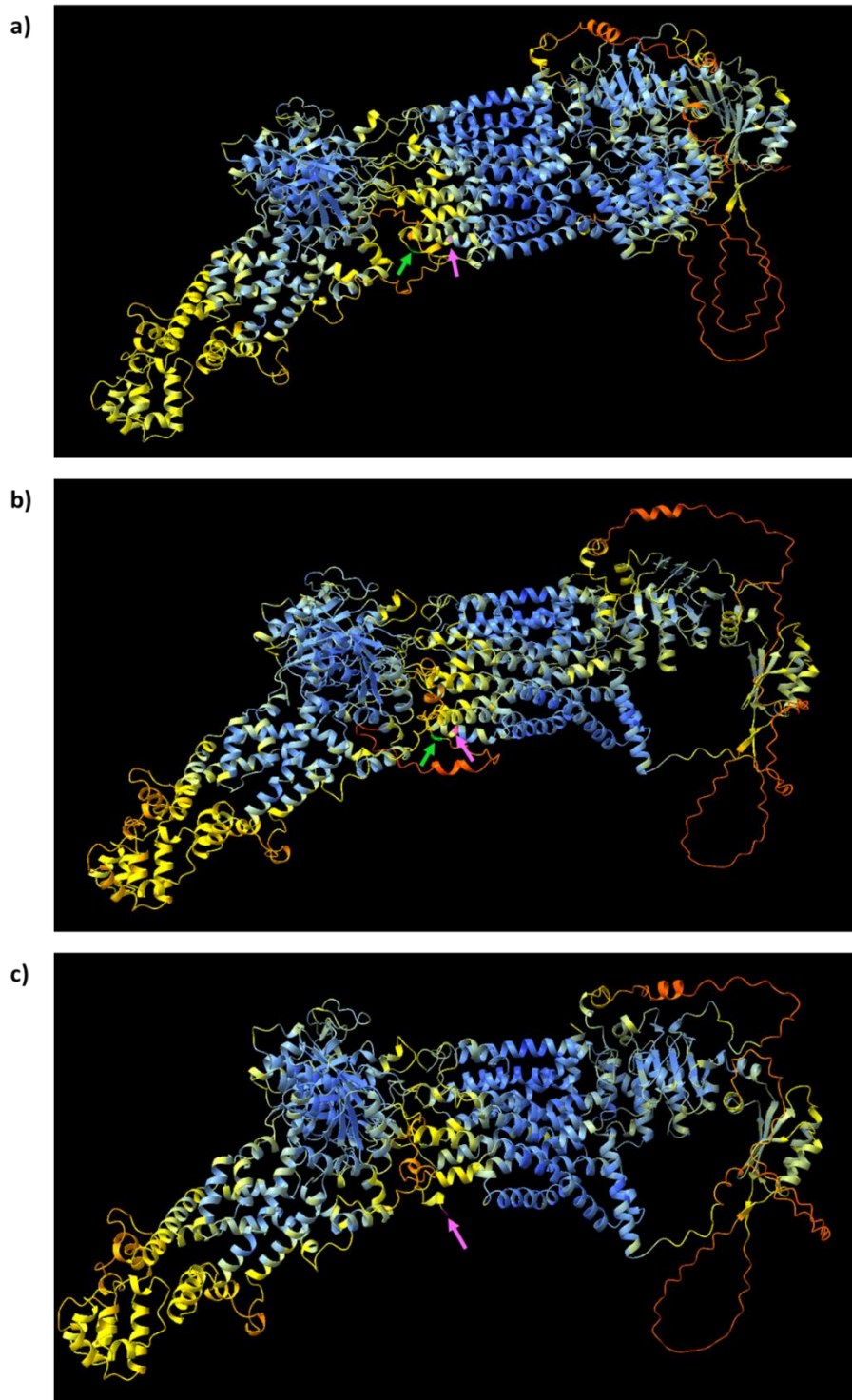


Figure 5.14. AlphaFold protein folding predictions for (a) wildtype compared to the amino acid sequence produced as a result of the *ABCA4* c.5584+5del variant; (b) p.(Glu1863Glyfs*33) and (c) p.(Phe1825*). The green residue also marked by the green arrow is residue 1863. The pink residue highlighted by the pink arrow is residue 1825. Their positions are indicated in each protein prediction image.

Table 5.1 Detailed overview of the classification of two splice-altering variants, according to ACMG/AMP guidelines along with SVI ClinGen Splicing group recommendations.

Gene	Transcript	Variant		Population data			Computational and predictive data			Segregation data			Allelic data			PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	
		c.	p.	ACMG guideline	Comments	Score	ACMG guideline	Comments	Score	ACMG guideline	Comments	Score	ACMG guideline	Comments	Score									
ABCA4	NM_000350.3	c.5584+5del	p.?	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Splicing assay (RNA) data demonstrating that a variant leads to aberrant splicing profile	Multimodal splice defect, where both mutant transcripts p.(Glu1863Glyfs*33) and p.(Phe1825*) are predicted to undergo NMD, and proportionally little WT transcript is produced	PVS1_strong	N/A	Only have one affected sibling who carries the variant	N/A	N/A	Have not been able to confirm phasing for certain as both affected family members have the same genotype.	N/A	0	4	0	1	0	0	0	5	Hot VUS
RPGR	NM_001034853.1	c.1307G>A	p.Gly436Asp	Absent or extremely low frequency in population databases	Not in gnomAD or in literature. 5 likely pathogenic/p athogenic reports on LOVD.nl and 1 report on ClinVar with no additional info.	PM2_supporting	Splicing assay (RNA) data demonstrating that a variant leads to aberrant splicing profile	No residual wildtype transcript was observed, with both mutant transcripts p.(Glu416Serfs*30) and p.(Val354Serfs*30) predicted to undergo NMD	PVS1	Co-segregation with disease in multiple affected family members	5 affected males (1pt each), 2 unaffected males (1pt each). Only using these males and two carrier females as we have their genotype confirmed, but it is noted that there are 6 obligate carrier females. While PP4 was considered in the application of PP1 as per PMID: 38103548, will not apply it here due to phenotypic overlap with other genes, but have confirmed with clinicians that RPGR is appropriate for this pedigree.	PP1_strong	N/A	X-linked variation	N/A	8	4	0	1	0	0	0	13	Pathogenic

Each coloured heading represents a different group of data used. Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per ACMG/AMP guidelines and ACGS updated recommendations. The classification is listed in the final column. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria). See Materials and methods section 4.2.6 for *in silico* tool thresholds.

5.4 Discussion

In this chapter of the thesis, we have explored the functional consequences of one known and one novel variant in the *RPGR* and *ABCA4* IRD-associated genes respectively, by utilising midigene splice assays. The *RPGR* c.1307G>A variant creates a cryptic branchpoint motif within exon 11 of the gene, leading to a 90nt deletion in exon 11. In contrast, the *ABCA4* c.5584+5del variant is a single base deletion +5 bases into intron 39 of the gene leading to the retention of intron 39 in one transcript, and the partial deletion of exon 39 with only 11nt retained from that exon in another transcript. We believe these two variants to be contributing to the ocular phenotype exhibited by the probands of two Irish families due to their impact on RNA splicing.

Variant c.1307G>A in *RPGR* has a SpliceAI score of 0.41, predicting an acceptor gain 30 bases downstream from the variant, as well as a 0.48 delta score indicating acceptor loss at its canonical acceptor site 61 bases upstream. The Branch Point score tool that is integrated into the Alamut visual software was altered from 0 in the wildtype scenario to 86.1, indicating the likely creation of a branchpoint motif 11 bases into exon 11 of *RPGR* (**Figure 5.8**). A midigene construct containing a 9.5kb fragment encompassing exons 10-13 of *RPGR* was utilised, with fragments isolated from an affected proband and an unaffected wildtype sibling. Our investigation confirmed that the cryptic acceptor site 30 bases away from the variant is being utilised along with the cryptic branchpoint motif to excise intron 10 along with 90 bases of exon 11. This shifts the reading frame leading to the creation of a premature stop codon. This transcript is predicted to undergo NMD, in turn suggesting that no functional *RPGR* protein is predicted to be created. Following the most recent ACGS recommendations, we have been able to apply PVS1 criteria here as no residual wildtype transcript was observed in the RNA-splicing assay (Walker *et al.*, 2023) (**Table 5.1**).

RPGR-ORF15 is a major isoform of *RPGR* that is expressed specifically in the retina. All of the variants identified to date in association with an IRD or XLRP in *RPGR* have been associated with this *RPGR*-ORF15 isoform (Megaw, Soares and Wright, 2015). The open reading frame created at the C-terminus of *RPGR*-ORF15 consists of a repetitive glutamic acid and glycine-rich domain and a basic, highly conserved, C-terminal domain. The repetitive domain of ORF15 is a mutational hotspot, with two thirds of disease-causing mutations associated with XLRP being found in this region (Vervoort *et al.*, 2000). Mutations impacting the RCC1-like domain are associated with a more severe phenotype than those affecting ORF15 (Sharon *et al.*, 2003). Two resulting transcripts; p.(Glu416Serfs*30) and p.(Val354Serfs*30) were identified from the mutant midigene assay of *RPGR* c.1307G>A,

with no residual wildtype produced. The AlphaFold imaging allowed us to visualise the predicted truncation of the protein by this branchpoint variant (**Figure 5.9**). However as mentioned previously both transcripts are predicted to undergo NMD. While we cannot fully estimate the severity and prognosis of the resulting phenotype caused by this variant from such *in vitro* splice assays, the complete loss of RPGR activity could align with the progressive and severe nature of the XLRP phenotype observed in all affected male members of the 4.5 pedigree, along with the carrier female imaging showing disease characteristics in one of the patients, 2.5 (**Supplemental 5.2**).

Previous studies have identified variants associated with ocular phenotypes that lead to abolishment of the branchpoint lariat structure within an intron (Kuivenhoven *et al.*, 1996; Corradi *et al.*, 2022; Fadaie *et al.*, 2022; Rodríguez-Hidalgo *et al.*, 2023; Fernández-Suárez *et al.*, 2025). For example, in 1996, a variant within intron 4 of the *LCAT* gene was found to destroy the branchpoint motif leading to a null allele in the patient, causing fish-eye disease (Kuivenhoven *et al.*, 1996). Similarly and more recently, a study identified an intronic variant in *BBS1* that disrupts a branchpoint motif, and leads to the partial or complete deletion of exon 8, in a BBS affected family (Fadaie *et al.*, 2022). Additionally, two branchpoint variants have been identified in *ABCA4* (c.859-25A>G and c.6480-35A>G), where midigene splice assays revealed them to have severe and moderately severe effects respectively (Corradi *et al.*, 2022; Rodríguez-Hidalgo *et al.*, 2023). The *ABCA4* c.859-25A>G variant was identified as a Palestinian founder mutation, that abolishes the branchpoint motif in intron 7 leading to exon 8 skipping and two partial intron 7 inclusions. As for the *ABCA4* c.6480-35A>G variant, here the branchpoint motif in intron 47 was abolished leading to partial intron 47 inclusion.

These *BBS1* and *ABCA4* variants are the first branchpoint variants identified in genes associated with IRDs. It is of note, that variants involved in altered branchpoint motifs are very rare and are not widely reported on. Indeed, while all of the above-mentioned variants lead to the destruction of a branchpoint motif, each displays a varying complex combination of resulting splice defects. The destruction of a branchpoint motif sequence could lead to the employment of a non-canonical branchpoint elsewhere in the intron, meaning there could be a level of intron inclusion in the resulting mRNA. It has also been seen that the creation of a branchpoint sequence within an intron could also lead to pseudoexon inclusion, if there are strong cryptic splice acceptor and donor sites within the area that could be employed (De Klein *et al.*, 1998). The branchpoint consensus sequence is an essential splice motif as it allows for the formation of a lariat structure, aiding the splicing machinery in identifying the

intron that is to be excised from the pre-mRNA. Despite its vital role, variants associated with branchpoint splice defects are less often reported in the literature. This is believed to be due to their varied location within the intron with respect to the acceptor site. When categorised, the majority of branchpoint motifs were identified between 18-34 nucleotides into an intron away from an acceptor site (Gao *et al.*, 2008), with these consensus sequences having also been found up to 400 nucleotides away from a canonical acceptor site (Gooding *et al.*, 2006; Leman *et al.*, 2020).

As mentioned previously, the adenosine nucleotide is the most conserved residue in the branchpoint structure. The c.1307G>A variant leads to the creation of the branchpoint consensus sequence within an exon due to the introduction of this vital adenosine. Alamut Visual Plus utilises an integrated tool to predict branchpoint formation and destruction, as was evidenced in **Figure 5.8(a)**. This tool provides a predictive score between 0 and 100 on the strength of a branchpoint sequence. For the c.1307G>A *RPGR* variant, the wildtype branchpoint score is 0, which has then become 86.1 in the presence of the variant. This *in silico* prediction from Alamut Visual Plus along with the delta scores provided by SpliceAI have accurately been able to predict the multimodal consequences, later confirmed by the *in vitro* midigene splice assay, of the creation of a cryptic branchpoint within an exon. Of note, to the best of our knowledge, this is the first pathogenic variant reported that leads to creation of a branchpoint variant within an exon that is associated with an IRD. It is very likely that this type of variant may be less frequently reported on due to the fact that such variants may present as missense variants with poor pathogenicity via *in silico* predictions and therefore are categorised as VUS. The observation of such a variant aids in informing the field, suggesting that missense variants with low pathogenicity should be reanalysed to ensure there are no potential effects on splicing such as creation of a novel branchpoint.

The novel *ABCA4* non-canonical splice site variant c.5584+5del was identified through smMIPs sequencing as outlined in Chapter 1, in a patient with classical STGD1. This variant had intriguing SpliceAI scores with a donor loss score of 0.88 predicted 6bp away, and 0.27 donor gain predicted -1bp away (**Figure 5.13(b)**). These *in silico* scores indicate potential multimodal splice defects occurring due to this variant, where previously variants in IRD genes at the +5 position in the donor site motif have led to various splice defects such as intronic inclusion and single/multiple exon skipping (Weisschuh, Buena-Atienza and Wissinger, 2021). Indeed, through midigene splice assays we have been able to confirm this *ABCA4* variant results in two incorrectly spliced transcripts, one where intron 39 has been retained and another where all but 11nt of exon 39 have been deleted. AlphaFold imaging

enabled visualisation of the truncated protein transcripts that result from the +5 deletion, however both transcripts are predicted to ultimately undergo NMD (**Figure 5.14**). Even if NMD was evaded, the two resulting truncated proteins would be missing the NBD and transmembrane domains critical for ATPase activity and N-Ret-PE binding, as well as the correct folding and localisation of the ABCA4 protein product (Molday *et al.*, 2022b). In comparison to the wildtype midigene construct, little to no wildtype transcript remains. This residual amount of *ABCA4* activity is not predicted to save the phenotype, and therefore we have been able to apply the PVS1_Strong criteria (**Table 5.1**), based on recent recommendations (Walker *et al.*, 2023). We have reduced the criteria strength by one level in order to be more stringent due to the small amount of residual wildtype and the non-retinal specific nature of the cell type used in the assay. It would be of interest in the future to evaluate the correlation between the level of mutant transcript expression and disease severity/progression in order to fully classify this *ABCA4* variant. The classical STGD1 phenotype, with onset typically occurring in the second decade of life, is usually caused by the combination of a severe and mild allele, or two moderately severe alleles (Cremers *et al.*, 2020; Cornelis *et al.*, 2022a). Previously the c.[2588G>C;5603A>T] complex allele has been classified as a fully penetrant mild variant, where it is causative of disease if in a compound heterozygous state with a second severe allele (Zernant *et al.*, 2017; Runhart *et al.*, 2018). While additional data is needed, the proposed multimodal splicing defect, along with previous studies that have analysed splice mutations at this same +5 location, it could be estimated that the c.5584+5del may act as a severe variant (Kaltak *et al.*, 2024). Long read sequencing to confirm phasing of this novel variant may add to the classification evidence, along with genotype-phenotype correlation studies where additional patients carrying this variant are required for the correlation to be informative. This combination of a proposed severe variant along with a well-categorised mild allele would fit into the previously described genotype-phenotype model for our patients with classical STGD1.

As discussed, an *in vitro* midigene assay in HEK293 is the most routine functional analysis assay for splice-altering variants (Sangermano *et al.*, 2018; Khan *et al.*, 2020; Qian *et al.*, 2021a; Corradi *et al.*, 2023). HEK293 kidney cells represent a good primary approach to explore splicing where there is no access to fresh patient blood, or stem cell facilities. A study undertaken in 2018 carried out midigene analyses for 47 different *ABCA4* noncanonical splice variants (Khan *et al.*, 2020). An entire wildtype *ABCA4* library of overlapping midigene inserts was created, allowing analysis of the functional effects of all splice variants identified, through mutagenesis of specific inserts. As with most assays,

midigene analyses have their limitations. For example, it is often the case that a splice defect is not seen as midigene studies are typically undertaken in HEK293 cells, which differ significantly in expression profile to the target retinal cells. Many of the splice variants being evaluated are selectively expressed in retinal cell types, and therefore are not naturally seen in HEK293 cells. It could be that certain splice defects are only observed in a retinal cell type, as indeed there is evidence from prior studies that seemingly silent candidate splice variants in HEK293 cells can affect splicing when an ocular cell type is used for the midigene assay (Parfitt *et al.*, 2016). At present, there are no immortal retinal cell lines that are sufficiently robust for this kind of experimentation. For this reason, it is often a good approach to assess the functional consequences of candidate splice altering variants in patient-derived photoreceptor precursor cells (PPCs). Such a functional study was undertaken for the *ABCA4* deep-intronic variant, c.4539+2028C>T, as mentioned in previous chapters (Albert *et al.*, 2018). The PPCs were produced from Stargardt disease (STGD1) patient fibroblasts, and were successful in demonstrating that the splice altering variant leads to pseudoexon inclusion, due to altered recruitment of splice enhancers.

As research progresses, retinal organoids have also become a highly successful tool in functional studies (Watson and Lako, 2023; Galindo-Cabello *et al.*, 2025). Organoids are 3D human stem-cell derived model systems, and have been seen to be a promising way forward in clinical translational research. To make retinal organoids, fresh patient blood samples or skin biopsies are required to generate induced pluripotent stem cells (iPSC), and indeed such organoids can take up to a year to culture into 3D structures from iPSCs. Studies in organoids may be undertaken to compare whether a splice defect is heightened in the organoid when compared to the effect of the splice variant in PPCs or HEK293 cells as, in principle, organoids have a more accurate gene expression profile, and splice defects that were missed when using assays involving less disease relevant cell types may hopefully be picked up in a retinal organoid. For example, such a study was undertaken on the *ABCA4* c.5196+1137G>A intronic variant (Khan *et al.*, 2020). The team observed an increase in mRNA transcripts that contained a pseudoexon inclusion in the retinal organoid, but not in a midigene splicing assay in HEK293 cells. It was concluded that this was due to the specific genetic expression profile seen in the more retinal lineage of the organoid.

Given the rapid emergence of potential gene therapies for *RPGR*-linked retinal degenerations as outlined below, the need to accurately diagnose patients is imperative. Understanding the pathogenicity of candidate variants may enable patients, where relevant and with appropriate consent, to join clinical trials or to receive an *RPGR* targeted therapy, once they are FDA

and/or EMA approved. For completeness, and to highlight the relevance of accurate genetic diagnoses for IRDs, some of the gene therapies currently in clinical trial for *RPGR* IRDs are briefly outlined below. At this point in time, the most applicable therapeutic strategy that could be recommended for a coding splice-altering variant such as this candidate variant in *RPGR*, would be gene replacement therapy. In principle such an approach is independent of the specific mutant involved as *RPGR*-associated IRDs are inherited in an X-linked manner. As it stands currently, there is no treatment for XLRP other than supportive care for patients. Indeed, as per the clinical trials submitted to clinicaltrials.gov ((*ClinicalTrials.gov*, 2025), search terms; RPGR/XLRP, Accessed; July 2025), there are numerous trials employing adeno-associated virus (AAV) vectors at various phases of trial. The predominant approach is gene replacement therapy coupled with codon optimisation to ensure the correct splicing and translation of the purine-rich *RPGR*-ORF15 region, leading to more stable *RPGR* cDNA.

Recently, there are 4 active clinical trials in various phases being carried out by Beacon therapeutics utilising rAAV2tYF-GRK1-RPGR (NCT06333249, NCT06275620, NCT03316560) or AGTC-501 (NCT04850118). AGTC-501 is a therapeutic approach that employs an AAV2 capsid, AAV2tYF, that has been optimised to enhance both its stability and transduction efficiency as a vector. This is coupled with the *RPGR*-ORF15 wildtype gene under the control of the *GRK1* promoter. Preliminary results are promising, with significant improvement in visual function and retinal sensitivity noted in treated eyes (Birch *et al.*, 2024). AAV2/5.hRKp.RPGR is based on the AAV2/5 serotype. This approach uses a shortened version of the human *RPGR*-ORF15, where 10% of the ORF15 sequence is deleted, and packaged into an AAV2/5 capsid with the *RPGR* transgene under the control of a *GRK1* promoter. This treatment approach also seems efficient, with approximately 50% of treated patients displaying improved retinal sensitivity and visual function in comparison to the control group (Michaelides *et al.*, 2024). This approach is currently being investigated in three phase III trials by Janssen Research & Development, LLC (NCT04671433, NCT04794101, NCT05926583). The final approach that must be mentioned to highlight the challenges faced when developing a gene therapy for XLRP caused by *RPGR* is BIIB112. This therapy employs an AAV8 vector in conjunction with a codon-optimised human *RPGR*-ORF15 sequence, which had proven to be stable in previous models of disease, and the preliminary results were reported in 2020 (Cehajic-Kapetanovic *et al.*, 2020). While initially promising, BIIB112 has failed to meet the desired clinical outcomes, and in fact 28% of treated patients experienced serious treatment-emergent adverse effects. Additionally,

approximately 33% of patients demonstrated a statistically significant improvement in visual function through 12 months (Krusenstiern *et al.*, 2023).

While there is no established treatment or cure for *ABCA4*-associated retinopathies like STGD1, various therapeutic strategies have been increasingly proposed in recent years. There are, at present, 39 clinical trials listed on clinicaltrials.gov for Stargardt disease, all at various different stages ((*ClinicalTrials.gov*, 2025), search terms; *ABCA4*/Stargardt, Accessed; July 2025). The preclinical and clinical trials currently underway targeting the management of STGD1 have adopted a number of promising approaches including; small molecule therapy, gene augmentation therapy, stem cell therapy/cell replacement and antisense oligonucleotides (AONs). At the genetic level, gene augmentation and AONs work to ameliorate disease symptoms and stop further vision loss by restoring correct protein function. Gene augmentation approaches allow for the delivery of healthy *ABCA4* coding sequence to the retinal cells, but this has proven to be a challenge due to the large size of the gene. Standard adeno-associated virus (AAV) delivery methods typically used in gene replacement therapies have been insufficient in their packaging capabilities and capacities for *ABCA4*. However, where these typical approaches have failed, alternative strategies such as dual-AAV and non-viral vector delivery have shown promise at the preclinical level. Numerous animal studies to date have shown the successful use of a dual-AAV vector system to deliver the two halves of the *ABCA4* gene, which then recombine within the host target cells (Dyka *et al.*, 2019; McClements *et al.*, 2020; Riedmayr *et al.*, 2023). When compared to other delivery systems tested in *ABCA4* knockout mice, this method resulted in the active wildtype protein's correct localisation to the photoreceptor cells. Currently there is a Phase I/II trial being carried out by SpliceBio named the ASTRA study, to analyse the efficacy and safety of a subretinal injection of the SB-007 gene therapy which utilises this dual-AAV approach to restore the expression of *ABCA4* protein in patients with STGD1 (NCT06942572). As part of the eligibility criteria for this study, the patient must have a diagnosis of STGD1 caused by bi-allelic pathogenic, or likely pathogenic variants in *ABCA4* which have been confirmed by a clinically accredited laboratory. The first patient was dosed with this promising treatment on the 13th of March 2025.

AONs have also been shown to have good therapeutic potential for IRD genes that harbour splice altering variants as they can alter splicing by binding to specific splicing motifs in the pre-mRNA (Xue and MacLaren, 2020). There are currently no AON-based therapies available on the market for IRD treatment. However, this AON approach is being tested in phase I/II trials to rescue a common deep-intronic variant in *CEP290* in LCA patients

(NCT03140969)(Russell *et al.*, 2022). As well as this, AON methodology is being employed in the Ulteversen therapy to modulate the vast number of pathogenic variants found in exon 13 of *USH2A* by leading to therapeutic exon skipping (NCT06627179). AONs appear to be a promising therapeutic approach to rescue splice altering and deep-intronic variants in *ABCA4*. Utilising AONs to correct splicing and allow for higher levels of wildtype protein to be produced has been proven to be effective in *ABCA4* in numerous studies (Kaltak, de Bruijn, *et al.*, 2023; Corradi *et al.*, 2024; Suárez-Herrera *et al.*, 2024). In fact, a recent study carried out on a non-canonical splice site variant in *ABCA4*, c.5584+5G>A, at the same DNA coding position as our variant has evaluated the efficacy of utilising the QR-1011 AON to rescue defective splicing (Kaltak *et al.*, 2024). They found that the QR-1011 AON did not rescue splicing of this variant to the same level as other variants analysed, which had a seemingly less severe effect on splicing. They hypothesised that the level of wildtype restoration varies due to both the severity of this variant's impact on splicing and the higher propensity of the +5 position of the donor site consensus to tend toward its wildtype nucleotide. In terms of the severity, it is likely that the AONs could perform more favourably in a retinal organoid, as QR-1011 has been shown to result in a clinically relevant level of rescue in retinal organoids when used to ameliorate the severe splicing defect of common pathogenic *ABCA4* variant c.5461–10T>C (Kaltak, de Bruijn, *et al.*, 2023). Further investigation into the potential effects of AON rescue on the *ABCA4* c.5584+5del variant could yield interesting results, potentially similar to those seen of similarly severe variants impacting the canonical donor site. However, it is of note that for many such AON approaches, on-going relatively frequent administration of the therapeutic modality is required. Delivery approaches employed for AON therapies will also greatly influence efficacy of therapies (for further discussion of this topic see (Godfrey *et al.*, 2017; Kuijper *et al.*, 2021)).

Ongoing and future clinical trials highlight the immense progress that has been made to date and is currently being made in the gene therapy field for IRDs, including for X-linked RP caused by *RPGR* mutations. Furthermore, as outlined above, the development of such therapies serves to highlight the vital importance of genetic diagnosis and exploration of the pathogenicity of novel candidate variants in IRD genes in order for patients to be able to receive such treatments in the future. Functional studies are clearly extremely important as per the research described in this chapter of the thesis. However, such studies are extremely time consuming and costly, and hence this represents a challenge for IRDs, and indeed other genetic conditions, where a significant proportion of patients have VUS which require

further investigation. The lack of sufficient expertise and funding within health services to support such activities represents a current barrier to many patients in obtaining definitive diagnoses, as will be discussed further in the following General Discussion section of this doctoral thesis (Section 6.).

6. Discussion

6.1 Working to resolve the unresolved and the progress of Target 5000

Recent years have seen the rise in NGS technologies, with DNA sequencing becoming a routine part of patient diagnosis in many medical fields. As sequencing technologies become more sophisticated, disease-causing variant detection has become more efficient for hereditary diseases such as IRDs. Nevertheless, even with such progression being made in the sequencing field, there is still a large population of IRD patients that remains genetically unresolved and without a complete molecular diagnosis. This shows that while we continue to expand our knowledge of IRD pathogenesis, there is still a lot that is not known or understood about these genetic disorders. In an effort to contribute to this growing knowledge base, this doctoral thesis has focused on sequencing, functional and computational work with the aim of expanding the diagnostic capability of Target 5000. The utilisation of targeted sequencing for some studies presented in this thesis has not only allowed us to highlight the benefits of such methods of sequencing, but has also allowed us to analyse key positives and negatives of more comprehensive forms of sequencing such as WES and WGS. It has been a privilege to contribute to our understanding of genetic architecture of IRDs in Ireland, the progression of Target 5000 and to expanding our ability to provide precise and comprehensive genetic diagnoses to the IRD patients of Ireland.

Chapter 3 focuses on analysing 223 Irish iMD patients, utilising smMIPs targeted sequencing of 105 iMD associated genes along with a select few AMD risk loci, deep intronic variants and regulatory sequences previously associated with disease. This panel was designed by our collaborators in the Cremers Lab at RadboudUMC, where I was able to perform library preparation, filtering and identification of candidate variants using a bespoke pipeline during secondments in their Institute. Chapter 3 provides an overview of the first landscape study that encapsulates a spectrum of iMD phenotypes in the Irish population, where the patients who were sequenced using the MD-smMIPs panel had either not had a previous form of NGS or had remained unresolved following other targeted sequencing approaches. This cost-efficient sequencing approach achieved a ~33% diagnostic yield for iMDs. 72 patients are considered to be genetically resolved, with some having already received confirmatory sequencing in a clinically accredited lab. Within the scope of this study, we have discovered 9 novel variants thought to be causative of disease in various iMD genes and were able to classify them where appropriate. The identification of a novel splice-altering variant in *ABCA4* has allowed us to contribute to the growing spectrum of

ABCA4 variation, with functional studies being carried out and described in Chapter 5. We have also been able to highlight some interesting cases such as a large novel deletion in *CDH3*, where our patient is the second patient globally to have a deletion incorporating exons 12 and 13 of the gene. Additionally, we have described a rare carrier female phenotype associated with *RS1*, as well as discussed the hurdles that were encountered when analysing 4 patients with variants located within the opsin gene cluster.

Following on from the smMIPs study, it was important to draw attention to a WES case study in order to comment on the effectiveness of each sequencing approach, as well as to highlight a family affected with BBS. Chapter 3B outlines the first described *BBS7* pedigree in Ireland where we have identified compound heterozygous variants in the *BBS7* gene employing WES. Within this subchapter we have described in detail the multifaceted BBS phenotypes experienced by two sisters with the same *BBS7* genotype, allowing us in turn to analyse and classify one known and one novel *BBS7* variant. As BBS shares extensive phenotypic overlap with other ciliopathies and IRD syndromes, it is vital that genetic testing be adopted into the diagnostic pathway in order to ensure a definitive clinical diagnosis and optimal disease management plan for each patient. We have therefore been able to contribute to the expanding knowledge regarding the pathogenesis of BBS with the identification of novel disease-causing variants in known genes.

Chapter 4 describes a collection of Target 5000 family studies that have allowed us to improve both the classification of IRD variants, as well as expand our understanding of the involvement of these variants in disease phenotype, severity and progression. The 13 families outlined within the chapter have provided interesting perspectives on the complexities of IRDs as clinically and genetically heterogeneous disorders; for example, the variable penetrance associated with *CDHR1* variants and hypomorphic *ABCA4* alleles, among others. Furthermore, we were able to analyse novel variants using phasing and segregation analyses and *in silico* tools to predict pathogenicity enabling thorough classification using criteria we could apply from the ACMG/ACGS guidelines. Similarly, the ClinGen VCEP for *USH2A* was utilised and discussed, evidencing the value in gene and condition specific classification guidelines. Analysis of multiple *ABCA4* families was completed, using the severity of known variants, genotype-phenotype correlation models and consulting with the clinical team thereby allowing in-depth clinical and molecular diagnoses to be reached. Such studies underpin the value of characterising a VUS as thoroughly as possible to try to establish pathogenicity, as it may impact a patient's eligibility

for participating in a clinical trial or gene therapy, where an accurate genetic diagnosis may be critical.

Chapter 5 highlights the importance of *in silico tools* and *in vitro* functional assays in the classification of disease-causing variants. Not only do such assays deepen our understanding of the mechanism of disease, they also allow VUS classifications to potentially be up- or down-graded creating a clearer genetic diagnosis for the affected patient. As outlined in the chapter, eligibility criteria for emerging clinical trials and gene therapies typically indicate the need for a complete genetic diagnosis for each patient before they are considered for recruitment into a trial or treatment with an approved therapy. Some of the most elusive variants are variants that are predicted to impact splicing, however such splice variants can typically be assessed by functional assays. Two such variants were analysed using midigene assays in Chapter 5, with results indicating little to no wildtype transcript remaining as a result of each variant respectively. While it is indeed the case that these assays were completed in HEK293 cells and not in a retinal specific cell line or indeed *in vivo*, they can often be a good preliminary indicator of aberrant splicing, with patient-derived photoreceptor precursor cells (PPCs), or retinal organoids being more ideal retinal models for *in vitro* assays.

The studies described within this thesis serve to underpin the basis of disease in the IRD population of Ireland encapsulated by the Target 5000 study. Additionally, these studies have employed the most effective sequencing methods on a case-by-case basis, allowing us to provide comprehensive and detailed genetic diagnoses to IRD patients to aid in providing medical knowledge, as well as clarity on disease prognosis and family planning. Each patient that remains unresolved following a form of targeted sequencing that was undertaken as part of these thesis studies will progress to a more comprehensive form of sequencing such as WGS (Liu *et al.*, 2024b; Zeuli *et al.*, 2024a). International collaborative efforts and multidisciplinary teams have been paramount in achieving the results outlined thus far, and hopefully I have been able to highlight the vast importance of such teamwork and knowledge sharing to the future of the IRD field. As part of the research arm of Target 5000, through close collaboration with scientific and clinical teams in recruitment hospitals, we have had the opportunity to create connections to specialists worldwide to seek further expertise when needed. Having a greater understanding of IRDs is not only beneficial for patients in our Target 5000 cohort, but is also vital for both ocular researchers and clinicians in providing more comprehensive genetically informed care to patients globally.

6.2 *What are we missing?*

A historical bias has been observed in sequencing studies, where specific emphasis is placed on the coding regions of the genome as previously discussed, by employing sequencing methods such as WES and target capture as preliminary forms of analysis. This common theme tends to extend to many medical genetic testing projects, not just IRDs. However, it is often the case that when employing such targeted sequencing methods, many cases can still go unresolved. Using targeted methods means that from the outset, in principle, we are not capturing all of the genomic information. Intronic and regulatory regions of genes, and indeed many other noncoding genomic regions, have not been assessed at all. Despite these facts, it remains both an efficient and cost-effective manner of preliminary genetic screening, with 68% of surveyed European countries choosing targeted panel sequencing as their first genetic testing approach in genetic diagnosis for IRDs (Paudel, Daly, Moran, *et al.*, 2024).

With the evolution of NGS technologies, WGS has become both more cost and time effective giving us the opportunity to increase our diagnostic yield and provide patients with inherited disorders a better chance of receiving a genetic diagnosis, appropriate care and improving their lifestyle. While it remains that WGS is the most comprehensive form of sequencing, and potentially does allow us to make observations on primary causes of disease and on broader contributors to disease such as modifier loci, I do believe that the work I have presented within this thesis effectively underpins the significant benefits of the utilisation of smMIPs sequencing as a preliminary form of genetic testing. smMIPs sequencing is not only cost-effective but also produces a manageable amount of data, without the potential of encountering secondary findings. smMIPs panels are easily updated and altered, and in an evolving field such as IRD research, this adaptability is a useful characteristic as panels can be modified efficiently and without overwhelming workload. Designing a smMIPs panel to incorporate candidate genes and loci as they are discovered and associated with IRD phenotypes offers a promising way to provide effective first-tier sequencing to IRD patients. Therefore, it is a more streamlined process than WES, or particularly WGS, where excessive storage issues, data management, workload and secondary findings pose great barriers to timely genetic diagnoses for patients. Additionally, smMIPs targeted sequencing enables high quality sequencing traces and coverage, with multiple tiled probes allowing for confident variant calling. Nevertheless, WGS would be the most suitable method of sequencing to use for patients that remain unresolved even after targeted forms of sequencing. Patients remain unresolved for several potential reasons, including novel genes and variants that have not been identified or associated with IRDs previously, regulatory

variants that have not been well explored that may impact gene expression, and the current standard/quality of NGS and filtration thresholds applied. WGS could allow us to identify previously elusive regulatory genetic variation, as well as large structural (SV) or copy number variants (CNV) where breakpoints might not be captured by a targeted panel, modifier loci, and novel genotype-phenotype correlations where the locus has not been previously analysed. However, many of the non-coding variants would remain classified as a VUS and would require additional functional studies to establish their impact on the RNA and/or protein, and potential pathogenicity.

It is important to note, that Target 5000 initially reported a diagnostic yield of approximately 68-70% (Dockery *et al.*, 2017; Whelan *et al.*, 2020a). This statistic was obtained from a completely naïve cohort that had not yet received any form of NGS, therefore the original targeted panel of 254 IRD genes was effective in identifying the genetic cause of disease in a large number of patients (Carrigan *et al.*, 2016). The patient cohort that was described in Chapter 3 of this doctoral thesis consists of a mixture of iMD patients that had either received an alternative form of preliminary sequencing, or had not received any form of NGS. While it is generally the trend observed within iMD cohort studies that the diagnostic rate is lower than in other IRD subsets, we believe that the 33% diagnostic yield observed in our iMD cohort is likely to be in part due to the inclusion of previously unresolved patients who remained unresolved after multiple forms of targeted sequencing and appear to have less well defined or more complex causes of disease. For patients such as these, the strategy moving forward could be to expand the panel of genes and loci that are analysed to include novel candidate variants and intronic regions, or WGS.

When analysing variants in candidate genes that have been newly associated with an IRD, many potential hurdles to diagnosis may be faced. Predictive models may not be completely informative due to lack of information available for the novel locus, therefore *in vitro* and animal modelling are often required to prove the involvement of the variant in an IRD (Cremers *et al.*, 2018). Similarly, it can be difficult to determine causality of variants in these novel candidate genes especially as they may be quite rare and hence the association has not been identified in patients sooner. The novel variants reported in this thesis may represent new ocular phenotypes associated with known IRD genes. Furthermore, it is important to consider that in the ocular disease field, we are gaining a greater understanding that not only the primary genetic defect may affect ocular disease phenotypes, but indeed, modifier loci can also influence the clinical presentation of disease (Dockery *et al.*, 2021). Additionally, the clinical overlap observed between iMD and AMD poses another barrier to molecular

diagnosis. While iMDs present much earlier and more rarely, an AMD phenotype presents later in life, becoming more prevalent with age (Colijn *et al.*, 2017). It remains likely that some iMD patients where symptoms manifested later in life may have received a misdiagnosis of AMD, or vice versa. It would be of interest to analyse potential modifiers that may lead to either a later or earlier onset of both iMD and AMD, in order to further illuminate what other factors may be at play. As observed in rare cases and outlined in Chapter 3, variants in AMD risk factor genes such as *CFH* have been identified in patients with early onset macular drusen, and have sometimes been associated with a Mendelian inheritance pattern (Taylor *et al.*, 2019). A reanalysis in some cases for patients with AMD and iMD may be beneficial, along with continued large-scale studies such as the macular smMIPs study presented in this thesis, in order to further explore genetic factors underlying macular degenerations.

Throughout this thesis, particular emphasis has been placed on variants in IRD genes that have potential impacts on splicing, such as deep-intronic and splice site variants. However, there exist other kinds of pathogenic non-coding variants identified in IRD genes that we have not encountered thus far, but that cannot be ruled out as contributing to disease severity and missing heritability. Some patients may carry pathogenic variants within cis-regulatory regions of IRD genes. The 5' UTR of a gene typically contains regulatory motifs such as promoter elements, enhancers, and transcription factor binding sites, making it vital in transcription, post-transcriptional control and correct gene expression (Rydzek, Łyś and Makałowska, 2023). Variants within these regions have been associated with disease in a small number of IRD genes, such as *CHM*, *EYS*, *PRPF31*, *NMNAT1* and *MERTK* among others (Ruberto *et al.*, 2021; Daich Varela, Bellingham, *et al.*, 2023; Dueñas Rey *et al.*, 2024; Hayman *et al.*, 2025). Additionally, mutations in promoter sequences involved in regulation of transcription, and often comprising large regions of DNA upstream of the 5'UTR, may also be implicated in IRDs. While this seems to be a rare occurrence, it is likely that some Irish patients may harbour similar variants that were not detected as the sequencing panels used do not cover all of these regions. The MD-smMIPs panel utilised in Chapter 3 was indeed designed to cover the 5' UTRs of the 105 genes covered; however, we did not identify any variation in these regions as causative in our patients. The fact remains that even if identified, the interpretation criteria for such complex variants are still in their infancy, with guidelines recently published for non-coding variation recommending that further study is needed to train *in silico* tools and encompass the diverse mechanisms of pathogenicity associated with regulatory regions such as the 5' UTR (Ellingford *et al.*, 2022). *Cis-*

regulatory regions such as promoters and the 5' and 3' UTRs are indeed understudied, where variants in these non-coding regions may represent novel genetic causes of disease and are definitely worth exploring in existing sequencing data where these regions have been captured but potentially overlooked. However, while existing exon-centric data sets may have 5'UTR and 3'UTR sequences included, in many cases promoter regions are not present and therefore remain largely understudied as yet.

IRDs are viewed as largely Mendelian in nature, often caused by variations in a single gene. However, as reviewed in Chapter 3, this widely held view has begun to shift with the identification of hypomorphic variants displaying varied levels of penetrance, along with potential digenic inheritance patterns, tri-allelism and modifier loci acting at the genetic level to contribute to the phenotypic variability observed in IRDs (Dryja *et al.*, 1997; Eichers *et al.*, 2004; Burkard *et al.*, 2018; Frecer *et al.*, 2019; Lee *et al.*, 2021b; Zernant *et al.*, 2022; Lisbjerg *et al.*, 2023; Ford and Petersen-Jones, 2025b). Modifier loci implicated in IRDs thus far have been seen to alter dominance, penetrance and expression leading to varied effects on phenotype and disease severity. The effects of modifiers remain difficult to quantify, with their identification also posing a significant challenge. Modifiers can be *cis*- or *trans*- acting, whereby the variant believed to be modifying disease is either within the same gene or in a second gene outside of the primary causative gene. A good example to discuss both *cis*- and *trans*- modifiers is the *ABCA4* gene. The most common variants in *ABCA4* are categorised as mild variants when they occur alone, and they are c.5603A>T and c.5882G>A. Both of these variants present with reduced penetrance, as was discussed in Chapters 3 and 4. A recent study in 2021 identified a deep intronic variant in *ABCA4*, c.789-784C>T, that was found to occur *in cis* with the common c.5882G>A variant, and was seen to alter the phenotypic severity of disease (Lee *et al.*, 2021b). When compared, patients carrying only the common hypomorphic c.5882G>A variant presented with much milder and uniform clinical phenotypes than patients where c.789-784C>T was present as part of a complex allele with c.5882G>A. Additionally, c.5882G>A homozygotes also displayed increased penetrance of disease where c.789-784C>T was present even just on one allele. Similarly, as shown in Chapter 3 with four patients carrying this allele, the most common complex *ABCA4* allele found in patients with STGD1 is c.[2588G>C;5603A>T], where c.2588G>C has been classified as benign when found alone (Runhart *et al.*, 2018). This complex allele accounts for ~50% of all complex alleles encountered in patients with an *ABCA4*-retinopathy. Both of these variants are required to be present on the same allele to render it fully penetrant (Zernant *et al.*, 2017). Approximately 10% of disease-causing alleles found in *ABCA4*

consist of more than one variant, where one variant within the complex is believed to act as a *cis*-modifier (Zhang *et al.*, 2015). A number of complex alleles have been identified within *ABCA4* and encountered throughout this doctoral study, where a hypomorphic variant was found along with a *cis*-acting benign variant or with a splice altering variant; c.[1622T>C;3113C>T] and c.[5461-10T>C;5603A>T] to name just two (Zernant *et al.*, 2017; Khan *et al.*, 2020).

Trans-modifiers of *ABCA4* have also become a popular area of study, and we have discussed a few throughout this thesis. While *ABCA4* genotype-phenotype studies have informed the current proposed disease model, individuals with the same combination of *ABCA4* variants frequently exhibit different clinical phenotypes. This can in part be explained by *trans*-modifiers. Phenocopies of *ABCA4* present as the most likely candidates for *trans*-modifiers of disease, where genes such as *PRPH2*, *ROM1*, and *PROM1* have a similar functionality in maintaining photoreceptor disc morphogenesis, structure and integrity (Stuck, Conley and Naash, 2016; Yanardag *et al.*, 2024). As is often the case, variants in *PRPH2* lead to a phenotype that is clinically almost identical to *ABCA4* STGD1 disease, where approximately 10% of presumed STGD1/*ABCA4* patients, who are negative for *ABCA4* variants, indeed carry variants in *PRPH2* (Wolock *et al.*, 2019). As *PRPH2* co-localises with *ABCA4*, and *ROM1* often forms a complex with *PRPH2* in the photoreceptor OS discs (Goldberg and Molday, 1996; Molday *et al.*, 2022a), it is not unexpected that both *PRPH2* and *ROM1* have exhibited both digenic inheritance patterns and may act as modifiers in *ABCA4* disease. A study has analysed the hypothesis that phenocopy genes with overlapping molecular functions and clinical presentations act as modifiers to *ABCA4*-retinopathies; variants in seven *ABCA4* phenocopy genes were tested as potential *trans*-modifiers (Zernant *et al.*, 2022). They found that rare variants in *ROM1* and a particular common *PRPH2* haplotype were potential modifiers of *ABCA4* disease phenotypes and penetrance. When analysing their cohort of patients with only the c.5603A>T variant present in *ABCA4*, they found that the presence of the common *PRPH2* haplotype was significantly increased. Therefore, they suggested that the *PRPH2* haplotype has sufficient impact on the protein that this mechanism in turn increases the penetrance of the c.5603A>T variant in *ABCA4* leading to late-onset STGD1 disease phenotypes in patients. This was observed at a population level, where the authors also indicated that due to the current lack of research on *ROM1*, larger cohorts need to be explored to further understand the association of the *ROM1*/*PRPH2* variants as modifiers in *ABCA4* disease. Despite this, *ABCA4* and *PRPH2* pathogenic variants are often reported in the same patients, *PRPH2* and *ROM1* have been identified in a digenic

inheritance pattern in individuals with late-onset pattern dystrophy, and mouse models have validated that *ROM1* contributes to the phenotypic variability associated with *PRPH2*, further supporting the action of these genes as modifiers (Dryja *et al.*, 1997; Poloschek *et al.*, 2010; Strayve *et al.*, 2020). Identifying and understanding the underlying mechanisms of modifiers and their impact on molecular pathways vital to the retina could expand the landscape of therapeutics, with the identification of novel therapeutic targets. Modifier loci could also be used for early-identification and genetic testing to elucidate disease prognosis and severity later on in life. Modifiers remain a vital area of study not just in IRDs but in all heritable human disease, as we continue to expand our understanding of the genetic basis of rare and common diseases.

6.3 ‘Variants of Uncertain significance’: Insights into the classification of IRD variants

With advancements made in NGS technologies and increased access to comprehensive sequencing strategies, we have seen an exponential rise in genetic variant identification, not only in IRD-associated genes but in all hereditary diseases. While exciting, this comes with the challenge of classifying and interpreting the pathogenicity or benignity of these variants. Herein lies the complication, as the development of variant interpretation guidelines has not been able to keep pace with the rate at which these novel variants are being identified, leading to many variants being classified as VUS. As discussed throughout all three chapters of this doctoral thesis, VUS represent a significant issue in patient care pathways and treatment plans, as these variants are clinically inactionable. This means that patients often are ineligible for clinical trials or gene therapies as a VUS classification means there is conflicting or even insufficient evidence to definitively associate this variant with disease. VUS represent a large portion of variants identified in IRD genes, with >40% of all variants identified in IRD genes listed on ClinVar being classified as a VUS (Finnegan *et al.*, 2025).

Due to the high level of genetic heterogeneity exhibited by IRDs and the significant clinical overlap between conditions, variant interpretation is vital for accurate genetic diagnosis of such diseases. Currently, it is standard practice in most research and clinical labs globally to utilise ACMG/AMP guidelines for variant interpretation associated with Mendelian hereditary disorders (Richards *et al.*, 2015). As has been discussed previously in this PhD thesis, these ACMG/AMP guidelines provide a framework for variant classification based on a number of evidence types such as population, computational, functional, and allelic data, among others. These data are often readily available in open-source or public databases,

with carefully curated variant collection platforms such as LOVD.nl often serving as useful references (Fokkema *et al.*, 2021). Since the publication of these guidelines in 2015, it has become apparent that the lack of specificity of this framework has resulted in a lack of consistency in their use across institutions globally. Broadly, this means that different groups are sometimes interpreting candidate variants in different manners, leading to inconsistencies in classifications therefore creating uncertainty surrounding molecular diagnoses and treatment eligibility. This uncertainty opens up the possibility of misdiagnosis, posing a threat to clinical management and treatment not only for the patient but for their family as well. As discussed in Chapter 4, The Association for Clinical Genomic Science (ACGS) has over the years published suggested updates and annotations to the ACMG/AMP guidelines to adapt them for standardised usage on rare diseases in labs and clinics in the UK and Ireland (Ellard *et al.*, 2020; Durkie *et al.*, 2024). While these additional recommendations provide clarity on some of the issues that have been raised over the years by researchers and clinicians alike, there still remains a lot that is disease and gene specific that is not covered. Some such hurdles that have been encountered throughout this thesis will be discussed herein.

While the ACGS recommendations work to minimise the discrepancies in interpretation between institutions to a degree, it may be more prudent to introduce unique guidelines for IRD-associated variants which would take into account the nuances encountered with these specific genes and disorders. Such an endeavour has been pioneered by the curation panels set up by the Clinical Genome Resource (ClinGen) (Wright *et al.*, 2024). In an effort to provide recommendations for classifying monogenic candidate variants specific to a particular disease and to evaluate the evidence associating a particular disease with a genetic cause, ClinGen have put together Variant Curation panels of experts (VCEPs) and Gene Curation panels (GCEPs). One such set of expertly curated guidelines was described in Chapter 4, wherein recommendations from the genetic hearing loss VCEP (HL-EP) were utilised for the classification of variants in *USH2A* (Hearing Loss VCEP, 2022). Furthermore, the *ABCA4* VCEP is currently underway, with the proposed classification rules being tested in a pilot phase at the moment, as listed on the ClinGen website. The X-linked Inherited Retinal Disease VCEP was recently approved for seven X-linked IRD genes in April of 2025. As it stands now, the specifications for classifying variants in *RPGR* and *RS1* have been published in May 2025 in the ClinGen Criteria Specification Registry (CSpec) with others soon to follow (*Criteria Specification Registry*, accessed June 2025). These guidelines were reviewed and utilised in this doctoral thesis to classify variants encountered

in the *RPGR* and *RS1* genes. These panels have the potential to massively influence the way we categorise variants and analyse their pathogenicity with focus on the disease gene, variant and phenotype. Some IRD working groups are even developing population-tailored ACMG/AMP guidelines by adapting the criteria to ensure that disease-specific considerations and unique ethnic factors are taken into account when interpreting IRD candidate variants, in an effort to maintain consistent and accurate results (Fujinami *et al.*, 2024). Similarly, our collaborators in RadboudUMC, as part of an *ABCA4*-specialised research group, have processed and reclassified all 2,246 unique variants in *ABCA4* compiled on the LOVD.nl curated database using ACMG/AMP guidelines and the ACGS recommendations that they have efficiently adapted to incorporate the nuances *ABCA4*-associated retinopathies, once again to refine variant classification and allow for standardised approaches specific to one gene (Cornelis *et al.*, 2023). Such studies involve vast workloads, and require expertise and funding, with the large number of genes implicated in hereditary disease posing significant hurdle to progression. Updates to the ACMG/AMP guidelines are due shortly and may aid in resolving some of the issues posed over the last few years since their initial proposal.

Some of the challenges encountered throughout this doctoral project have been discussed thus far in each section respectively, regarding the classification of the identified candidate variants. This includes, but is not limited to the use of functional studies and the ability to apply criteria based on their suitability to the disease context, the strength at which to apply criteria where modified strengths are recommended, lack of access to segregation and allelic data, interpreting non-coding and splice-altering novel variants, and what to do in the event of rare occurrences of incomplete penetrance or X-linked female carrier phenotypes. As encountered in the functional studies chapter of this thesis (Chapter 5), when interpreting novel and known variants it is important to employ both *in silico* tools such as SpliceAI and Alamut Visual and pathogenicity prediction scores like AlphaMissense and REVEL, alongside functional studies where appropriate. The rise of deep learning and AI-based tools is an exciting prospect for many aspects of IRD molecular diagnosis, especially in pathogenicity prediction and interpretation of multimodal ocular imaging (Pontikos *et al.*, 2025). Tools such as SpliceAI, AlphaMissense and AlphaFold are trained on large genomic and protein databases, where SpliceAI is trained on RNA-sequencing data and AlphaMissense is trained on population and proteomics data (de Sainte Agathe *et al.*, 2023b; Kurtovic-Kozaric *et al.*, 2024). SpliceAI was used to obtain predictions on aberrant splicing for each variant identified within this doctoral thesis which was referenced in depth in

Chapter 5. While it is an efficient tool and successfully aided in the variant filtering process described in Chapter 3, there are also a few drawbacks to consider. A cutoff threshold of ≥ 0.2 was used for this study, but as we have seen with the *ATF6* c.479A>G (**Table 3.2**) variant identified in Chapter 3, a score below this may be worth exploring with functional studies, especially if all other evidences point to the variant being causative of disease in such cases. SpliceAI was not trained on RNA-sequencing data that were derived from retinal tissue, so this could have an impact on the tools ability to correctly predict the impact of variants that might alter retinal-specific splicing. This issue is being addressed in a recent study in the pre-print phase, where the authors have developed a promising retina-specific splice prediction tool that is trained on splice junction data derived from retinal RNA-sequencing data (Riepe *et al.*, 2025). It is the hope that this tool can be applied to large patient cohorts to aid in identifying novel splice-altering variants as well as improving the predictions of retinal-specific splice variants.

Alongside *in silico* predictions, functional studies are imperative in providing additional lines of evidence for variant pathogenicity. Previously, in the ACMG/AMP guidelines, the PS3/BS3 evidence was utilised for functional data. Additionally, in recent ACGS updates, it is recommended that PVS1 criteria be utilised for splice variants (canonical and non-canonical) when RNA splicing evidence is available, such as results obtained from a minor or mid-gene splice assay (Durkie *et al.*, 2024). ClinGen have established an SVI working group to provide recommendations for splicing variants, where a PVS1 decision tree similar to that published in 2018 has been altered to include a branch for splice variants where RNA studies have confirmed an aberrant splicing profile (Abou Tayoun *et al.*, 2018; Walker *et al.*, 2023). This decision tree amendment to include RNA functional studies for splice variants takes into account the ratio of wild type to mutant transcript produced, frame, domains within the protein and whether NMD is predicted to occur. The ClinGen splicing update recommends that PS3 criteria should only be used in cases where functional assays have shown the impact on the protein without the use of RNA-splicing assays. Similarly, due to the vague nature of the PS3 criteria description in the original ACMG/AMP guidelines, Brnich *et al.* have published further recommendations for PS3/BS3 application in an effort to better define how to decide if the functional assay and its results really allow for the application of these criteria (Brnich *et al.*, 2019). These additions suggest that the functional analysis used should take into account the disease mechanism, the number of controls required, and that sufficient statistical analyses should be carried out. While these suggestions are welcome, this can still be an issue where the disease mechanism for a gene

is not fully defined, therefore it can be unclear what assays to use and whether they do in fact allow for the application of the PS3/BS3 criteria once completed. ClinGen's VCEPs will undoubtedly aid in the decision-making process when it comes to designing functional assays to use on a gene specific basis, as each expert panel works to evaluate what assays are the most suitable for their gene(s) of interest, in turn providing recommendations on what strength criteria can be applied based on the outcome of the assays. Another limiting factor in the application of PS3/BS3 criteria is the lack of clarity on what may constitute a sufficient number of controls. Current guidelines indicate that at least 11 pathogenic and benign variant controls are required to allow even a moderate weighting of functional evidence. This often means that when classifying a variant, one cannot utilise previously published functional studies as 'functional/PS3 evidence' as they have not been designed with this number or these criteria in mind. Consideration of the Brnich *et al.* updates (Brnich *et al.*, 2019) and gene-specific VCEPs moving forward will aid in the standardised design and usage of functional assays to facilitate variant classification globally, further allowing for a better understanding and functional characterisation of VUS and pathogenic variants in hereditary disorders such as IRDs.

Historically, it has been the case that variants exhibiting reduced levels of penetrance and hypomorphic alleles have been difficult to classify using the standard ACMG guidelines. In the case of reduced penetrance variants, one difficulty complicating interpretation is that the population frequency may exceed the disease prevalence given that there are significant levels of reduced penetrance. Incomplete penetrance often poses an issue when analysing segregation and phasing data as the candidate variant may not segregate with disease in the typical manner. A well-known example of this can be seen in patient CI18, as described in Chapter 3, where MD-smMIPs sequencing identified the c.133C>T variant in *RP11* which is famously associated with reduced penetrance levels (**Table 3.1**) (Akahori *et al.*, 2010). This variant is present in family member CI19 who does not present with disease. In Chapter 4, we have a family presenting with late onset cone-rod dystrophy with macular involvement, where the variant c.562G>A in *CDHR1* has previously been identified as a hypomorphic allele (Farag *et al.*, 2024), with each individual in our pedigree that carries the variant manifesting ocular symptoms. Some more common examples of incomplete penetrance and hypomorphic alleles have been outlined throughout this doctoral thesis in the *ABCA4* gene, such as the c.5603A>T and c.5882G>A variants. The aforementioned variants are very well-known examples of such phenomena, but when a novel variant is identified, it can be difficult to categorise whether this variant is truly the cause of disease in the pedigree being analysed.

This poses a particular problem when targeted sequencing is used as the genetic screening method, as we cannot definitively say that there have been no other potential candidate variants identified in the patient. In terms of classifying such variants associated with incomplete penetrance, the ACMG/AMP guidelines offer little to no guidance, and no framework specifically addressing variants on the lower end of the penetrance spectrum has been designed. For such variants, the typical variant classification guidelines, which are designed to assume high penetrance consistent with monogenic disease, may lead to misclassification as it can be difficult to decide what evidences can be applied and at what strength. A prime example of this is seen with the application of the population frequency-based criteria (PM2/BA1/BS1). Low penetrance variants are more likely to be identified in ‘control’ population databases such as gnomAD, as not all people harbouring such variants will develop the associated phenotype. It can therefore be misleading to apply such strong benign criteria to variants assumed to have reduced penetrance, meaning that more appropriate frameworks with less stringent constraints on population data would be more suitable for low penetrance variants. ClinGen have assembled a working group for Low Penetrance/Risk alleles to address the classification of such variants that display low penetrance levels and where no clear segregation with disease can be pin pointed (Schmidt *et al.*, 2023). Some aspects of this framework have since been included in the updated ACGS recommendations (Durkie *et al.*, 2024), however this ClinGen group has predominantly worked initially on clearly defining risk/protection alleles and low penetrance variants, with a unique focus on risk allele terminology and classification methodology. Further updates from this working group are eagerly awaited in the hope that recommendations on the evaluation of low penetrance variants specifically be suggested in the near future (Schmidt *et al.*, 2025).

6.4 MDTs, IRD care pathways and the Irish Genomics strategy moving forward

Continued landscape sequencing studies will indeed expand our knowledge of the mechanism of disease and functionality of disease variants in IRDs, and the unique genetic background of IRDs in Ireland, as well as contributing to the global repertoire of variants identified in IRD genes thereby facilitating genotype-phenotype analyses and better disease prognosis categorisation. Broadly, this work aids in providing a comprehensive genetic diagnosis to patients. Genomic testing, as has been described within this thesis, can provide many benefits to IRD patients such as clarity on disease progression and prognosis, potential eligibility for emerging clinical trials and gene therapies, access to a multidisciplinary team

specialising in their care management which may be of particular importance in syndromic cases, and access to family planning, risk assessment services and genetic counselling.

While genetic testing of IRD patients is becoming part of the routine standard of care globally in ophthalmology clinics, it is often the case in many countries that access to such testing is not available for everyone (Ormond *et al.*, 2018). While the policies in countries surrounding genomic testing may differ, studies show that IRD patients, their families and care-givers predominantly agree that genetic testing should be offered to all impacted individuals if they should want it (Willis *et al.*, 2013). A more recent survey focused on patient experiences and understanding of IRD genetic testing, found that 73% of interviewees indicated their genetic counsellor and/or medical geneticist were the biggest influence on their understanding of test results, where most people indicated having no regrets towards genetic testing regardless of whether they received a conclusive diagnosis or not (Britten-Jones *et al.*, 2024). Frequent communication with the patient community is vital not only for researchers in heritable disease, but also for ophthalmologists, clinicians and charitable organisations that work to provide support for all aspects of the patient's life. In order to increase patient accessibility to genetic testing, it has been advocated by numerous European countries that the improvement of knowledge and awareness of general physicians towards genetic testing of IRDs would in turn increase the number of patients referred (Calzetti *et al.*, 2024). As diagnostic genetic testing for IRDs becomes more readily available, it is of vital importance to ensure that all individuals working in 'eye care' have a sufficient level of up-to-date genomics knowledge. Patients who undergo genetic testing report feeling a sense of empowerment and understanding of their test results through interaction with genetic counsellors, medical geneticists and other healthcare professionals with genetics expertise (Britten-Jones *et al.*, 2024). MDTs that foster communication between genetic counsellors and medical geneticists, along with clinicians, ophthalmologists and ocular genetics researchers, will undoubtedly increase the value of genetic testing in implementing the most optimal and comprehensive treatment plan on a patient-by patient basis, but also in providing the necessary medical and emotional supports to patients and their families.

Patient-specific collaborative MDTs for IRD patients in Ireland have been developed and utilised throughout the Target 5000 affiliated recruitment centres, with the Mater Misericordiae University Hospital publishing findings in a recent collection of case reports (Conway *et al.*, 2024b). Throughout my doctoral studies, I have been able to attend patient-specific MDT meetings to observe and contribute on behalf of the research arm of Target

5000 in Trinity. The need for such collaborative effort in IRD and heritable disease diagnosis and management cannot be understated. It is through close communication as part of an MDT between ophthalmologists, retinal specialists, molecular and clinical geneticists, genetic counsellors and additional interdisciplinary experts for syndromic cases that effective resolution and management of complex IRD cases was achieved. While it is clear that MDT frameworks carry great benefits for patients, their families, and the wider scientific community of IRDs, there are significant hurdles that must be overcome to implement these types of diagnostic teams for each patient. Some such hurdles include the increased workload and lack of workforce available, the funding required to access genetic testing and required experts, expertise available at each site dealing with IRDs nationally and globally, and appropriate patient referral at the initial clinical diagnosis (Murro *et al.*, 2023; Conway *et al.*, 2024b). Despite these challenges, MDT frameworks have undoubtedly facilitated unique patient-centred care and can lead to more effective diagnoses with a higher level of confidence. Additionally, MDTs inform the wider field of ocular genetics as contributions from interdisciplinary experts can enable novel genotype-phenotype associations to be elucidated that were previously overlooked. Such findings could benefit both the understanding of the disease as well as bring more global benefit to future patient diagnosis. Patient care pathways should always place the individual and their wellbeing at the centre of the whole process. MDTs are created with the intention of providing a ‘person-centred’ healthcare approach, where continued support is provided to the patient from different specialists to address their physical and mental wellbeing (McVeigh *et al.*, 2019). It is for the above outlined reasons that genetic counselling for patients is imperative, both pre- and post-genetic testing for IRDs. Genetic counselling provides patients and families with an explanation of potential outcomes of genetic testing in advance, as well as providing information after testing regarding potential risk(s) for offspring to inform family planning, implications of a molecular diagnosis on vision decline/prognosis, and potential treatment options if applicable.

Genetic counselling is a valuable and necessary process, which by definition, integrates the assessment of chance of disease occurrence/reoccurrence, education on core genetic testing principles such as inheritance patterns, disease management, available resources and current research, as well as post-test counselling to allow patients a greater understanding of their condition and promote informed decisions (Resta *et al.*, 2006). Recently, a study was undertaken to analyse the existing genomic strategies and genetic counselling services provided to IRD patients across 19 European countries, including Ireland (Paudel, Daly,

Moran, *et al.*, 2024). It was observed, that genetic counselling services are being offered to IRD patients in the majority of European countries, however they are being undertaken by clinicians and ophthalmologists who have a background in medical genetics, rather than someone qualified specifically in genetic counselling. Only 3/19 countries, the UK, Ireland and Belgium, specified that the counselling offered to IRD patients pre- and post-testing is undertaken by a genetic counsellor (Paudel, Daly, Moran, *et al.*, 2024). As a relatively small number of European countries offer higher education courses in genetic counselling, it is not surprising that there is only a limited number of genetic counsellors available to work with the large number of IRD patients who have received a genetic diagnosis. In Ireland, there are approximately 2.5 trained genetic counsellors per 1 million population (Abacan *et al.*, 2019), where the workforce recommendation, set out by the Royal College of Physicians UK in 1991 is 6–12 genetic counsellors per million population (‘Clinical Genetic Services in 1990 and beyond and Purchasers’ Guidelines to Genetic Services in the NHS: A Summary of Two Reports of Working Groups of the Clinical Genetics Committee of the Royal College of Physicians’, 1991). Given this recommendation was made in the early 90s, we must take into account the ever-growing population that has grossly increased since then as well as the proportionally higher number of genetic tests and diagnoses provided to patients as NGS becomes more efficient and mainstream. Recent studies have thoroughly outlined that the demand for genetic counsellors focused solely on IRDs as a subspecialty has increased greatly, in part due to the growth of knowledge in recent years of the genetic pathogenesis of IRDs, along with the large number of variants implicated in disease thus far (Black *et al.*, 2021; Alabek *et al.*, 2023; Paudel, Daly, Waters, *et al.*, 2024).

At the end of 2022, the National Strategy for Accelerating Genetic and Genomic Medicine in Ireland was published by the HSE National Genetics and Genomics Strategy Steering Group (National Strategy for Accelerating Genetic and Genomic Medicine in Ireland, Hse, 2023). Subsequently in 2023, the National Genetics and Genomics Office (NGGO) was established to drive the implementation of this strategy. This strategy has been formulated in an effort to streamline the care pathways for patients with heritable disease, where genetics and genomics services should play an integral part of the framework. The Irish genomics strategy endeavours to coordinate and expand the infrastructure that is currently in place to support enhancement of genomics services available to patients in Ireland. To do this, the strategy focuses on recruiting and educating a specialised workforce, and fostering communication between patients, researchers and healthcare professionals. Patient and Public Involvement (PPI) partnerships are vital when creating a genomics service in Ireland,

as the individuals who will avail of the services should be consulted at every step to ensure their expectations and needs are being met. Building on such pillars, it is vital that research is embedded into the national genomics strategy to facilitate the utilisation of the most up-to-date methodologies in current clinical practice. In 2021, Stephenson *et al.* described the current infrastructure and improvements to be made for IRD management in Ireland, through continued evolution and application of Target 5000 (Stephenson *et al.*, 2021). The Target 5000 project serves as a model example of collaboration between researchers and clinicians working to integrate genomics into routine medical practice in Ireland. Not only does routine genetic testing of IRD patients provide the potential of a valuable molecular diagnosis to the patient to inform their care, but it also allows research centres and laboratories to focus on innovation and broadening our understanding of the basis of disease through large-scale sequencing studies and functional analyses. It is often the case that patients will attend 3-5 different clinics/physicians before they receive a definitive genetic diagnosis, and in fact the majority of patients have indicated that it took more than 3 years to even be referred for genetic testing from when they first noticed vision problems (Paudel, Daly, Waters, *et al.*, 2024). National genomics strategies such as that driven by the NGGO in Ireland will undoubtedly allow for timely diagnoses and treatment plan implementation for patients, along with access to medical geneticists and genetic counsellors when needed. However, to achieve this, significant increases in government funding and supports will be required.

Within the *BBS7* case study chapter of this doctoral thesis (Chapter 3B), I have discussed the newly created National Genomic Test Directory ('National Genomic Test Directory Rare and Inherited Disease - Version 1', 2024). This Test Directory that has been compiled by the NGGO through collaborative efforts between clinical specialists and genomics researchers to assist with identification of the right test for the right patient at the right time to allow for early intervention/identification, as well as optimal genetic diagnosis and care management. This is welcome progress, as available genetic testing for the 'Ophthalmology' subspecialty has been included in this first version of the test directory, meaning that once a centralised National Genomic Processing Service is established that patients can receive genetic testing as part of routine healthcare. The implementation of the National Genomic Test Directory and the processing service are set to be established by the end of this year. Such services will be crucial in ending the reliance of clinics on commercial clinical testing partners such as Blueprint that are currently being used for IRD genetic testing and validation of research grade diagnoses by the Target 5000 recruitment hospitals.

6.5 Current landscape of IRD therapies and clinical trials

With the rise of gene therapies, and the increased number of clinical trials currently underway for IRDs (Hu *et al.*, 2021; Battu, Ratra and Gopal, 2022; clinicaltrials.gov, 2025), it is becoming increasingly more likely that some of these therapies may provide a realistic way forward for patient treatment, at least for a subset of IRD patients. As outlined in all Chapters of this doctoral thesis, depending on the nature of the therapy, many of these clinical trials are only open to IRD patients where the causative variants have been identified, and are classified as ‘likely pathogenic’ or ‘pathogenic’ in a particular IRD gene. This precision medicine approach to patient care underpins the benefit of having a genotypic diagnosis, as well as a comprehensive clinical evaluation including deep-phenotyping. Additionally, gene and variant agnostic therapeutic approaches are being explored and will be particularly relevant for IRDs whose genetic basis remains unresolved. As IRDs are predominantly monogenic in nature, they represent a prime candidate for gene therapy approaches that may slow the progression of disease or even restore levels of vision. A recent study found that there were 101 gene therapy clinical trials being undertaken up until the end of 2022 that targeted IRDs (Ameri, Kesavamoorthy and Bruce, 2025). Despite advancements and the number of promising trials, Luxturna™ remains the only FDA/EMA-approved gene therapy for an IRD. Luxturna™ represents a milestone in IRD treatment and was FDA approved in 2017 and EMA approved in 2018 for bi-allelic *RPE65* variants (Bennett *et al.*, 2016; Russell *et al.*, 2017). Multiple follow-up studies in different cohorts have been undertaken since then to evaluate the safety and efficacy of the drug, and analyse its impact in real-world settings (Maguire *et al.*, 2021; Deng *et al.*, 2022; Daruich *et al.*, 2025). In fact, Luxturna™ was approved only in August 2023 by the HSE for use in Ireland, with the first LCA patient treated in November 2024; a number of additional patients have been treated since then.

While gene augmentation or replacement therapies remain the most advanced and broadly applicable allowing for patients with any combination of pathogenic variants in a single gene to be treated, gene editing technologies and approaches have progressed rapidly in recent years, potentially allowing for further precise treatments to be developed. Advancements in CRISPR-Cas systems have emerged in recent years, with the use of prime and base genome editing becoming popular methods utilised as therapy approaches for IRDs showing promise in animal models and phase I/II trials (Jang *et al.*, 2022; Jo *et al.*, 2023). A serious concern for some such gene editing approaches remains the longevity of AAV vectors, which are

frequently used to deliver the therapies, since the longer the therapies remain in the body the higher the risk of off-target and immunological effects occurring (Ahmadian *et al.*, 2025).

As outlined within Chapter 5 of this thesis, RNA-based therapeutics are also showing great promise for correction of splice-altering variants at the post-transcriptional level through utilisation of AONs, with work on *ABCA4*/STGD1 disease and *CEP290*/LCA at the forefront (Collin *et al.*, 2012; Albert *et al.*, 2018; Russell *et al.*, 2022; Kaltak *et al.*, 2024). The Ulteversen therapy, which uses AONs to cause therapeutic skipping of exon 13 in *USH2A*, has shown promising results both in efficacy and safety (Yang *et al.*, 2025). The first individuals were dosed with this therapy in December of 2024 as part of Sepul Bio's Luna phase 2b clinical trial (NCT06627179). Such therapies are particularly beneficial for larger genes that cannot be packaged into AAV vectors, such as *ABCA4* and *USH2A*. To address such capacity issues, research groups and companies such as AAVantgarde Bio have developed dual-AAV technologies for large gene delivery. In their most recent press release, AAVantgarde presented positive clinical data from their AAVB-081 program for Usher 1B, which works to deliver healthy copies of the *MYO7A* gene into retinal cells using dual-AAV8 vector methodologies (AAVantgarde Bio Press Release, 2025). The first phase I/II trial patient was dosed September of 2024, with no serious drug-related adverse effects being reported to date.

So far, we have given particular attention to gene-specific therapies, as the importance of timely and accurate genetic diagnosis has been a key focus of the work described in this doctoral thesis. Due to the heterogeneity observed amongst IRDs it must be mentioned that gene-agnostic therapy options are also being explored within the research community targeting overarching molecular pathways and regulators of these pathways common to multiple IRDs rather than single disease-causing genes. One such regulator that has been analysed as a broad-spectrum therapy target is *NR2E3*. This gene is a photoreceptor-specific hormone receptor gene involved in photoreceptor differentiation and maintenance, phototransduction, inflammation, and cell homeostasis networks within the retina (Cheng *et al.*, 2004). *NR2E3* has been widely studied in mouse models of RP, displaying promising results with reduced levels of retinal degeneration observed due to improved survival of photoreceptors and retinal structure, as well as overall improved retinal homeostasis and correct genetic expression profile restoration (Li *et al.*, 2021). Multiple clinical trials at various different phases are currently focused on utilising *NR2E3* as a gene-agnostic broad-spectrum therapy for RP, LCA, STGD1 and AMD with geographic atrophy (NCT06388200, NCT05203939, NCT05956626, NCT06018558). Such therapeutic approaches appear to be

most effective if administered in early stages of disease, similar to the gene-specific approaches, as they stand a better chance of restoring cellular processes and functions before the onset of too much retinal atrophy.

In summary, gene therapies continue to transform the treatment of IRDs and ocular disorders with the future looking promising, where undoubtedly emerging advancements and novel approaches are sure to improve the sensitivity, specificity, and safety of personalised medicine. Utilising MDTs, research labs and accredited genotyping services alike can accommodate for clinical phenotyping, research level genotyping, as well as clinically accredited validation of these findings, genetic counselling for patients and families, and access to clinical trials and treatments as appropriate.

7. Concluding remarks and future directions

The field of IRDs, particularly in recent decades, has revealed a significant level of genetic heterogeneity inherent in these conditions and a great diversity of mutations implicated in disease. Great strides have been made in illuminating the genetic pathogenesis of IRDs, as described throughout this doctoral thesis. Landscape genetic sequencing studies and in vitro functional assays, such as those described within the scope of this thesis, will undoubtedly continue to broaden our knowledge and understanding of the molecular mechanisms underlying IRDs. Targeted forms of sequencing have been employed in a cohort of Irish iMD patients in an effort to illuminate some of the missing heritability identified in iMDs and to provide the first iMD Irish landscape study. While the analyses presented as part of the targeted sequencing have provided novel and valuable insights into the molecular basis of iMDs in a subset of Irish patients, we have also been able to highlight the substantial levels of heterogeneity both in the clinical and genetic presentation across the cohort. An interesting avenue for future work, beyond the scope of this PhD, would be to stratify the patients within the macular study according to a few key features such as age of onset, disease severity or even extraocular features. Previous such sequencing studies have suggested that earlier-onset and more classically clinically defined macular dystrophies are more likely to have a genetic cause, whereas later-onset or atypical presentations often remained unresolved. Therefore, it could be the case that the majority of patients we were able to resolve using the iMD-smMIPs panel all cluster within the same range for age of onset. Similarly, it would be interesting to see if the patients presenting with an age of onset closer to 55 years make up the majority of the unresolved cohort. Within the unresolved group, would we tend to see that patients with later age of onset have no candidate variants identified or potentially variation identified in AMD risk factor loci? Additionally, for patients where variants have been identified in AMD risk factor loci, clinical re-evaluation could be beneficial before sending their sample off for more comprehensive genome sequencing. A re-evaluation could reveal a misdiagnosis of earlier onset AMD, where some IRD genes mimic AMD symptoms. It would be prudent to analyse the unresolved cohort for any extraocular symptoms patients might display, that were not noted upon first clinical assessment. This could hint towards a syndrome with a retinal phenotype only occurring as a less frequent symptom of the syndrome. In such situations the macular smMIPs panel might not encapsulate genetic loci associated with such syndromes.

Applying a stratified framework based on age of onset and clinical phenotype to the Irish MD cohort could identify whether any potential trends previously seen in MD studies are also present here. It might also allow us to clarify whether current targeted sequencing and

candidate variant filtering approaches might be less effective for particular patient groups identified through this stratification, where inherited causes appear less likely. This begs the question, is it possible that discovery bias is influencing our current knowledge on the landscape of iMD and AMD, and the genetic background of both disorders? Could it be that early-onset, severe, and clearly inherited cases are preferentially studied due to volume of some monoallelic cases, reinforcing the idea of iMDs and IRDs as predominantly Mendelian disorders. Stratification by clinical parameters, incorporating late-onset macular disease, could aid in addressing potential bias, as well as distinguish between genuinely unresolved iMD cases following a Mendelian inheritance pattern and MD cases in which alternative genetic and environmental mechanisms may be operating. This further analysis could provide insights into the genetic architecture of different macular dystrophy subtypes, where early onset iMDs are typically associated with fully penetrant variants in known disease genes, and late-onset iMDs may involve hypomorphic/mild alleles, incomplete penetrance, or more complex modes of inheritance that are not readily captured by standard analysis methods. Future studies building on the MD-smMIPs project in this way could therefore integrate detailed age of onset and phenotypic stratification with expanded genetic analyses as discussed previously including; the assessment of modifier loci, variants regulatory regions and non-Mendelian forms of inheritance. Applying these approaches to clinically defined subgroups of IRDs might improve diagnostic yield and contribute to our understanding of the disease spectrum of iMD and AMD. These additional layers of analysis represent a valuable direction for future research arising from the results described within this doctoral thesis.

Novel variants have been identified and characterised, not only in the iMD cohort but within numerous IRD pedigrees in Target 5000, providing a greater understanding of the genetic pathogenesis of iMDs/IRDs and with the ultimate goal of helping to improve genetic diagnosis for IRD patients. The data presented within these studies will help to inform further variant classifications and genotype-phenotype analyses, along with adding to the global repertoire of IRD pathogenic variants. Moving forward, it is the goal to perform in-depth analyses of DNA sequences data together with family cascade studies for each Target 5000 pedigree, as well as completing additional functional studies to categorise novel variants identified. It would be of great interest to complete long-read sequencing studies on some of the patients that remain unresolved after other targeted forms of sequencing. This provides an opportunity to complete phasing analysis readily for all patients where family members are not available to participate, to investigate further into variants that are enriched in the

Irish IRD population, as well as potentially lead to the identification of complex variation in the regulatory regions of IRD genes that still remain elusive. Haplotype studies would be of great benefit with such a large country-specific IRD cohort, as haplotype analysis can add population-level and historical context that standard sequencing analyses often miss such as the identification of founder mutations and informing complex genotype-phenotype correlations. Functional studies are invaluable in our understanding of the pathogenesis of variants identified in IRDs, however further work remains to be undertaken in order for these studies to fully recapitulate the conditions and expression profile of the retina. Due to the vast number of disease-causing variants identified in over 350 IRD-associated genes, conducting comprehensive functional studies for each of them remains a significant hurdle. For the functional studies presented within this thesis focusing on the evaluation of the impact(s) of novel variants on splicing, it would be of great interest to re-run these experiments with patient-derived RNA samples where applicable, as well as utilising organoids as a model tissue to more completely represent the gene expression patterns present in the target retinal tissue. Comparing such results to those derived from the *in vitro* Midigene assays and protein modelling studies described herein would be of great value for both our understanding of the disease mechanism and the investigation of the validity of the utilised functional assay results. Indeed, high throughput functional assays have been recently developed for variants in IRD genes such as *RPE65* and *RHO* (Wan *et al.*, 2019; Pormehr *et al.*, 2025), where a greater focus on development and implementation of such functional studies will greatly aid in defining causative variants in the future.

Now as comprehensive forms of sequencing rise in popularity, such as WGS, additional resources and expertise must be invested to interpret identified candidate genes and variants in a way that is meaningful both to the field and to patients. Data sharing and open communication between research and clinical teams, and amongst global IRD scientists and clinicians, is paramount to expedite this progress. While many challenges still exist, it is evident that this is only the beginning and that there is much left to do in order to further understand the complexities of IRDs including the true involvement of known, novel and to-be-discovered genetic variants and the associated diversity of mechanisms of disease. For patients and families diagnosed with an IRD, providing a thorough genetic diagnosis in principle allows them to access appropriate clinical trials and therapies, personalised care pathways and provides greater overall quality of life with autonomy over family planning and healthcare decisions given an expanded understanding of their disease cause, prognosis and progression.

8. Supplemental materials

Chapter 3

3.1 List of all variants identified in patients considered not solved. (Records divided into 2 tables below, 1/2). Total gnomAD allele frequency is noted for each variant along with the ACMG/AMP classification as appears on Franklin and the allele count within this study.

Gene	Allele cDNA	Allele genomic	Allele protein	gnomAD-ALLAF	Franklin ACMG Classification	Allele count
ABCA4	c.589G>C	1:g.94564529C>G	p.Asp197His	0.0079543% (22)	Likely pathogenic	1
ABCA4	c.2588G>C	1:g.94517254C>G	p.Gly863Ala	0.42951% (1214)	Pathogenic	1
ABCA4	c.4139C>T	1:g.94496666G>A	p.Pro1380Leu	0.023363% (66)	Pathogenic	1
ABCA4	c.4685T>C	1:g.94487490A>G	p.Ile1562Thr	0.12482% (353)	Likely pathogenic	1
ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS	10
ABCA4	c.5929G>A	1:g.94473266C>T	p.Gly1977Ser	0.00079764% (2)	Pathogenic	1
ABCA4	c.6446G>A	1:g.94466425C>T	p.Arg2149Gln	0.0056563% (16)	Likely pathogenic	1
ABCA4	c.[4222T>C;4918C>T]	1:g.[94496583A>G; 1:g.94486896G>A]	p.[Trp1408Arg; p.Arg1640Trp]	0.0015913% (4) / 0.0031819% (8)	Likely pathogenic	1
ABCA4	c.[2588G>C;5603A>T]	1:g.[94517254C>G; 94476467T>A]	p.[Gly863Ala,Gly863del ;Asn1868Ile]	0.42951% (1214) / 4.2191% (11928)	Likely pathogenic	1
AIPL1	c.1127C>T	17:g.6328808G>A	p.(Pro376Leu)	0% (0)	VUS	1
ATF6	c.67C>T	1:g.161736217C>T	p.Leu23=	0.00079863% (2)	VUS	1
ATF6	c.742G>A	1:g.161771895G>A	p.Val248Ile	0.0049515% (14)	VUS	1
CABP4	c.695G>A	11:g.67225885G>A	p.Arg232Gln	0.00079803% (2)	VUS	1
CACNA1F	c.209G>A	X:g.49088206C>T	p.Arg70Gln	0.0027388% (5)	VUS	1
CACNA1F	c.5863G>A	X:g.49061635C>T	p.Asp1955Asn	0.0073537% (15)	VUS	1
CACNA2D4	c.98G>T	12:g.2027542C>A	p.Arg33Leu	0.0042583% (11)	VUS	1
CACNA2D4	c.2089C>T	12:g.1965241G>A	p.Arg697Trp	0.0060571% (17)	VUS	1
CACNA2D4	c.2307C>A	12:g.1955795G>T	p.Ser769Arg	0.018766% (50)	VUS	1
CACNA2D4	c.2406C>A	12:g.1953632G>T	p.Tyr802*	0.042284% (118)	Pathogenic	1
CDH3	c.247-2A>T	16:g.68712035A>T	p.?	not in gnomad?	Likely pathogenic	1
CDHR1	c.1133G>A	10:g.85968099G>A	p.Arg378Gln	0.11079% (313)	VUS	1
CDHR1	c.1894A>G	10:g.85972958A>G	p.Lys632Glu	not in gnomad?	VUS	1
CERKL	c.191G>A	2:g.182521543C>T	p.Ser64Asn	0.0081696% (22)	VUS	1
CERKL	c.769C>T	g.182423344G>A	p.(Arg257*)	0.03319% (93)	Pathogenic	1
CFAP410	c.218G>C	21:g.45753071C>G	p.Arg73Pro	0.032545% (90)	Pathogenic	1
CFAP410	c.811_814del	21:g.45750533_45750536del	p.(Asn271Argfs*5)	0.0027286% (4)	VUS	1
CNGA3	c.607T>C	2:g.99008367T>C	p.Trp203Arg	not in gnomad?	Likely pathogenic	1
CNGA3	c.667C>T	2:g.99008427C>T	p.Arg223Trp	0.0070789% (20)	Pathogenic	1
CNGB3	c.1148del	8:g.87656009del	p.Thr383IlefsTer13	0.175% (493)	Pathogenic	1
CNNM4	c.458G>A	2:g.97427194G>A	p.Arg153Gln	0% (0)	VUS	1
CNNM4	c.502T>C	2:g.97427238T>C	p.Ser168Pro	0.00039862% (1)	VUS	1
CNNM4	c.1660G>T	2:g.97463363G>T	p.Ala554Ser	0.0042514% (12)	VUS	1
CRB1	c.498_506del	1:g.197297979_197297987del	p.(Ile167_Gly169del)	0.061557% (174)	Pathogenic	1
CRB1	c.2843G>A	1:g.197403836G>A	p.(Cys948Tyr)	0.02027% (57)	Pathogenic	1
DRAM2	c.518-6T>G	1:g.111662587A>C	p.?	0.00040045% (1)	VUS	1
EYS	c.5186A>C	6:g.65300574T>G	p.Lys1729Thr	0.0045591% (7)	VUS	1
EYS	c.6952G>A	6:g.64694379C>T	p.Glu2318Lys	not in gnomad?	VUS	1
EYS	c.7723+5G>A	6:g.64497993C>T	p.?	0.0031867% (1)	VUS	1
EYS	c.9299_9302del	6:g.64430626_64430629del	p.Thr3100Lysfs*26	0.0043009% (8)	Likely pathogenic	1
FBN2	c.1571G>T	5:g.127727743C>A	p.Gly524Val	0.00079679% (2)	VUS	1
FBN2	c.3296G>A	5:g.127680124C>T	p.(Arg1099His)	0.0091932% (26)	VUS	1
FBN2	c.4631A>G	5:g.127653927T>C	p.Asn1544Ser	0.0031861% (1)	VUS	1
FBN2	c.7808T>C	5:g.127609564A>G	p.Phe2603Ser	0.0088507% (25)	Likely pathogenic	1
GUCA1A	c.557G>A	6:g.42147092G>A	p.(Gly186Asp)	not in gnomad?	VUS	1

3.1 List of all variants identified in patients considered not solved. (records continued from previous page, 2/2)

Gene	Allele cDNA	Allele genomic	Allele protein	gnomAD-ALL AF	Franklin ACMG Classification	Allele count
GUCY2D	c.49T>C	17:g.7906414T>C	p.Cys17Arg	0.0033883% (5)	VUS	1
GUCY2D	c.1297C>G	17:g.7909951C>G	p.Arg433Gly	0.0039902% (11)	VUS	1
IFT81	c.461T>C	12:g.110570383T>C	p.Leu154Ser	0.0010956% (3)	VUS	1
IFT81	c.599A>G	12:g.110573145A>G	p.Gln200Arg	0.00040051% (1)	VUS	1
IMPG2	c.270A>T	3:g.101038492T>A	p.Lys90Asn	0.0015943% (4)	VUS	1
IMPG2	c.284A>T	3:g.101038478T>A	p.(Glu95Val)	not in gnomad?	VUS	1
IMPG2	c.829-1G>T	3:g.100988418C>A	p.?	0.00079758% (2)	Pathogenic	1
IMPG2	c.1680T>G	3:g.100963495A>C	p.Tyr560Ter	not in gnomad??	Pathogenic	1
IMPG2	c.3056G>A	3:g.100951802C>T	p.Cys1019Tyr	not in gnomad?	VUS	1
IRX1	c.115T>G	5:g.3596334T>G	p.Ser39Ala	not in gnomad?	VUS	1
IRX1	c.118T>G	5:g.3596337T>G	p.Ser40Ala	not in gnomad??	VUS	1
KCNV2	c.517C>T	9:g.2718256C>T	p.Leu173Phe	0% (0)	VUS	1
KCNV2	c.1148G>T	9:g.2718887G>T	p.Arg383Leu	0.034479% (96)	VUS	1
MFRP	c.491_492insT	11:g.119216279_119216280insA	p.(Asn167Glnfs*34)	0.0028604% (8)	Pathogenic	1
MFRP	c.705C>T	11:g.119215651G>A	p.Phe235=	0.0059235% (16)	VUS	1
MFRP	c.1124+1G>T	11:g.119214525C>A	p.?	0.0060646% (17)	Pathogenic	1
MFS8	c.1205C>T	4:g.128842824G>A	p.Ser402Leu	0.0074276% (21)	VUS	1
NMNAT1	c.676A>G	g.10042595A>G	p.(Ile226Val)	not in gnomad?	VUS	1
OTX2	c.380G>A	14:g.57268967C>T	p.(Arg127Gln)	0.0063646% (18)	VUS	1
PDE6C	c.2335T>C	10:g.95422368T>C	p.Phe779Leu	not in gnomad??	VUS	1
PITPNM3	c.1257C>T	17:g.6377753G>A	p.(Asp419=)	0.0078802% (22)	VUS	1
PITPNM3	c.1878G>C	17:g.6371557C>G	p.Gln626His	0.1616% (455)	VUS	1
PRDM13	c.673G>C	6:g.100061184G>C	p.Glu225Gln	0.0065598% (13)	VUS	1
RDH12	c.284G>A	14:g.68191912G>A	p.Arg95Gln	0.0035787% (9)	VUS	1
RIMS1	c.2136T>G	6:g.72957725T>G	p.Ser712Arg	not in gnomad?	VUS	1
RIMS1	c.2872C>T	6:g.72967929C>T	p.Arg958Cys	not in gnomad?	VUS	1
RIMS1	c.3048A>C	6:g.72968809A>C	p.Gln1016His	0.0014343% (4)	VUS	1
RIMS1	c.3272C>T	6:g.72984117C>T	p.Ala1091Val	0.0010702% (3)	VUS	1
ROM1	c.793G>A	11:g.62381932G>A	p.Ala265Thr	0.027595% (78)	VUS	1
RP1L1	c.593C>T	g.10480119G>A	p.(Thr198Met)	0.0072308% (18)	VUS	1
RP1L1	c.1052C>T	8:g.10470556G>A	p.(Ala351Val)	0.00071513% (2)	VUS	1
RP1L1	c.4864C>T	8:g.10466744G>A	p.Arg162Trp	0.0070289% (17)	VUS	1
RP1L1	c.5334_5335del	8:g.10466274_10466275del	p.Asn1778LysfsTer2	0.0017795% (5)	Likely pathogenic	1
RP1L1	c.6797C>A	8:g.10464811G>T	p.Ser226Tyr	not in gnomad?	VUS	1
RP1L1	c.7203G>T	g.10464405C>A	p.(*2401Tyrext*5)	0.00040074% (1)	VUS	1
RPGRIP1	c.3367G>A	14:g.21811222G>A	p.Val449Ile	0.00040382% (1)	VUS	1
RS1	c.167C>T	X:g.18674790G>A	p.Ser56Phe	not in gnomad?	VUS	1
TTLL5	c.92T>C	14:g.76135776T>C	p.Met31Thr	0.014855% (42)	VUS	1
TTLL5	c.781C>T	14:g.76184244C>T	p.Arg261Trp	0.0092119% (26)	VUS	1
TTLL5	c.993C>T	14:g.76186168C>T	p.Thr311=	0.044631% (126)	VUS	1
TTLL5	c.1409G>A	14:g.76211846G>A	p.Arg470Gln	0.0053071% (15)	VUS	1
TTLL5	c.1912C>T	14:g.76232608C>T	p.Arg638Cys	0.01381% (39)	VUS	1
TTLL5	c.2057A>G	14:g.76238118A>G	p.His686Arg	0.0045978% (13)	VUS	1

3.2 Segregation and confirmational sanger sequencing carried out for ‘solved’ patients and participating family members. (Records divided into 3 tables below, 1/3). Variants 1-4 and the zygosity confirmed through Sanger sequencing are listed, along with family members if available. (Het = heterozygous, Het/Dominant = heterozygous for dominant-acting variant, Hom WT = homozygous for wildtype allele, Hom Mut = homozygous for candidate variant, Hemi = for X-linked conditions where male patient is found to carry variant on the X chromosome).

Status	Patient	gene	Variant 1 c.	Zygosity	Variant 2 c.	Zygosity	Variant 3 c.	Zygosity	Variant 4 c.	Zygosity
Very Likely Solved	1462	ABCA4	c.161G>A	HET	c.4222C>T	HET	c.4918C>T	HET		
Very Likely Solved	CJ13	BEST1	c.73C>T	HET / DOMINANT						
Very Likely Solved	CC33	BEST1	c.886A>C	HET / DOMINANT						
Very Likely Solved	CI51	BEST1	c.652C>T	HET / DOMINANT						
Possibly solved	CH13	CACNA1F	c.3146G>T	HET / CARRIER FEMALE						
Very Likely Solved	CC31	BEST1	c.203A>G	HET / DOMINANT						
Very Likely Solved	CI31	BEST1	c.886A>C	HET / DOMINANT						
Affected father of CI31	CI37	BEST1	c.886A>C	HET / DOMINANT						
Very Likely Solved	CF78	BEST1	c.203A>G	HET / DOMINANT						
Very Likely Solved	CA48	BEST1	c.652C>T	HET / DOMINANT						
Very Likely Solved	CI4	ABCA4	c.4540-2A>G	HET	c.5882G>A	HET				
Very Likely Solved	CC5	BEST1	c.103G>A	HET / DOMINANT						
Very Likely Solved	R68	BEST1	c.652C>T	HET / DOMINANT						
Affected mother of R68	R69	BEST1	c.652C>T	HET / DOMINANT						
Very Likely Solved	CJ25	BEST1	c.294G>T	HET / DOMINANT						
Very Likely Solved	CC78	OPN1LW	c.283del	HEMI						
Unaffected mother of CC78	CC79	OPN1LW	c.283del	HET						
Very Likely Solved	CH67	CFAP410	c.218G>C	HET	c.460_469del	HET				
Unaffected father of CH67	CH68	CFAP410	c.218G>C	HOM WT	c.460_469del	HET				
Unaffected mother of CH67	F52	CFAP410	c.218G>C	HET	c.460_469del	HOM WT				
Very Likely Solved	CJ17	OPN1LW	c.607T>C	HEMI						
Unaffected mother of CJ17	CJ18	OPN1LW	c.607T>C	HET						
Very Likely Solved	CG80	KCNV2	c.1381G>A	HOM MUT						
Affected sister of CG80	CH1	KCNV2	c.1381G>A	HOM MUT						
Unaffected sister of CG80	CH2	KCNV2	c.1381G>A	HET						
Unaffected father of CG80	CI52	KCNV2	c.1381G>A	HET						
Unaffected brother of CG80	CI53	KCNV2	c.1381G>A	HET						
Very Likely Solved	R25	CFAP410	c.355C>T	HET	c.33_34insAGCTGCACAGCGTGCA	COULDN'T SEQUENCE				
Unaffected mother of R25	R26	CFAP410	c.355C>T	HOM WT	c.33_34insAGCTGCACAGCGTGCA	COULDN'T SEQUENCE				
Unaffected father of R25	CG69	CFAP410	c.355C>T	HET	c.33_34insAGCTGCACAGCGTGCA	COULDN'T SEQUENCE				
Very Likely Solved	CI64	CRB1	c.2129A>T	HOM MUT						
Unaffected mother of CI64	CI65	CRB1	c.2129A>T	HET						
Very Likely Solved	CI26	BEST1	c.73C>T	HOM MUT						
Possibly solved	G59	PROM1	c.1339C>G	HET / DOMINANT						
Possibly solved	H72	KCNV2	c.257C>G	HET	c.1148G>T	HET				
Very Likely Solved	1666	PRPH2	c.457A>G	HET / DOMINANT						
Very Likely Solved	1688	ABCA4	c.161G>A	HET	c.4139C>T	HET				
Very Likely Solved	CG48	DRAM2	c.700_706del	HOM MUT						
Very Likely Solved	CI79	ABCA4	c.2588G>C	HET	c.3322C>T	HET	c.5603A>T	HET		

3.2 Segregation and confirmational sanger sequencing carried out for 'solved' patients and participating family members. (records continued from previous page, 2/3)

Status	Patient	gene	Variant 1 c.	Zygoty	Variant 2 c.	Zygoty	Variant 3 c.	Zygoty	Variant 4 c.	Zygoty
Very Likely Solved	CI68	CDH3	c.1570+229_2003-860del	HOM MUT						
Unaffected mother of CI68	CI67	CDH3	c.1570+229_2003-860del	HET						
Affected sister of CI68	CI69	CDH3	c.1570+229_2003-860del	HOM MUT						
Affected brother of CI68	CI70	CDH3	c.1570+229_2003-860del	HOM MUT						
Very Likely Solved	CG42	PROM1	c.1117C>T	HET / DOMINANT						
Very Likely Solved	CH14	GUCA1A	c.296A>G	HET / DOMINANT						
Possibly solved	CH71	RS1	c.327G>C	HET / CARRIER FEMALE						
Unaffected sister of CH71	CI73	RS1	c.327G>C	HOM WT						
Possibly solved	CI56	EYS	c.2822G>T	HET	c.4991C>T	HET	c.6632C>T	HET		
Very Likely Solved	CF34	CFAP410	c.218G>C	HOM MUT						
Very Likely Solved	CI18	RP1L1	c.133C>T	HET / DOMINANT						
Unaffected mother of CI18	CI19	RP1L1	c.133C>T	HET / DOMINANT						
Very Likely Solved	CD45	PRPH2	c.584G>A	HET / DOMINANT						
Affected father of CD45	CF43	PRPH2	c.584G>A	HET / DOMINANT						
Very Likely Solved	CJ31	ABCA4	c.735T>G	HET	c.2588G>C	HET	c.5603A>T	HET		
Very Likely Solved	CH50	ABCA4	c.1622T>C	HOM MUT	c.3113C>T	HOM MUT				
Very Likely Solved	CJ79	ABCA4	c.1622T>C	HOM MUT	c.3113C>T	HOM MUT				
Unaffected father of CJ79	CJ80	ABCA4	c.1622T>C	HET	c.3113C>T	HET				
Unaffected mother of CJ79	CK1	ABCA4	c.1622T>C	HET	c.3113C>T	HET				
Very Likely Solved	CH32	ABCA4	c.161G>A	HET	c.4577C>T	HET				
Unaffected mother of CH36	CH36	ABCA4	c.161G>A	HOM MUT	c.4577C>T	HET				
Very Likely Solved	CH42	ABCA4	c.5882G>A	HOM MUT						
Very Likely Solved	CG77	ABCA4	c.1805G>A	HOM MUT						
Possibly solved	CH43	ABCA4	c.2588G>C	HET	c.5584+5del	HET	c.5603A>T	HET		
Very Likely Solved	CH33	PRPH2	c.394del	HET						
Very Likely Solved	CH30	ABCA4	c.1622T>C	HET	c.3113C>T	HET	c.5882G>A	HET		
Very Likely Solved	CJ35	ABCA4	c.3323G>A	HET	c.5461-10T>C	HET	c.5603A>T	HET		
Unaffected mother of CJ35	CJ36	ABCA4	c.3323G>A	HET	c.5461-10T>C	HOM WT	c.5603A>T	HET		
Very Likely Solved	CJ47	ABCA4	c.2588G>C	HET	c.4326C>A	HET	c.5603A>T	HET		
Very Likely Solved	CH46	ABCA4	c.3323G>A	HET	c.6229C>T	HET				
Unaffected father of CH46	CH47	ABCA4	c.3323G>A	HOM WT	c.6229C>T	HET				
Unaffected mother of CH46	CH48	ABCA4	c.3323G>A	HET	c.6229C>T	HOM WT				
Very Likely Solved	CB72	NMNAT1	c.53A>G	HET	c.769G>A	HET				
Affected brother of CB72	S23	NMNAT1	c.53A>G	HET	c.769G>A	HET				
Unaffected father of CB72	CF51	NMNAT1	c.53A>G	HOM WT	c.769G>A	HET				
Very Likely Solved	CH35	BEST1	c.652C>T	HET / DOMINANT						
Very Likely Solved	CH63	PRPH2	c.903_906del	HET / DOMINANT						
Very Likely Solved	CH28	ABCA4	c.3323G>A	HOM MUT						

3.2 Segregation and confirmational sanger sequencing carried out for 'solved' patients and participating family members. (records continued from previous page, 3/3)

Status	Patient	gene	Variant 1 c.	Zygotity	Variant 2 c.	Zygotity	Variant 3 c.	Zygotity	Variant 4 c.	Zygotity
Very Likely Solved	CG35	CNGA3	c.101+1G>A	HET	c.967G>C	HET				
Possibly solved	CF76	IMPG2	c.392G>A	HET / DOMINANT						
Very Likely Solved	CA64	CNGB3	c.1148del	HOM MUT						
Unaffected father of CA64	CA65	CNGB3	c.1148del	HET						
Very Likely Solved	CC21	CNGB3	c.1148del	HOM MUT						
Affected brother of CC21	CD65	CNGB3	c.1148del	HOM MUT						
Unaffected mother of CC21	CD66	CNGB3	c.1148del	HET						
Very Likely Solved	CJ60	ABCA4	c.1253T>C	HET	c.3056C>T	HET				
Unaffected mother of CJ60	CJ61	ABCA4	c.1253T>C	HET	c.3056C>T	HOM WT				
Very Likely Solved	CJ56	CRB1	c.2639A>G	HOM MUT						
Very Likely Solved	CJ75	RP1L1	c.2107C>T	HET / DOMINANT						
Possibly solved	CJ78	ABCA4	c.872C>T	HET	c.1531C>T	HET	c.3482G>A	HET	c.5882G>A	HET
Very Likely Solved	CK2	BEST1	c.475C>T	HET / DOMINANT						
Very Likely Solved	CK6	ABCA4	c.5371dup	HET	c.5882G>A	HET				
Very Likely Solved	CK13	RDH12	c.698T>A	HET	c.869T>G	HET				
Unaffected mother of CK13	CK14	RDH12	c.698T>A	HOM WT	c.869T>G	HET				
Very Likely Solved	CK18	BEST1	c.722C>A	HET						
Possibly solved	CK68	CRB1	c.3997G>A	HET	c.2129A>T	HET				
Very likely solved	CK38	TULP1	c.1496-6C>A	HOM MUT						
Very likely solved	CK40	ABCA4	c.161G>A	HET	c.5603A>T	HOM WT	c.903_906del	HET / DOMINANT		
Very Likely Solved	CK41	ABCA4	c.161G>A	HET	c.5603A>T	HET	c.903_906del	HOM WT		
Very likely solved	CK50	CDHR1	c.783G>A	HET	c.297+1G>A	HET				
Very likely solved	CK51	ABCA4	c.1805G>A	HET	c.3322C>T	HET				
Very likely solved	CK56	KCNV2	c.1354G>C	HET	c.1381G>A	HET				
Affected brother of CK56	CK57	KCNV2	c.1354G>C	HET	c.1381G>A	HET				
Unaffected father of CK56	CL9	KCNV2	c.1354G>C	HET	c.1381G>A	HOM WT				
Unaffected mother of CK56	CL10	KCNV2	c.1354G>C	HOM WT	c.1381G>A	HET				
Unaffected brother of CK56	CL11	KCNV2	c.1354G>C	HOM WT	c.1381G>A	HOM WT				
Unaffected sister of CK56	CL12	KCNV2	c.1354G>C	HOM WT	c.1381G>A	HET				
Possibly solved	CL2	CFH	c.1291T>A	HET						
Affected mother of CL2	CL2X	CFH	c.1291T>A	HET						
Possibly solved	O14	ATF6	c.479A>G	HET	c.950G>A	HET				
Unaffected father of O14	CB15	ATF6	c.479A>G	HET	c.950G>A	HOM WT				
Unaffected mother of O14	CB16	ATF6	c.479A>G	HOM WT	c.950G>A	HET				
Possibly solved	P24	PROM1	c.2436G>A	HET						

3.3 All patients considered not solved (Records divided into 4 tables below, 1/4). Patients considered not solved are listed along with any candidate variants that were identified but that do not resolve the genetic diagnosis. (N/A = no candidate variants identified).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALL AF	Franklin ACMG (Allele 1)
1612	MD-BV	M	Unsolved	Monoallelic	ATF6	c.742G>A	1:g.161771895G>A	p.Val248Ile	0.0049515% (14)	VUS
CB59	MD-BV	M	Unsolved	Primary finding	IMPG2	c.3056G>A	3:g.100951802C>T	p.Cys1019Tyr	not in gnomad?	VUS
CB59	MD-BV	M		Monoallelic	MFSD8	c.1205C>T	4:g.128842824G>A	p.Ser402Leu	0.0074276% (21)	VUS
CI50	MD-BV	F	Unsolved	Primary finding	FBN2	c.1571G>T	5:g.127727743C>A	p.Gly524Val	0.00079679% (2)	VUS
CG31	MD-BV	F	Unsolved	N/A						
CH72	MD-BV	F	Unsolved	Monoallelic	CACNA2D4	c.2089C>T	12:g.1965241G>A	p.Arg697Trp	0.0060571% (17)	VUS
CI16	MD-BV	F	Unsolved	Monoallelic	CERKL	c.847C>T	g.182423344G>A	p.(Arg283*)	0.03319% (93)	Pathogenic
CI16	MD-BV	F		Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CJ26	MD-BV	M	Unsolved	Primary finding	FBN2	c.7808T>C	5:g.127609564A>G	p.Phe2603Ser	0.008507% (25)	Likely pathogenic
CG41	MD-BV	F	Unsolved	N/A						
CH7	MD-BV	F	Unsolved	Monoallelic	CACNA2D4	c.98G>T	12:g.2027542C>A	p.Arg33Leu	0.0042583% (11)	VUS
CD5	MD-BV	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CD11	MD-BV	M	Unsolved	Monoallelic	IMPG2	c.829-1G>T	3:g.100988418C>A	p.?	0.00079758% (2)	Pathogenic
CD11	MD-BV	M		Monoallelic	RIMS1	c.2136T>G	6:g.72957725T>G	p.Ser712Arg	not in gnomad?	VUS
CD11	MD-BV	M		Monoallelic	CNGA3	c.667C>T	2:g.99008427C>T	p.Arg223Trp	0.0070789% (20)	Pathogenic
CJ14	MD-BV	M	Unsolved	N/A						
1411	CCRD	NN	Unsolved	N/A						
1346	CCRD	NN	Unsolved	N/A						
E38	CCRD	M	Unsolved	N/A						
L11	CCRD	F	Unsolved	N/A						
G57	CCRD	M	Unsolved	N/A						
CA20	CCRD	F	Unsolved	N/A						
CG56	CCRD	F	Unsolved	N/A						
CD56	CCRD	F	Unsolved	N/A						
K34	CCRD	M	Unsolved	Monoallelic	CACNA2D4	c.2406C>A	12:g.1953632G>T	p.Tyr802Ter	0.042284% (118)	Pathogenic
G68	CCRD	M	Unsolved	Primary finding	RP1L1	c.5334_5335del	8:g.10466274_10466275del	p.Asn1778LysfsTer2	0.0017795% (5)	Likely pathogenic
CC59	CCRD	M	Unsolved	Primary finding	RP1L1	c.4864C>T	8:g.10466744G>A	p.Arg1622Trp	0.0070289% (17)	VUS
CC59	CCRD	M		Monoallelic	CERKL	c.191G>A	2:g.182521543C>T	p.Ser64Asn	0.0081696% (22)	VUS
CC55	CCRD	M	Unsolved	Primary finding	RIMS1	c.3048A>C	6:g.72968809A>C	p.Gln1016His	0.0014343% (4)	VUS
CC55	CCRD	M		Monoallelic	TTL5	c.781C>T	14:g.76184244C>T	p.Arg261Trp	0.0092119% (26)	VUS
CE42	CCRD	M	Unsolved	N/A						
CI43	CCRD	M	Unsolved	Monoallelic	CDHR1	c.1894A>G	10:g.85972958A>G	p.Lys632Glu	not in gnomad?	VUS
CI43	CCRD	M		Monoallelic	CNNM4	c.458G>A	2:g.97427194G>A	p.Arg153Gln	0% (0)	VUS
1560	CCRD	M	Unsolved	N/A						
1570	CCRD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
O33	CCRD	M	Unsolved	N/A						
O39	CCRD	F	Unsolved	N/A						
I53	CCRD	M	Unsolved	N/A						
CB39	CCRD	F	Unsolved	N/A						
R34	CCRD	F	Unsolved	N/A						

3.3 All patients considered not solved (Records continued from previous page, 2/4).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALLAF	Franklin ACMG (Allele 1)
G50	CCRD	F	Unsolved	N/A						
L20	CCRD	M	Unsolved	N/A						
CE43	CCRD	F	Unsolved	N/A						
1670	CCRD	M	Unsolved	Primary finding	FBN2	c.3296G>A	5:g.127680124C>T	p.(Arg1099His)	0.0091932% (26)	VUS/likely benign
CF8	CCRD	M	Unsolved	Primary finding	ROM1	c.793G>A	11:g.62381932G>A	p.Ala265Thr	0.027595% (78)	VUS
CD74	CCRD	M	Unsolved	Primary finding	GUCY2D	c.49T>C	17:g.7906414T>C	p.Cys17Arg	0.0033883% (5)	VUS/Likely pathogenic
CD74	CCRD	M	Unsolved	Monoallelic	CFAP410	c.218G>C	21:g.45753071C>G	p.Arg73Pro	0.082545% (90)	Pathogenic
CE29	CCRD	F	Unsolved	N/A						
1349	CCRD	F	Unsolved	Primary finding	IRX1	c.115T>G	5:g.3596334T>G	p.Ser39Ala	not in gnomad?	VUS
1349	CCRD	F	Unsolved	Monoallelic	CNNM4	c.1660G>T	2:g.97463363G>T	p.Ala554Ser	0.0042514% (12)	VUS
O4	CCRD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CE52	STGD	F	Unsolved	Monoallelic	RPGRI1	c.3367G>A	14:g.21811222G>A	p.Val449Ile	0.00040382% (1)	VUS
CE59	MD	F	Unsolved	N/A						
CI57	STGD	F	Unsolved	Primary finding	RP1L1	c.6797C>A	8:g.10464811G>T	p.Ser2266Tyr	not in gnomad?	VUS
G33	STGD	M	Unsolved	Monoallelic	ABCA4	c.2588G>C	1:g.94517254C>G	p.Gly863Ala	0.42951% (1214)	Pathogenic
CB25	MD	F	Unsolved	N/A						
CD62	MD	M	Unsolved	Monoallelic	CDH3	c.2280+8G>A	16:g.68729834G>A	p.?	0.0019938% (5)	VUS
1413	MD	NN	Unsolved	N/A						
1477	MD	F	Unsolved	N/A						
1508	MD	M	Unsolved	N/A						
1342	MD	NN	Failed	Failed						
1405	MD	NN	Unsolved	Monoallelic	RP1L1	c.7203G>T	g.10464405C>A	p.(*2401Tyrxt *5)	0.00040074% (1)	VUS
CA80	MD	F	Unsolved	N/A						
S49	MD	F	Unsolved	Monoallelic	CNNM4	c.502T>C	2:g.97427238T>C	p.Ser168Pro	0.00039862% (1)	VUS
S64	MD	M	Unsolved	N/A						
CA10	MD	M	Unsolved	N/A						
I31	MD	F	Unsolved	Monoallelic	IFT81	c.461T>C	12:g.110570383T>C	p.Leu154Ser	0.0010956% (3)	VUS
O42	MD	F	Unsolved	Primary finding	RIMS1	c.3272C>T	6:g.72984117C>T	p.Ala1091Val	0.0010702% (3)	VUS
F61	MD	M	Unsolved	N/A						
154	MD	M	Unsolved	Monoallelic	PITPNM3	c.1878G>C	17:g.6371557C>G	p.Gln626His	0.1616% (455)	VUS
154	MD	M	Unsolved	Monoallelic	ATF6	c.67C>T	1:g.161736217C>T	p.Leu23=	0.00079863% (2)	VUS
1495	MD	M	Unsolved	N/A						
CF80	MD	F	Unsolved	Monoallelic	RS1	c.167C>T	X:g.18674790G>A	p.Ser56Phe	not in gnomad?	VUS
1574	MD	M	Unsolved	Monoallelic	TTLL5	c.1912C>T	14:g.76232608C>T	p.Arg638Cys	0.01381% (39)	VUS
1574	MD	M	Unsolved	Monoallelic	EYS	c.7723+5G>A	6:g.64497993C>T	p.?	0.0031867% (1)	VUS
1584	MD	F	Unsolved	Monoallelic	CNGA3	c.607T>C	2:g.99008367T>C	p.Trp203Arg	not in gnomad?	Likely pathogenic
1584	MD	F	Unsolved	Monoallelic	TTLL5	c.2057A>G	14:g.76238118A>G	p.His686Arg	0.0045978% (13)	VUS
1584	MD	F	Unsolved	Monoallelic	CACNA1F	c.5863G>A	X:g.49061635C>T	p.Asp1955Asn	0.0073537% (15)	VUS
1686	MD	F	Unsolved	Primary finding	GUCY2D	c.2318T>C	17:g.7917252T>C	p.Met773Thr	0.018745% (53)	Likely benign
CI45	MD	M	Unsolved	Primary finding	GUCA1A	c.557G>A	6:g.42147092G>A	p.(Gly186Asp)	not in gnomad?	VUS

3.3 All patients considered not solved (Records continued from previous page, 3/4).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALLAF	Franklin ACMG (Allele 1)
CG55	MD	F	Unsolved	N/A						
CG79	MD	F	Unsolved	N/A						
CJ23	MD	F	Unsolved	Monoallelic	CFAP410	c.811_814del	21:g.45750533_45750536del	p.(Asn271Argfs*5)	0.0027286% (4)	Likely pathogenic
R70	MD	F	Unsolved	N/A						
CF32	MD	F	Unsolved	Monoallelic	IMPG2	c.270A>T	3:g.101038492T>A	p.Lys90Asn	0.0015943% (4)	VUS
CJ7	MD	M	Unsolved	Monoallelic	MFRP	c.1124+1G>T	11:g.119214525C>A	p.?	0.0060646% (17)	Pathogenic
CE78	MD	M	Unsolved	N/A						
CE16	MD	M	Unsolved	Primary finding	GUCY2D	c.1297C>G	17:g.7909951C>G	p.Arg433Gly	0.0039902% (11)	VUS
CH45	MD	F	Unsolved	Monoallelic	CDH3	c.247-2A>T	16:g.68712035A>T	p.?	not in gnomad?	Likely pathogenic
CJ10	MD	F	Unsolved	Primary finding	RP111	c.593C>T	g.10480119G>A	p.(Thr198Met)	0.0072308% (18)	VUS
CJ10	MD	F		Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CJ10	MD	F		Monoallelic	IMPG2	c.284A>T	3:g.101038478T>A	p.(Glu95Val)	not in gnomad?	VUS
CF39	MD	F	Unsolved	Monoallelic	EYS	c.6952G>A	6:g.64694379C>T	p.Glu2318Lys	not in gnomad?	VUS
CF39	MD	F		Monoallelic	IFT81	c.599A>G	12:g.110573145A>G	p.Gln200Arg	0.00040051% (1)	VUS
CE36	MD	M	Unsolved	Primary finding	RIMS1	c.2872C>T	6:g.72967929C>T	p.Arg958Cys	not in gnomad?	VUS
CG64	MD	F	Unsolved	Monoallelic	CRB1	c.498_506del	1:g.197297979_197297987del	p.(Ile167_Gly169del)	0.061557% (174)	Pathogenic
CC15	MD	M	Unsolved	N/A						
CJ40	MD	F	Unsolved	Monoallelic	CDHR1	c.1133G>A	10:g.85968099G>A	p.Arg378Gln	0.11079% (313)	VUS
CC14	MD	F	Unsolved	Monoallelic	TTL5	c.933C>T	14:g.76186168C>T	p.Thr311=	0.044631% (126)	VUS
CD72	MD	M	Unsolved	N/A						
CJ44	MD	F	Unsolved	N/A						
CD31	MD	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CD73	MD	F	Unsolved	N/A						
CF71	MD	F	Unsolved	Monoallelic	EYS	c.5186A>C	6:g.65300574T>G	p.Lys1729Thr	0.0045591% (7)	VUS
CD40	MD	F	Unsolved	Monoallelic	ABCA4	c.4685T>C	1:g.94487490A>G	p.Ile1562Thr	0.12482% (353)	Pathogenic
CC69	MD	F	Unsolved	N/A						
CH6	MD	F	Unsolved	Monoallelic	ABCA4	c.6446G>A	1:g.94466425C>T	p.Arg2149Gln	0.0056563% (16)	Likely pathogenic
CG33	MD	M	Unsolved	Primary finding	CACNA1F	c.209G>A	X:g.49088206C>T	p.Arg70Gln	0.0027388% (5)	VUS
CG51	MD	M	Unsolved	N/A						
CA19	MD	M	Unsolved	N/A						
CE57	MD	M	Unsolved	Monoallelic	TTL5	c.1409G>A	14:g.76211846G>A	p.Arg470Gln	0.0053071% (15)	VUS
CE58	MD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CJ74	MD	M	Unsolved	Primary finding	FBN2	c.4631A>G	5:g.127653927T>C	p.Asn1544Ser	0.0031861% (1)	VUS
CF19	MD	M	Unsolved	N/A						
CE49	MD	F	Unsolved	Primary finding	PRDM13	c.673G>C	6:g.100061184G>C	p.Glu225Gln	0.0065598% (13)	VUS
CH27	MD	M	Unsolved	Monoallelic	DRAM2	c.518-6T>G	1:g.111662587A>C	p.?	0.00040045% (1)	VUS
CF38	MD	F	Unsolved	Monoallelic	KCNV2	c.1148G>T	9:g.2718887G>T	p.Arg383Leu	0.034479% (96)	VUS
CC63	MD	F	Unsolved	Monoallelic	CACNA2D4	c.2307C>A	12:g.1955795G>T	p.Ser769Arg	0.018766% (50)	VUS
CJ6	MD	F	Unsolved	N/A						

3.3 All patients considered not solved (Records continued from previous page, 4/4).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele 1 cDNA	Allele 1 genomic	Allele 1 protein	gnomAD-ALL AF	Franklin ACMG (Allele 1)
Ci20	MD	M	Unsolved	N/A						
1559	MD	F	Unsolved	Monoallelic	CRB1	c.2843G>A	1:g.197403836G>A	p.(Cys948Tyr)	0.02027% (57)	Pathogenic
S42	MD	M	Failed	Failed						
1321	STGD	NN	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
H32	STGD	F	Unsolved	Primary finding	RP1L1	c.1052C>T	8:g.10470556G>A	p.(Ala351Val)	0.00071513% (2)	VUS
CF45	STGD	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CG68	STGD	M	Unsolved	N/A						
P47	STGD	F	Unsolved	Monoallelic	ABCA4	c.[4222T>C;4918C>T]	1:g.[94496583A>G; 1:g.94486896G>A]	p.[Trp1408Arg; p.Arg1640Trp]	0.0015913% (4) / 0.0031819% (8)	Pathogenic
R14	STGD	M	Unsolved	Monoallelic	ABCA4	c.[2588G>C;5603A>T]	1:g.[94517254C>G; 94476467T>A]	p.[Gly863Ala; Gly863del; Asn1868Ile]	0.42951% (1214) / 4.2191% (11928)	Pathogenic
1475	STGD	F	Unsolved	N/A						
CG45	MD-BV	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	4.2191% (11928)	VUS
CG45	MD-BV			Monoallelic	TTL5	c.92T>C	14:g.76135776T>C	p.Met31Thr	0.014855% (42)	VUS
CH19	ACH	NN	Unsolved	Monoallelic	OTX2	c.380G>A	14:g.57268967C>T	p.(Arg127Gln)	0.0063646% (18)	VUS
CH19	ACH	NN		Monoallelic	AIPL1	c.1127C>T	17:g.6328808G>A	p.(Pro376Leu)	not in gnomad??	VUS
CJ59	MD	F	Unsolved	Monoallelic	NMNAT1	c.676A>G	g.10042595A>G	p.(Ile226Val)	not in gnomad?	VUS/Likely pathogenic
CJ66	CCRD	M	Unsolved	N/A						
CK7	STGD	M	Unsolved	Monoallelic	CFB	c.1625-6C>A	6:g.31918390C>A	p.(?)	0.0016233% (4)	VUS
CK12	MD-BV	F	Unsolved	Monoallelic	MFRP	c.491_492insT	11:g.119216279_119216280insA	p.(Asn167Glnfs*34)	0.0028604% (8)	Pathogenic
CK19	MD	F	Unsolved	Monoallelic	ABCA4	c.4139C>T	1:g.94496666G>A	p.Pro1380Leu	0.023363% (66)	Pathogenic
CK20	MD	M	Unsolved	N/A						
CK35	MD	M	Unsolved	N/A						
CK36	MD	F	Unsolved	Monoallelic	PDE6C	c.2335T>C	10:g.95422368T>C	p.Phe779Leu	not in gnomad??	VUS
CK37	MD	M	Unsolved	N/A						
CK39	MD	F	Unsolved	N/A						
CK46	MD	M	Unsolved	N/A						
CK47	MD	M	Unsolved	Primary finding	IRX1	c.118T>G	5:g.3596337T>G	p.Ser40Ala	not in gnomad??	VUS
CK52	MD	F	Unsolved	N/A						
CK62	CCRD	M	Unsolved	N/A						
CK65	MD	M	Unsolved	Monoallelic	IMPG2	c.1680T>G	3:g.100963495A>C	p.Tyr560Ter	not in gnomad??	Likely pathogenic
CK66	MD	M	Unsolved	Monoallelic	IMPG1	c.1796G>A	6:g.76660307C>T	p.Arg599Gln	0.0028585% (8)	VUS
CK29	MD	F	Unsolved	Monoallelic	RDH12	c.284G>A	14:g.68191912G>A	p.Arg95Gln	0.0035787% (9)	VUS
CK71	MD	M	Unsolved	Primary finding	MFRP	c.705C>T	11:g.119215651G>A	p.Phe235=	0.0059235% (16)	VUS
CK75	MD	M	Unsolved	N/A						
CK76	MD	F	Unsolved	N/A						
CK79	MD	F	Unsolved	Primary finding	EYS	c.9299_9302del	6:g.64430626_64430629del	p.Thr310Lysfs*26	0.0043009% (8)	Likely pathogenic
CK79	MD	F	Unsolved	Primary finding	EYS	c.6079-8_6079-3del	6:g.65016994_65016999del	p.?	0.11721% (144)	VUS
CK80	MD	F	Unsolved	Monoallelic	CNGB3	c.1148del	8:g.87656009del	p.Thr383IlefsTer13	0.175% (493)	Pathogenic
CK80	MD	F	Unsolved	Monoallelic	KCNV2	c.517C>T	9:g.2718256C>T	p.Leu173Phe	0% (0)	VUS
G39	CCRD	F	Unsolved	Primary finding	PITPNM3	c.1257C>T	17:g.6377753G>A	p.(Asp419=)	0.0078802% (22)	VUS
G27	STGD	F	Unsolved	Monoallelic	ABCA4	c.589G>C	1:g.94564529C>G	p.Asp197His	0.0079543% (22)	Likely pathogenic
H67	CCRD	F	Unsolved	N/A						
G18	STGD	F	Unsolved	N/A						
F67	ACH	M	Unsolved	N/A						
CJ64	STGD	F	Unsolved	Monoallelic	ABCA4	c.5929G>A	1:g.94473266C>T	p.Gly1977Ser	0.00079764% (2)	Likely pathogenic
CG7	STGD	M	Unsolved	Monoallelic	CABP4	c.695G>A	11:g.67225885G>A	p.Arg232Gln	0.00079803% (2)	VUS

3.4 Patients considered not solved, with mono-allelic variants identified in a single gene (Records divided into 2 tables below, 1/2). (Variant label; Primary finding = variant identified in a gene associated with multiple inheritance patterns in IRDs but remains unconvincing cause given available evidence, Monoallelic = single variant identified in a gene associated with autosomal recessive disease.)

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele cDNA	Allele genomic	Allele protein	Franklin ACMG
1612	MD-BV	M	Unsolved	Monoallelic	ATF6	c.742G>A	1:g.161771895G>A	p.Val248Ile	VUS
CI50	MD-BV	F	Unsolved	Primary finding	FBN2	c.1571G>T	5:g.127727743C>A	p.Gly524Val	VUS
CH72	MD-BV	F	Unsolved	Monoallelic	CACNA2D4	c.2089C>T	12:g.1965241G>A	p.Arg697Trp	VUS
CJ26	MD-BV	M	Unsolved	Primary finding	FBN2	c.7808T>C	5:g.127609564A>G	p.Phe2603Ser	Likely pathogenic
CH7	MD-BV	F	Unsolved	Monoallelic	CACNA2D4	c.98G>T	12:g.2027542C>A	p.Arg33Leu	VUS
CD5	MD-BV	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
K34	CCRD	M	Unsolved	Monoallelic	CACNA2D4	c.2406C>A	12:g.1953632G>T	p.Tyr802Ter	Pathogenic
G68	CCRD	M	Unsolved	Primary finding	RP1L1	c.5334_5335del	8:g.10466274_10466275del	p.Asn1778LysfsTer2	Likely pathogenic
1570	CCRD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
1670	CCRD	M	Unsolved	Primary finding	FBN2	c.3296G>A	5:g.127680124C>T	p.(Arg1099His)	VUS
CF8	CCRD	M	Unsolved	Primary finding	ROM1	c.793G>A	11:g.62381932G>A	p.Ala265Thr	VUS
O4	CCRD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CE52	STGD	F	Unsolved	Monoallelic	RPGRIP1	c.3367G>A	14:g.21811222G>A	p.Val449Ile	VUS
CI57	STGD	F	Unsolved	Primary finding	RP1L1	c.6797C>A	8:g.10464811G>T	p.Ser2266Tyr	VUS
G33	STGD	M	Unsolved	Monoallelic	ABCA4	c.2588G>C	1:g.94517254C>G	p.Gly863Ala	Pathogenic
1405	MD	NN	Unsolved	Monoallelic	RP1L1	c.7203G>T	g.10464405C>A	p.(*2401Tyrext*5)	VUS
S49	MD	F	Unsolved	Monoallelic	CNNM4	c.502T>C	2:g.97427238T>C	p.Ser168Pro	VUS
I31	MD	F	Unsolved	Monoallelic	IFT81	c.461T>C	12:g.110570383T>C	p.Leu154Ser	VUS
O42	MD	F	Unsolved	Primary finding	RIMS1	c.3272C>T	6:g.72984117C>T	p.Ala1091Val	VUS
CF80	MD	F	Unsolved	Monoallelic	RS1	c.167C>T	X:g.18674790G>A	p.Ser56Phe	VUS
CI45	MD	M	Unsolved	Primary finding	GUCA1A	c.557G>A	6:g.42147092G>A	p.(Gly186Asp)	VUS
CJ23	MD	F	Unsolved	Monoallelic	CFAP410	c.811_814del	21:g.45750533_45750536del	p.(Asn271Argfs*5)	Likely pathogenic
CF32	MD	F	Unsolved	Monoallelic	IMPG2	c.270A>T	3:g.101038492T>A	p.Lys90Asn	VUS
CJ7	MD	M	Unsolved	Monoallelic	MFRP	c.1124+1G>T	11:g.119214525C>A	p.?	Pathogenic
CE16	MD	M	Unsolved	Primary finding	GUCY2D	c.1297C>G	17:g.7909951C>G	p.Arg433Gly	VUS
CH45	MD	F	Unsolved	Monoallelic	CDH3	c.247-2A>T	16:g.68712035A>T	p.?	VUS/Likely pathogenic
CE36	MD	M	Unsolved	Primary finding	RIMS1	c.2872C>T	6:g.72967929C>T	p.Arg958Cys	VUS
CG64	MD	F	Unsolved	Monoallelic	CRB1	c.498_506del	1:g.197297979_197297987del	p.(Ile167_Gly169del)	Pathogenic
CJ40	MD	F	Unsolved	Monoallelic	CDHR1	c.1133G>A	10:g.85968099G>A	p.Arg378Gln	VUS
CC14	MD	F	Unsolved	Monoallelic	TTLL5	c.933C>T	14:g.76186168C>T	p.Thr311=	VUS

3.4 Patients considered not solved, with mono-allelic variants identified in a single gene (Records continued from previous page, 2/2).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele cDNA	Allele genomic	Allele protein	Franklin ACMG
CD31	MD	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CF71	MD	F	Unsolved	Monoallelic	EYS	c.5186A>C	6:g.65300574T>G	p.Lys1729Thr	VUS
CD40	MD	F	Unsolved	Monoallelic	ABCA4	c.4685T>C	1:g.94487490A>G	p.Ile1562Thr	Pathogenic
CH6	MD	F	Unsolved	Monoallelic	ABCA4	c.6446G>A	1:g.94466425C>T	p.Arg2149Gln	Likely pathogenic
CG33	MD	M	Unsolved	Primary finding	CACNA1F	c.209G>A	X:g.49088206C>T	p.Arg70Gln	VUS
CE57	MD	M	Unsolved	Monoallelic	TTL5	c.1409G>A	14:g.76211846G>A	p.Arg470Gln	VUS
CE58	MD	F	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CI74	MD	M	Unsolved	Primary finding	FBN2	c.4631A>G	5:g.127653927T>C	p.Asn1544Ser	VUS
CE49	MD	F	Unsolved	Primary finding	PRDM13	c.673G>C	6:g.100061184G>C	p.Glu225Gln	VUS
CH27	MD	M	Unsolved	Monoallelic	DRAM2	c.518-6T>G	1:g.111662587A>C	p.?	VUS
CF38	MD	F	Unsolved	Monoallelic	KCNV2	c.1148G>T	9:g.2718887G>T	p.Arg383Leu	VUS
CC63	MD	F	Unsolved	Monoallelic	CACNA2D4	c.2307C>A	12:g.1955795G>T	p.Ser769Arg	VUS
1559	MD	F	Unsolved	Monoallelic	CRB1	c.2843G>A	1:g.197403836G>A	p.(Cys948Tyr)	Pathogenic
1321	STGD	NN	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
H32	STGD	F	Unsolved	Primary finding	RP1L1	c.1052C>T	8:g.10470556G>A	p.(Ala351Val)	VUS
CF45	STGD	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
P47	STGD	F	Unsolved	Monoallelic	ABCA4	c.[4222T>C;4918C>T]	1:g.[94496583A>G; 1:g.94486896G>A]	p.[Trp1408Arg; p.Arg1640Trp]	Pathogenic
R14	STGD	M	Unsolved	Monoallelic	ABCA4	c.[2588G>C;5603A>T]	1:g.[94517254C>G; 94476467T>A]	p.[Gly863Ala;Gly863del;Asn1868Ile]	Pathogenic
CJ59	MD	F	Unsolved	Monoallelic	NMNAT1	c.676A>G	g.10042595A>G	p.(Ile226Val)	VUS/Likely pathogenic
CK12	MD-BV	F	Unsolved	Monoallelic	MFRP	c.491_492insT	11:g.119216279_119216280insA	p.(Asn167Glnfs*34)	Pathogenic
CK19	MD	F	Unsolved	Monoallelic	ABCA4	c.4139C>T	1:g.94496666G>A	p.Pro1380Leu	Pathogenic
CK36	MD	F	Unsolved	Monoallelic	PDE6C	c.2335T>C	10:g.95422368T>C	p.Phe779Leu	VUS
CK47	MD	M	Unsolved	Primary finding	IRX1	c.118T>G	5:g.3596337T>G	p.Ser40Ala	VUS
CK65	MD	M	Unsolved	Monoallelic	IMPG2	c.1680T>G	3:g.100963495A>C	p.Tyr560Ter	Likely pathogenic
CK29	MD	F	Unsolved	Monoallelic	RDH12	c.284G>A	14:g.68191912G>A	p.Arg95Gln	VUS
CK71	MD	M	Unsolved	Primary finding	MFRP	c.705C>T	11:g.119215651G>A	p.Phe235=	VUS
G39	CCRD	F	Unsolved	Primary finding	PITPNM3	c.1257C>T	17:g.6377753G>A	p.(Asp419=)	VUS
G27	STGD	F	Unsolved	Monoallelic	ABCA4	c.589G>C	1:g.94564529C>G	p.Asp197His	Likely pathogenic
CJ64	STGD	F	Unsolved	Monoallelic	ABCA4	c.5929G>A	1:g.94473266C>T	p.Gly1977Ser	Likely pathogenic
CG7	STGD	M	Unsolved	Monoallelic	CABP4	c.695G>A	11:g.67225885G>A	p.Arg232Gln	VUS

3.5 Patients considered not solved, with mono-allelic variants identified in multiple genes
(Variant label; Primary finding = variant identified in a gene associated with multiple inheritance patterns in IRDs but remains unconvincing cause given available evidence, Monoallelic = single variant identified in a gene associated with autosomal recessive disease).

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label	Gene	Allele cDNA	Allele genomic	Allele protein	Franklin ACMG
CB59	MD-BV	M	Unsolved	Primary finding	IMPG2	c.3056G>A	3:g.100951802C>T	p.Cys1019Tyr	VUS
CB59	MD-BV	M		Monoallelic	MFS2D8	c.1205C>T	4:g.128842824G>A	p.Ser402Leu	VUS
CI16	MD-BV	F	Unsolved	Monoallelic	CERKL	c.847C>T	g.182423344G>A	p.(Arg283*)	Pathogenic
CI16	MD-BV	F		Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CD11	MD-BV	M	Unsolved	Monoallelic	IMPG2	c.829-1G>T	3:g.100988418C>A	p.?	Pathogenic
CD11	MD-BV	M		Monoallelic	RIMS1	c.2136T>G	6:g.72957725T>G	p.Ser712Arg	VUS
CD11	MD-BV	M		Monoallelic	CNGA3	c.667C>T	2:g.99008427C>T	p.Arg223Trp	Pathogenic
CC59	CCRD	M	Unsolved	Primary finding	RP1L1	c.4864C>T	8:g.10466744G>A	p.Arg1622Trp	VUS
CC59	CCRD	M		Monoallelic	CERKL	c.191G>A	2:g.182521543C>T	p.Ser64Asn	VUS
CC55	CCRD	M	Unsolved	Primary finding	RIMS1	c.3048A>C	6:g.72968809A>C	p.Gln1016His	VUS
CC55	CCRD	M		Monoallelic	TLL5	c.781C>T	14:g.76184244C>T	p.Arg261Trp	VUS
CI43	CCRD	M	Unsolved	Monoallelic	CDHR1	c.1894A>G	10:g.85972958A>G	p.Lys632Glu	VUS
CI43	CCRD	M		Monoallelic	CNNM4	c.458G>A	2:g.97427194G>A	p.Arg153Gln	VUS
CD74	CCRD	M	Unsolved	Primary finding	GUCY2D	c.49T>C	17:g.7906414T>C	p.Cys17Arg	VUS/Likely pathogenic
CD74	CCRD	M		Monoallelic	CFAP410	c.218G>C	21:g.45753071C>G	p.Arg73Pro	Pathogenic
1349	CCRD	F	Unsolved	Primary finding	IRX1	c.115T>G	5:g.3596334T>G	p.Ser39Ala	VUS
1349	CCRD	F		Monoallelic	CNNM4	c.1660G>T	2:g.97463363G>T	p.Ala554Ser	VUS
I54	MD	M	Unsolved	Monoallelic	PITPNM3	c.1878G>C	17:g.6371557C>G	p.Gln626His	VUS
I54	MD	M		Monoallelic	ATF6	c.67C>T	1:g.161736217C>T	p.Leu23=	VUS
1574	MD	M	Unsolved	Monoallelic	TLL5	c.1912C>T	14:g.76232608C>T	p.Arg638Cys	VUS
1574	MD	M		Monoallelic	EYS	c.7723+5G>A	6:g.64497993C>T	p.?	VUS
1584	MD	F	Unsolved	Monoallelic	CNGA3	c.607T>C	2:g.99008367T>C	p.Trp203Arg	Likely pathogenic
1584	MD	F		Monoallelic	TLL5	c.2057A>G	14:g.76238118A>G	p.His686Arg	VUS
1584	MD	F		Monoallelic	CACNA1F	c.5863G>A	X:g.49061635C>T	p.Asp1955Asn	VUS
CJ10	MD	F	Unsolved	Primary finding	RP1L1	c.593C>T	g.10480119G>A	p.(Thr198Met)	VUS
CJ10	MD	F		Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CJ10	MD	F		Monoallelic	IMPG2	c.284A>T	3:g.101038478T>A	p.(Glu95Val)	VUS
CF39	MD	F	Unsolved	Monoallelic	EYS	c.6952G>A	6:g.64694379C>T	p.Glu2318Lys	VUS
CF39	MD	F		Monoallelic	IFT81	c.599A>G	12:g.110573145A>G	p.Gln200Arg	VUS
CG45	MD-BV	M	Unsolved	Monoallelic	ABCA4	c.5603A>T	1:g.94476467T>A	p.Asn1868Ile	VUS
CG45	MD-BV			Monoallelic	TLL5	c.92T>C	14:g.76135776T>C	p.Met31Thr	VUS
CH19	ACH	NN	Unsolved	Monoallelic	OTX2	c.380G>A	14:g.57268967C>T	p.(Arg127Gln)	VUS
CH19	ACH	NN		Monoallelic	AIPL1	c.1127C>T	17:g.6328808G>A	p.(Pro376Leu)	VUS
CK79	MD	F	Unsolved	Primary finding	EYS	c.6079-8_6079-3del	6:g.65016994_65016999del	p.?	VUS
CK79	MD	F		Primary finding	EYS	c.9299_9302del	6:g.64430626_64430629del	p.Thr3100Lysfs*26	Likely pathogenic
CK80	MD	F	Unsolved	Monoallelic	CNGB3	c.1148del	8:g.87656009del	p.Thr383IlefsTer13	Pathogenic
CK80	MD	F		Monoallelic	KCNV2	c.517C>T	9:g.2718256C>T	p.Leu173Phe	VUS

3.6 Unresolved patients with no candidate variants identified

Patient Barcode	Submitted Phenotype	Gender	Status	Variant Label
CG31	MD-BV	F	Unsolved	N/A
CG41	MD-BV	F	Unsolved	N/A
CJ14	MD-BV	M	Unsolved	N/A
1411	CCRD	NN	Unsolved	N/A
1346	CCRD	NN	Unsolved	N/A
E38	CCRD	M	Unsolved	N/A
L11	CCRD	F	Unsolved	N/A
G57	CCRD	M	Unsolved	N/A
CA20	CCRD	F	Unsolved	N/A
CG56	CCRD	F	Unsolved	N/A
CD56	CCRD	F	Unsolved	N/A
CE42	CCRD	M	Unsolved	N/A
1560	CCRD	M	Unsolved	N/A
O33	CCRD	M	Unsolved	N/A
O39	CCRD	F	Unsolved	N/A
I53	CCRD	M	Unsolved	N/A
CB39	CCRD	F	Unsolved	N/A
R34	CCRD	F	Unsolved	N/A
G50	CCRD	F	Unsolved	N/A
L20	CCRD	M	Unsolved	N/A
CE43	CCRD	F	Unsolved	N/A
CE29	CCRD	F	Unsolved	N/A
CE59	MD	F	Unsolved	N/A
CB25	MD	F	Unsolved	N/A
CD62	MD	M	Unsolved	N/A
1413	MD	NN	Unsolved	N/A
1477	MD	F	Unsolved	N/A
1508	MD	M	Unsolved	N/A
CA80	MD	F	Unsolved	N/A
S64	MD	M	Unsolved	N/A
CA10	MD	M	Unsolved	N/A
F61	MD	M	Unsolved	N/A
1495	MD	M	Unsolved	N/A
1686	MD	F	Unsolved	N/A
CG55	MD	F	Unsolved	N/A
CG79	MD	F	Unsolved	N/A
R70	MD	F	Unsolved	N/A
CE78	MD	M	Unsolved	N/A
CC15	MD	M	Unsolved	N/A
CD72	MD	M	Unsolved	N/A
CJ44	MD	F	Unsolved	N/A
CD73	MD	F	Unsolved	N/A
CC69	MD	F	Unsolved	N/A
CG51	MD	M	Unsolved	N/A
CA19	MD	M	Unsolved	N/A
CF19	MD	M	Unsolved	N/A
CJ6	MD	F	Unsolved	N/A
CI20	MD	M	Unsolved	N/A
CG68	STGD	M	Unsolved	N/A
1475	STGD	F	Unsolved	N/A
CJ66	CCRD	M	Unsolved	N/A
CK7	STGD	M	Unsolved	N/A
CK20	MD	M	Unsolved	N/A
CK35	MD	M	Unsolved	N/A
CK37	MD	M	Unsolved	N/A
CK39	MD	F	Unsolved	N/A
CK46	MD	M	Unsolved	N/A
CK52	MD	F	Unsolved	N/A
CK62	CCRD	M	Unsolved	N/A
CK66	MD	M	Unsolved	N/A
CK75	MD	M	Unsolved	N/A
CK76	MD	F	Unsolved	N/A
H67	CCRD	F	Unsolved	N/A
G18	STGD	F	Unsolved	N/A
F67	ACH	M	Unsolved	N/A

3.7 Previous screening methods for 46 patients. (T5K = Target 5000 target capture panel of 254 IRD genes).

Patient Barcode	Submitted Phenotype	Gender	Form of prescreening	Status
1411	CD	NN	T5K 254 gene panel	Unsolved
1346	CD	NN	T5K 254 gene panel	Unsolved
E38	CD	M	T5K 254 gene panel	Unsolved
L11	CD	F	T5K 254 gene panel	Unsolved
G57	CD-AR	M	T5K 254 gene panel	Unsolved
CA20	CD	F	T5K 254 gene panel	Unsolved
1560	CRD	M	T5K 254 gene panel	Unsolved
1570	CRD	F	T5K 254 gene panel	Unsolved
O33	CRD	M	T5K 254 gene panel	Unsolved
O39	CRD	F	T5K 254 gene panel	Unsolved
I53	CRD	M	T5K 254 gene panel	Unsolved
CB39	CRD	F	T5K 254 gene panel	Unsolved
R34	CRD	F	T5K 254 gene panel	Unsolved
G50	CRD	F	T5K 254 gene panel	Unsolved
L20	CRD	M	T5K 254 gene panel	Unsolved
1349	CRD	F	T5K 254 gene panel	Unsolved
O4	CRD	F	T5K 254 gene panel	Unsolved
G33	FFM	M	T5K 254 gene panel	Unsolved
CB25	GMA	F	T5K 254 gene panel	Unsolved
1413	MD	NN	T5K 254 gene panel	Unsolved
1508	MD	M	T5K 254 gene panel	Unsolved
CA80	MD	F	T5K 254 gene panel	Unsolved
S49	MD	F	T5K 254 gene panel	Unsolved
S64	MD	M	T5K 254 gene panel	Unsolved
CA10	MD	M	T5K 254 gene panel	Unsolved
I31	MD	F	T5K 254 gene panel	Unsolved
O42	MD	F	T5K 254 gene panel	Unsolved
F61	MD	M	T5K 254 gene panel	Unsolved
I54	MD	M	T5K 254 gene panel	Unsolved
1321	STGD	NN	T5K 254 gene panel	Unsolved
H32	STGD	F	T5K 254 gene panel	Unsolved
1475	STGD	F	T5K 254 gene panel	Unsolved
CK7	STGD	M	T5K 254 gene panel	Unsolved
CK12	MD-BV	F	T5K 254 gene panel	Unsolved
CK20	MD	M	T5K 254 gene panel	Unsolved
O14	ACH	M	T5K 254 gene panel	Possibly solved
G39	CD	F	T5K 254 gene panel	Unsolved
H67	CRD	F	T5K 254 gene panel	Unsolved
CG56	CD	F	WES	Unsolved
CE43	CRD	F	WES	Unsolved
CF80	MD	F	WES	Possibly solved
F67	ACH	M	WES	Unsolved
G27	STGD	F	ABCA4 smMIPs and T5K 254 gene panel	Unsolved
P24	STGD	F	ABCA4 smMIPs and T5K 254 gene panel	Possibly solved
G18	STGD	F	ABCA4 smMIPs and T5K 254 gene panel	Unsolved
CG7	STGD	M	ABCA4 smMIPs and WES	Unsolved

3.8 List of disease genes and loci included in the MD-smMIPs panel (Records divided into 2 tables below, 1/2). (adapted from (Hitti-Malin *et al.*, 2022)). ‘X’ marks inheritance pattern associated with phenotypes listed for each gene.

Gene/Locus	iMD, iMD/RP-LCA or AMD	Phenotype 1	Phenotype 2	Phenotype 3	Autosomal Recessive (AR)	Autosomal Dominant (AD)	X-Linked
ABCA1	AMD						
ABCA4	iMD/RP-LCA	STGD1	RP	CD/CRD	X		
ADAM19	AMD						
ADAM9	iMD	CD/CRD			X		
AIP1	iMD/RP-LCA	LCA (AR)	CD/CRD (AD)		X	X	
APOE	AMD						
ARHGEF18	iMD/RP-LCA	RP			X		
ARMS2	AMD						
ATF6	iMD	ACHM			X		
BCAR1	AMD						
BEST1	iMD/RP-LCA	MD (AD)	RP (AR or AD)		X	X	
C1QTNF5	iMD	MD				X	
C2	AMD						
C21orf2 (CFAP410)	iMD	CD/CRD			X		
C2ORF71 (PCARE)	iMD/RP-LCA	RP			X		
C3	AMD						
C8ORF37	iMD/RP-LCA	RP	CD/CRD	BBS	X		
C9	AMD						
CABP4	iMD/RP-LCA	CSNB	LCA		X		
CACNA1F	iMD	CSNB	CD/CRD				X
CACNA2D4	iMD	CD/CRD			X		
CD46	AMD						
CD55	AMD						
CDH3	iMD				X		
CDHR1	iMD	CD/CRD			X		
CEP250	iMD	USH			X		
CEP78	iMD	USH	CRD		X		
CERKL	iMD/RP-LCA	RP	CD/CRD		X		
CETP	AMD						
CFB	AMD						
CFH	iMD/AMD						
CFHR1	AMD						
CFHR2	AMD						
CFHR3	AMD						
CFHR4	AMD						
CFHR5	AMD						
CFI	AMD						
CLN3	iMD/RP-LCA	RP	CRD		X		
CNGA3	iMD	ACHM	CD/CRD		X		
CNGB3	iMD	ACHM	CD/CRD		X		
CNNM4	iMD	CD/CRD			X		
COL4A5	AMD						
COL8A1	AMD						
CRB1	iMD/RP-LCA	LCA (AR)	RP (AR)	Other (AD)	X	X	
CRX	iMD/RP-LCA	LCA (AR or AD)	RP (AD)		X	X	
CTNNA1	iMD	MD				X	
CYMD locus	iMD	CYMD				X	
DRAM2	iMD	MD			X		
EFEMP1	iMD	MD				X	
ELOVL4	iMD	MD				X	
ESRRA	AMD						
EYS	iMD/RP-LCA	RP			X		
FBN2	iMD/AMD	MD				X	
GNAT2	iMD	ACHM			X		
GUCA1A	iMD	CD/CRD				X	
GUCY2D	iMD/RP-LCA	LCA (AR)	CD/CRD (AD)		X	X	
HK1	iMD/RP-LCA	RP	MD		X		
HMCN1	iMD	MD				X	
HTRA1	AMD						
IFT81	iMD	CRD			X		
IMPG1	iMD	MD (AR or AD)			X	X	
IMPG2	iMD/RP-LCA	RP			X		

3.8. List of disease genes and loci included in the MD-smMIPs panel (Records continued from previous page, 2/2).

Gene/Locus	iMD, iMD/RP-LCA or AMD	Phenotype 1	Phenotype 2	Phenotype 3	Autosomal Recessive (AR)	Autosomal Dominant (AD)	X-Linked
<i>IRX1/MCDR3</i> locus	iMD	NCMD				X	
<i>KCNV2</i>	iMD	ACHM	CD/CRD		X		
<i>KIR2DL4</i>	AMD						
<i>LIPC</i>	AMD						
<i>MFSD8</i>	iMD	MD			X		
<i>NMNAT1</i>	iMD/RP-LCA	LCA			X		
<i>OPN1LW</i>	iMD						
<i>OPN1MW</i>	iMD						
<i>OPN1SW</i>	iMD	Colour blindness				X	
<i>OTX2</i>	iMD/RP-LCA	LCA	MD			X	
<i>PDE6C</i>	iMD	ACHM	CD/CRD		X		
<i>PDE6H</i>	iMD	ACHM	CD/CRD		X		
<i>PHF12</i>	AMD						
<i>PITPNM3</i>	iMD	CD/CRD				X	
<i>POC1B</i>	iMD	CRD			X		
<i>PRDM13/MCDR1</i> locus	iMD	MD				X	
<i>PROM1</i>	iMD/RP-LCA	RP (AR)	CD/CRD (AD)	MD (AD)	X	X	
<i>PRPH2</i>	iMD/RP-LCA	MD	CD/CRD	RP		X	
<i>PUS7</i>	AMD						
<i>RAB28</i>	iMD	CD/CRD			X		
<i>RAX2</i>	iMD	CD/CRD			X		
<i>RDH12</i>	iMD/RP-LCA	LCA (AR)	RP (AD)		X	X	
<i>RDH5</i>	iMD	CSNB	CD/CRD		X		
<i>RIMS1</i>	iMD	CD/CRD				X	
<i>RLBP1</i>	AMD/RP-LCA	RP			X		
<i>ROM1</i>	iMD/RP-LCA	RP				X	
<i>RP1</i>	iMD/RP-LCA	RP (AR or AD)			X	X	
<i>RP1L1</i>	iMD/RP-LCA	RP (AR)	MD (AD)		X	X	
<i>RPGR</i>	iMD/RP-LCA	RP	CD/CRD	MD			X
<i>RPGRIP1</i>	iMD/RP-LCA	LCA	CD/CRD		X		
<i>RPGRorf15</i>	iMD						
<i>RS1</i>	iMD	RS					X
<i>RXFP2</i>	AMD						
<i>SCN10A</i>	AMD						
<i>SLC16A8</i>	AMD						
<i>SPEF2</i>	AMD						
<i>TACC2</i>	AMD						
<i>TIMP3</i>	AMD/RP-LCA	SFD				X	
<i>TLCD3B</i>	iMD	CRD			X		
<i>TTL5</i>	iMD	CD/CRD			X		
<i>TULP1</i>	iMD/RP-LCA	LCA	RP		X		
<i>UNC119</i>	iMD	CD/CRD				X	
<i>VP513B</i>	AMD						

Inheritance abbreviations: Autosomal dominant (AD); autosomal recessive (AR). **Disease abbreviations:** Achromatopsia (ACHM); age-related macular degeneration (AMD); Bardet-Biedl syndrome (BBS); cone dystrophy (CD); cone-rod dystrophy (CRD); congenital stationary night blindness (CSNB); cystoid macular dystrophy (CYMD); inherited macular degeneration (iMD); inherited retinal degeneration (IRD); Leber congenital amaurosis (LCA); macular degeneration (MD); North Carolina macular dystrophy (NCMD) retinitis pigmentosa (RP); Sorsby's fundus dystrophy (SFD); retinoschisis (RS); Stargardt disease (STGD); *ABCA4* -associated Stargardt disease (STGD1); Usher syndrome (USH).

3.9 ACMG/AMP variant classification of 9 novel candidate variants. (Records divided into 3 tables below, 1/3). Each coloured heading represents a different group of data used. Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per guidelines. The classification is listed in the final column. See Materials and methods section 4.2.6 for *in silico* tool thresholds. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria).

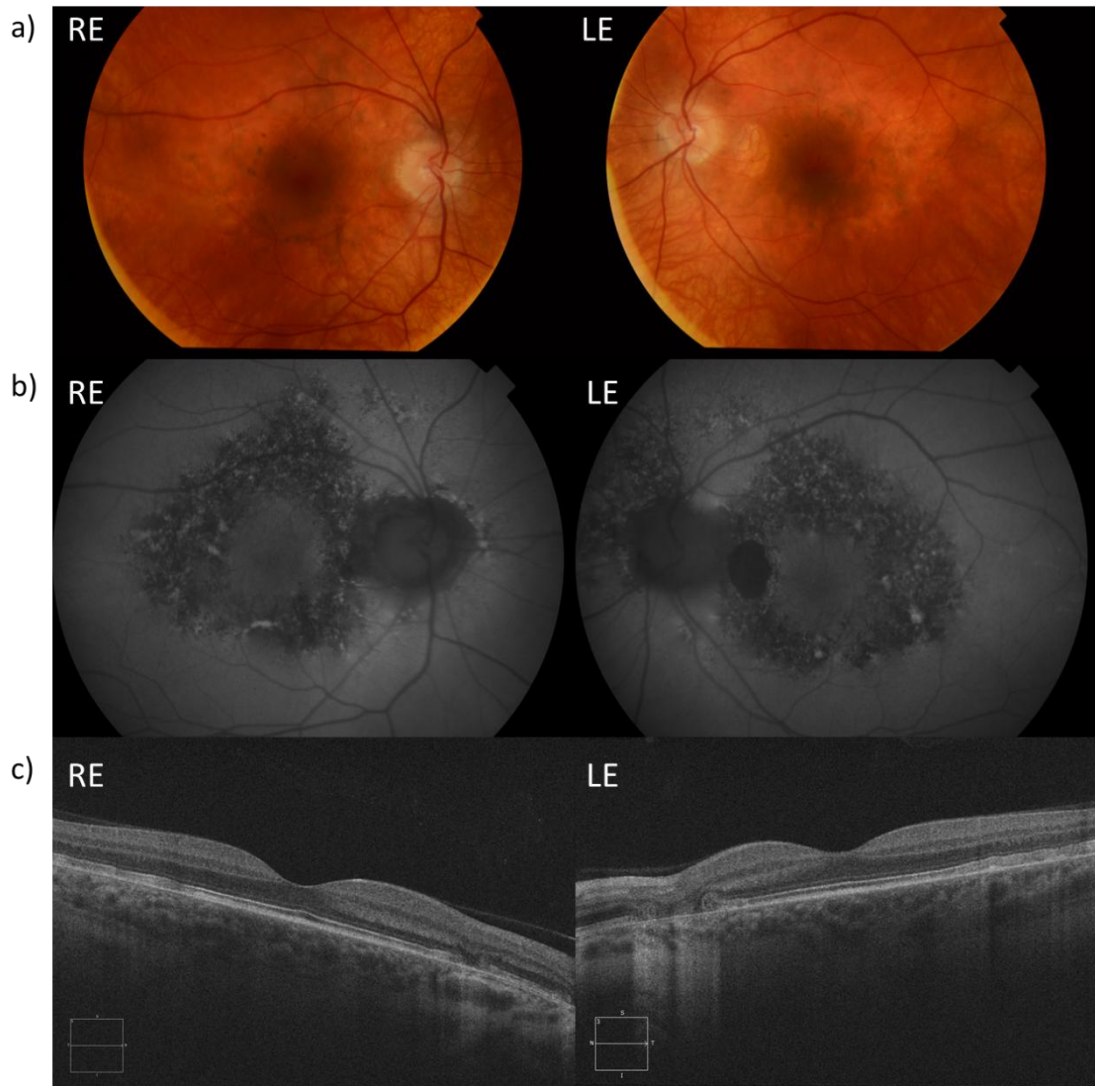
Gene	Transcript	Variant		Population data			Computational and predictive data		
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score
<i>ATF6</i>	NM_007348.4	c.479A>G	p.Lys160Arg	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Multiple lines of computational evidence suggests no impact on gene/gene product	SpliceAI - 0.17 donor loss. This score below the 0.2 threshold. REVEL- 0.03, Alphasense-0.068.	BP4_strong
<i>BEST1</i>	NM_004183.4	c.203A>G	p.Tyr68Cys	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl or in literature. One report on ClinVar with no additional info. We have 2 unrelated patients in Target 5000 with this variant.	PM2_supporting	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	REVEL- 0.92, Alphasense- 0.424, MetaLR- 0.98.	PP3_moderate
<i>BEST1</i>	NM_004183.4	c.294G>T	p.Glu98Asp	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Same amino acid change as an established pathogenic variant	Same amino acid change at position 98 (Glu to Asp) as another variant c.294G>C. PMID: 31456290 and 31465755 report this c.294G>C variant as pathogenic/likely pathogenic.	PS1
<i>CFA4P10</i>	NM_004928.3	c.460_469del	p.Gly154TyrfsTer5	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant deletes 9 bases in exon 5, leading to a premature stop to be created 5 amino acids downstream. Resulting mRNA is predicted to undergo NMD, as variant is not located in last exon or last 50bp, it is in exon 5/7	PVS1
<i>DRAM2</i>	NM_001349881.1	c.700_706del	p.(Ser234Glyfs*9)	Absent or extremely low frequency in population databases	Total allele frequency is 0.00001056 in gnomAD v4.1.0, with no homozygotes. Suggested cutoff threshold is 0.00056 based on most frequent pathogenic variant (c.737T>C).	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant is located in the final exon of the gene (exon 10), so is not predicted to undergo NMD. Variant removes more than 10% of the protein (approx. 12 %). There are 4 other pathogenic variants located in the region downstream of this variant, suggesting it is important to protein function.	PVS1_Strong
<i>KCNV2</i>	NM_133497.4	c.1354G>C	p.Ala452Pro	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	Revel-0.86, Alphasense-0.977.	PP3_moderate
<i>OPN1LW</i>	NM_020061.6	c.283del	p.Leu95TrpfsTer6	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Frameshift means resulting mRNA is predicted to undergo NMD as variant is not located in last exon or last 50bp, it is in exon 2/6	PVS1
<i>RS1</i>	NM_000330.4	c.327G>C	p.Gly109=	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	SpliceAI - acceptor gain 0.29, acceptor loss, donor gain 0.01.	PP3

3.9 ACMG/AMP variant classification of 9 novel candidate variants. (Records continued from previous page, 2/3).

Gene	Transcript	Variant		Functional data			Segregation data		
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score
<i>ATF6</i>	NM_007348.4	c.479A>G	p.Lys160Arg	N/A	N/A	N/A	N/A	N/A	N/A
<i>BEST1</i>	NM_004183.4	c.203A>G	p.Tyr68Cys	N/A	N/A	N/A	N/A	N/A	N/A
<i>BEST1</i>	NM_004183.4	c.294G>T	p.Glu98Asp	Mutational hotspot or well-studied functional domain without benign variation	35 pathogenic/likely pathogenic variation reported on Franklin within a 65bp region with little/no benign variation, but nothing definitive known about domain	PM1_supporting	N/A	N/A	N/A
<i>CFAP410</i>	NM_004928.3	c.460_469del	p.Gly154TyrfsTer5	N/A	N/A	N/A	N/A	N/A	N/A
<i>DRAM2</i>	NM_001349881.1	c.700_706del	p.(Ser234Glyfs*9)	N/A	N/A	N/A	N/A	Parents are first cousins	N/A
<i>KCNV2</i>	NM_133497.4	c.1354G>C	p.Ala452Pro	N/A	N/A	N/A	Co-segregation with disease in multiple affected family members	Using PMID:38103548, we have one proband CK56 with one affected sibling (CK57-2 pts).	PP1_moderate
<i>OPN1LW</i>	NM_020061.6	c.283del	p.Leu95TrpfsTer6	N/A	N/A	N/A	N/A	Mother (CC79) confirmed heterozygous carrier and remains unaffected	N/A
<i>RS1</i>	NM_000330.4	c.327G>C	p.Gly109=	N/A	N/A	N/A	N/A	N/A	N/A

3.9 ACMG/AMP variant classification of 9 novel candidate variants. (Records continued from previous page, 3/3).

Gene	Transcript	Variant		Allelic data			PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	
		c.	p.	ACMG guideline	Comment	Score									
<i>ATF6</i>	NM_007348.4	c.479A>G	p.Lys160Arg	N/A	Cannot use this criteria here as we only have one proband with this variant and it has been confirmed in trans with a second VUS.	N/A	0	0	0	1	0	-4	0	-3	Likely benign
<i>BEST1</i>	NM_004183.4	c.203A>G	p.Tyr68Cys	N/A	Dominant acting variant	N/A	0	0	2	1	0	0	0	3	VUS
<i>BEST1</i>	NM_004183.4	c.294G>T	p.Glu98Asp	N/A	Dominant acting variant	N/A	0	4	0	2	0	0	0	6	Likely pathogenic
<i>CFAP410</i>	NM_004928.3	c.460_469del	p.Gly154TyrfsTer5	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with a pathogenic variant (CH67)	PM3	8	0	2	1	0	0	0	11	Pathogenic
<i>DRAM2</i>	NM_001349881.1	c.700_706del	p.(Ser234Glyfs*9)	For recessive disorders, detected in trans with a pathogenic variant	Only 1 homozygote (CG48).	PM3_supporting	0	4	0	2	0	0	0	6	Likely pathogenic
<i>KCNV2</i>	NM_133497.4	c.1354G>C	p.Ala452Pro	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with a pathogenic variant (CK56), with same genotype noted in affected sibling.	PM3	0	0	6	1	0	0	0	7	Likely pathogenic
<i>OPN1LW</i>	NM_020061.6	c.283del	p.Leu95TrpfsTer6	N/A	X-linked variation	N/A	8	0	0	1	0	0	0	9	Likely pathogenic
<i>RS1</i>	NM_000330.4	c.327G>C	p.Gly109=	N/A	X-linked variation	N/A	0	0	0	2	0	0	0	2	VUS



3.10 (a-c). Multimodal imaging for *EYS* patient CI56, where the images are annotated as follows; (a) Pseudocolour widefield fundus photos, (b) Fundus autofluorescence (FAF) and (c) Optical coherence tomography (OCT) images. (RE= right eye, LE= left eye).

3.11 List of all primers used to confirm candidate variants. Exons covered and product size of primer pair listed for each design.

Gene	exon(s) covered	forward	reverse	product size
ABCA4	3	TAAGAGTTAGGGGCTCAGC	GAGCTTGAGAGAAGAGGCCA	473bp
ABCA4	6	AAGGTCAATTCGTGAGGCT	TGGCTTTAATCCCATCTGCG	403bp
ABCA4	8	CGCCCGGCCACTCTGTGATTTT	TGGCCCTCTGTCCCTTTCCA	718bp
ABCA4	10, 11	ACAGGGAGGAAGGGAGGAAGGGT	CTAAGGGAGCTCTGGCCCACT	1401bp
ABCA4	11	AGGGGATGTAGGGATTCTTGT	TGGACTTGGGGAATGGGAT	517bp
ABCA4	12, 13	CTAAAGACATGGAAGCCTTGAGG	GAGTTGAGTCTTTGAGTTGG	901bp
ABCA4	17	ACCGTTTACATAGAGGGCCA	GGGAGCCTGAGAATAGCCAT	319bp
ABCA4	21	CACCCCTAGAAGCTCTCCTG	CAGTGAGCATCTTGATTGC	360bp
ABCA4	22	GTCACGTATAAACCCCTTCTG	GCACCAACCACTGCTGG	385bp
ABCA4	23	TCTTCTGCAATGGTCCCAG	ACTCCCTAGATGCTGACCAT	636bp
ABCA4	23, 24	GAACAACCTGTGTGACCTGCAG	ACTGTGTGTGGCAATGCTTT	1716bp
ABCA4	28	AGTGAAGTGGGAAGGTCAGG	CTGATTAGGAGGCTGCAGGA	329bp
ABCA4	29	GGCTTCTAGGAGGTAGTG	GGTGACATGCCATCAGAC	488bp
ABCA4	31	AATGGGGCCCTCAAATCAGA	AGTGAGAATCAGGAGATGCC	389bp
ABCA4	35	TCCAGCTACCAACCTTCTGCT	CCAAATCAGCATTCGCGGTGG	373bp
ABCA4	38	ACCCATGAAGTGGTCAAGGCA	GGTCGGGGTAGGGAGGAAGAA	499bp
ABCA4	39, 40	TAGTCTCTGGCCTGGCCTTGA	GTTCGGATGCCCTGAGCTGCC	867bp
ABCA4	39	CCCTAATCCTCTCAGCTGG	ACCTCCATTGCAAAACCCTAG	461bp
ABCA4	40	GCCAGGCTTTGAGAATGTAGA	CTGGAGAGGATTAGGGTGCC	456bp
ABCA4	42	TCTCATGTGGCTAGTGAAGA	TGTGCTGCCCATGTCAATA	352bp
ABCA4	45	AGGAGCCAGCAGGAGCCTGTT	TGTGCGCAGTCCCAAGTCAAGG	498bp
ABCA4	47	CTCTGCATGCATCCTGATCA	ACATCCCACAGGCAAGAGAT	437bp
ATF6	5	TGTGGGTCTCCTCTTTTCT	CCCTTCTCTCCACGCTAA	376bp
ATF6	8	ACATTTGGGTTTTAGAAGGCCT	GGGTCCCCTTCAAATGCTTT	567bp
BEST1	partial 2	GGCCATGACCATCACTTACAC	TTCTTTTCTCTCCCAGCCC	340bp
BEST1	3, 4	ATGTGGGATAGCATCGAGGC	TTGACTACACCCCACTCTG	1099bp
BEST1	partial 4	GGAAGCGCTGACATCATGG	CTTGAGACTGCGGTGCTGA	487bp
BEST1	6	CCTGGAGAAGAGGTGGGG	GGGAGCCACTTAGCTCTAG	260bp
BEST1	7	TGTGCAAGTCAGAACAGGC	TTCCGAGATTGGCTGTGGT	697bp
BEST1	8	GAGCCCTAAACTCTGCCTTTG	GAGGAGGGGAGGGTTGATC	342bp
CACNA1F	2	CCTGGTGACCTTTGCCCTTC	AACCCAGCTACTCCAGTTC	546bp
CACNA1F	27	CCCTCACTCAAGTCCCAAAA	TCCACCACTATGTCTGCTG	465bp
CDH3	exon4	TCAAGTGACCCTCCCAAGT	CCCCGTGATGCTGAGAAA	558bp
CDH3	12-14	GGTGATCATGGCCAACGTTT	TTACATCTCACCCACACCC	1083bp
CDHR1	partial 3	GTCTCTCCACTGTGTTCTCT	tcgttaaggagccactct	402bp
CDHR1	8	GCAAAGGAATGGTGCACTT	AGCTCATGGTCTAGTTGGG	536bp
CFAP410	5	GCCAGACCAGGTATTTGCAG	AGGTCCCATGAGTGCTTTT	431bp
CFAP410	6	TTGGGCAAAACATGGACCTG	CGTGATGCATTCACAAGC	500bp
CFH	9	TCTATAGACGTTGCCTGCCA	TTACCCAGACAATGATGCCG	407bp
CNGA3	2	CTACAAGAGACAGCAGAGGGT	GAAGTTGGCAGTGCTGTGAA	418bp
CNGA3	7	TGCATACTGTGTAGCCGTGA	GAACCTTCTTCAGCGTGTC	867bp
CNGB3	10	GCCAAATAGCATTTACCAAGCC	ACACTCTGTCAATCCAGTCA	453bp
CRB1	4	TTTGGTTGAGGCAGCACAAA	TGCATTCACTGGATTTTCAGCA	846bp
CRB1	partial 9	TTGTGTGCATGTGTGTGTGT	GGGAGAGTTTGGAGTCAGCA	325bp
CRB1	partial 12	TGCTATCAGACCATCCAGT	TGTCACCTTCCATTGGCCAC	631bp
CRB1	14	GCATGGCCCACTTTTCTCTT	GTGGGAGAAATTTGCACTGGT	854bp
DRAM2	partial 10	GTTAATAGGGCAAGGTGCAG	CCCATCACTCAGGGTTATGT	714bp
EYS	17, 18	AGGAGCATTTTGCTCAGGCACA	ACAGCAACATTAGCAACATCTCT	901bp
EYS	26	TGAAAGTGCTGGAGTTGCTG	CATCAAAATCAGGCTGCTGA	790bp
EYS	30	AGAACGTAGGAATGTGAAGCA	CAGGTTGTAAACAAATGGCAGGA	570bp
EYS	33	GGTGACATCTGTAGTCCCA	ATAGCACTCTACCAACCCC	470bp
EYS	43	TGATTCCCCGTAAGCAATGT	AGGTGGCTCAAAATGTAGGTGA	925bp
FBN2	11	GGGGCACACTGAATAGCATAC	GTTGCAACCATGTGTCTCAGT	475bp
FBN2	61	CCCTCTGTCCCAAAAGTGAT	GCAACCATGGTGTTCAGACA	645bp
GUCYA1A	3	GGCTATACAGGGCGTCTAGG	CAAGGAAGGGAAGGAACGTG	530bp
GUCY2D	12	TTGTCTCTCTAGCAACCCC	CACTCCAAAAGCCTCCACAG	527bp
IMP2	2	AACTGGAGGGCACTGGA AAA	TGCCTCACTCTCTTTTCT	620bp
IMP2	3	CCTAGGGCAGCTTACTCAG	CAGAAGGCCCTTGACTGAGA	459bp
IMP2	8	TCCTGAATCTTAACCTAGCAA	TCAATTTCTGACCTGGGTATGC	454bp
IMP2	13	TTACCAACCCTAGGCCATGA	GCAAAAGTCAACAATTGTCCCT	1542bp
KCNV2	partial 1	TGGAACACGACGGGAATGA	ATCTCCTCCACGGTGTGAG	1474bp
KCNV2	partial 1	TCTTCACGCTCGAGTACCTG	CTTTTGACATCCATCGCCGT	585bp
KCNV2	partial 1	ATCTTCCGATCTCTCAAGCT	AGGATCAGAAGCAAGCCT	308bp
KCNV2	2	GCCAAAGTCTTTCAGTCCA	CTTCAAGCAAACTCAGC	465bp
NMNA1	partial 2	TCCGAGAAAGCTGAAGTGGT	GCTAGGACTACAGGCACAGT	301bp
NMNA1	partial 5	GGAGTCTTTGCTGTTCCCA	GTGTTTCTGCAAAAGGGGC	342bp
OPN1LW	2	AGGGGAGACTGTCTGAGGAT	GCCTCCAGAACCCCTTACGTA	606bp
OPN1LW	4	ACCAAAATCCTACCCAAACGG	GCTGCTCCACTCTACTCTGT	751bp
PROM1	10	AAGATCTGAAGGCGCACACT	AGACAATGCTCTTAAAGGGGA	534bp
PROM1	12	TCCATCACAGCAGGATCTC	GAAGGCAAGGGGTGTGAAGG	488bp
PROM1	23	TCTTTGAAGACAGCACACC	TGCTGGTCCACATGACATT	338bp
PRPH2	partial 1	CCACCACTACTGCATTACG	CTGGCTCTTCATCCCAAGG	1067bp
PRPH2	partial 1	CCACCACTACTGCATTACG	CTGGCTCTTCATCCCAAGG	1067bp
PRPH2	2	CTAGAACAGTGACTGGCAGC	CTCCACTGAAGGCTGTTT	526bp
PRPH2	partial 3	CTTGCCAGCTTCTTCACAC	CCTCTCTGGGACCTCAATT	256bp
RDH12	5	ACTGTGAAAAGCCCGAAGTG	ACACCTCCGACATTCCCAATT	535bp
RDH12	8	CCTGCTGAATCCTGGGGTTA	TCATCAGGCACAACTCAGC	417bp
RDH12	9	ACTTCTGCTCGCCACTACTT	GGATCAGAGCAGGCAGGATT	501bp
RIMS1	12	AAGCAGACCTTTACCCATATGTG	TGTATTATTGAGGCTTCAAGG	500bp
RIMS1	26	GTGAACCTGTGTGTGGTGCA	CCAGCAAACTCACTCAGCA	531bp
ROM1	partial 1	GCAGCTCTATACCCTCCCTG	GTAATATGTGCCAGCCAAGC	594bp
RPGR	ORF15	TGGCCATAATCGGGTCACAT	ATCGCTTGTACAGAGATCCCA	1750bp
RP11	partial 4	CAGGGGATTCGTCCAGATTT	TGTCACCTGCTATTGTGGAG	621bp
RP11	partial 4	TCTCCAGGGCCTCTACACT	AGGGGAGAACACACTGATGG	691bp
RP11	partial 4	TCAGTTCATCTTTCGGGCT	TGCTGGCAGAAAGACTCAGA	585bp
RP11	partial 4	TTTGAGCAGGAGTCGATGT	CTTCTCTGCTCTGGGACTC	515bp
RP11	2	TGCTTCCATGCTGTTCACT	TGCCCAACTTTTGTCTTCC	943bp
RS1	4	GCCACCAAGCCAGTTAATT	AGCCACTTCCCTCTGCTTT	519bp
RS1	5	CAAAGCAAGCCAGGAAGA	AGTATAAGGGAAGCTGGCCA	539bp
TULP1	partial 15	AAACTGGAGAGGGCGATGG	AGAAGGATGGGTGTGAGT	260bp

3.12 List of primers used for *CDH3* deletion breakpoint analysis

Gene_primername	exon(s) covered	purpose of primer set design	forward	reverse	product size
CDH3_ex11-14	11-14	used to find breakpoints	CTGGATGTACTCGTCTCAAC	TTGTCACGGGTGTCATCTTC	10171bp
CDH3_ex11_BREAKPOINT	11	used to sequence over breakpoints in carrier mother and control sample	CTGGATGTACTCGTCTCAAC	GAGACAGGGTCTCTGTATGT	828bp
CDH3_ex14_BREAKPOINT	14	used to sequence over breakpoints in carrier mother and control sample	ACAACATCTGCTGGGAGGTT	CTCCTTGATCTTCCGCTTCT	544bp
CDH3_ex9-11	9-11	used to check surrounding sequence for sequence abnormalities	TCTCACACTGACAGTACGAG	GTTGAGACGAGTACATCCAG	2904bp
CDH3_ex14-15	14-15	used to check surrounding sequence for sequence abnormalities	AAGATGACACCCGTGACAAC	ACATGTCTGCCAGTCTCTTG	3054bp

3.13 List of primers used to analyse *OPN1LW* gene cluster

Gene	exon(s) covered	purpose of primer set design	forward	reverse	product size
OPN1LW	4	used initially to confirm presence of candidate variant in patients CJ17 and CJ18	ACCAAAATCCTACCCAACGG	GCTGCTCCACTCTACTCTGT	751bp
OPN1LW	3-4	specific to OPN1LW gene	ACCTTGCAAGCTCCTCCA	TAGCAGAGCATGATGATAGC	3717bp
OPN1GREEN (OPN1MW,-MW2,-MW3)	3-4	specific to green opsin genes	ACCTTGCAAGCCCCTCCG	TAGCAGAGCACGATGATGCT	3719bp

3.14 List of primers for *BBS7* case study

Gene	exon(s) covered	variant sequenced	forward	reverse	product size
BBS7	17	c.1876dupA,p.Ile626fs	AGGTGATCTGCCTCTTCTAGA	ACTGCATCTGGTCAACTCCT	338bp
BBS7	14	c.1458C>G,p.Tyr486*	TCCAACCTCAAACCAAGCCTCT	GGACAGCTTTCCAACCTCCTCT	476bp

Chapter 4

4.1 ABCA4 patients: Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision (Records divided into 2 tables below, 1/2).

Patient	Clinical phenotype	Age of Onset of Ocular phenotype	Nyctalopia? (yes/no, age of onset)	Visual Acuity (VA)				Electroretinogram (ERG)					
				Visual Acuity OD (right)	Visual Acuity OS (left)	Lens status (phakic/cataract/pseudophakic)	Age of onset	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)
1A	Pan-Retinal Degeneration	Early childhood (8 years)	Yes, 8 yrs	Hand Movement	Hand Movement	Normal	9 yrs	non recorable	non recorable	non recorable	non recorable	non recorable	non recorable
4A	Cone-Rod dystrophy	Early teens (13 years)	No	6/60	3/30	Normal	13 yrs	187	191	48.1	46	49.8	41.3
7A	Cone-Rod dystrophy	Late teens (16 years)	No	6/48	6/48	Normal	16 yrs	803	639	128	177	66.9	93.8
9A	STGD1	Adulthood	No	6/30	6/30	Normal	36 yrs	437	393	201	180	183	163
12A	STGD1	Adulthood	No	6/9.5	6/7.5	Normal	//	496	545	173	279	//	216
15A	STGD1	Late onset	Yes, 54 yrs	6/48	6/48	Bilateral pseudophakic	19 yrs	222	258	109	114	78.2	105
33A	STGD1	Late childhood/Early teens	No	6/48	6/38	Normal	13 yrs	340	462	124	137	57.2	62.8
34A	STGD1	Late childhood/Early teens	No	6/9.5	6/12	Normal	//	444	471	161	157	66.3	70.4
35A	RP/ ABCA4-retinopathy	//	//	//	//	//	//	//	//	//	//	//	//

4.1 ABCA4 patients: Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision (Records continued from previous page, 2/2).

	Electroretinograph (ERG)	Visual fields		Colour vision		
Patient	Any other details/comments on ERG	Peripheral or central field loss? (age of onset)	Any other details/comments on Visual fields	Abnormalities to colour vision (yes/no, age of onset)	Axis of colour dysfunction - OD (protan, deutan, tritan, diffuse)	Axis of colour dysfunction - OS (protan, deutan, tritan, diffuse)
1A	No recordable photoreceptor responses, aligning with visual fields.	Central (age 12)	No remaining field of vision could be found in left eye. A very thin area of visual field remains inferiorly in Right eye using V4e target	Yes	diffuse	diffuse
4A	The cone responses are significantly delayed in response time in both eyes.	Central (in 30s)	Central scotoma in both eyes	Yes	diffuse	protan
7A	Reduction in cone-isolated amplitudes suggests cone-dystrophy. Multifocal ERG could only record responses from right eye, where all 61 segments tested were attenuated.	Central (age 16)	Humphrey visual test shows bilateral central scotoma.	Yes	protan	deutan
9A	These ERG readings were taken 6 year prior to most recent imaging. Multifocal ERG shows all central foveal and parafoveal segments were attenuated, with periphery having normal responses.	Central (age 36)	Wider field of vision is normal but has lost a significant portion of their central visual fields. Central scotoma seen in both eyes	No	//	//
12A	Multifocal ERG responses attenuated at central targets in both eyes, but more extensive in the right eye. Fairly normal ERG readings, with cone responses reduced in right eye compared to left. No quality trace obtained for 30Hz flicker in the right eye.	Central	Very slight concentric constriction to the smallest target, meaning a reduction in sensitivity centrally	No	//	//
15A	Multifocal ERG; responses attenuated at central targets in both eyes, with responses slightly better preserved in the periphery. Not a great Multifocal, as have picked up some noisy signals.	Central	Central scotoma in both eyes.	Yes, 44 yrs	deutan	deutan
33A	The cone responses are significantly reduced in both eyes. Multifocal ERG shows all central foveal and parafoveal segments were attenuated, with segments in periphery being better preserved.	Central	Visual fields are largely normal. Mild central scotomas in both eyes to smallest target	Yes	protan	protan
34A	Multifocal ERG; responses attenuated at all 61 segments tested in both eyes.	Central	Central scotoma in both eyes.	Yes	//	diffuse
35A	Last tested in the 80s. EOG; significantly reduced light rise. The amplitude should increase in light rise but this is not seen on his test. ERG; shows attenuated cone response, no convincing rod responses. These are the clinician's notes on the older files.	Central / Peripheral	Grossly constricted in both eyes.	//	//	//

4.2 *CDHR1*, *GUCY2D* and *PROM1* patients: Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision (Records divided into 2 tables below, 1/2). (OD = right eye, OS = left eye).

Patient	Clinical phenotype	Age of Onset of Ocular phenotype	Nyctalopia? (yes/no, age of onset)	Visual Acuity (VA)				Electroretinograph (ERG)					
				Visual Acuity OD (right)	Visual Acuity OS (left)	Lens status (phakic/cataract/pseudophakic)	Age of onset	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)
37C	Cone-Rod dystrophy	Adulthood	Yes	6/24	6/24	Normal	45 yrs	101	152	32	38	18	14
40G	AD Cone-rod dystrophy	Early childhood	No	6/38	6/48	Normal	25 yrs	169	186	6.28	6.9	24.6	71.4
41G	AD Cone-rod dystrophy	Early childhood	No	6/60	Hand Movement	Normal	Early childhood	349	243	91.9	45	114	53.4
42G	AD Cone-rod dystrophy	Adulthood	Yes, 70 yrs	6/15	Counting Fingers	Cataract	40s	323	315	56.6	59.4	66.9	77.8
43G	AD Cone-rod dystrophy	Adulthood	No	6/48	6/60	Normal	33 yrs	207.8	212.3	59.53	57.42	46.09	50.42
44G	AD Cone-rod dystrophy	Early childhood	No	6/38	6/48	Normal	1 yr	295	283	27	33	19	23
47P	Rod-Cone/Panretinal degeneration	Adulthood	Yes	Counting Fingers	Counting Fingers	Cataract	30 yrs	non recorable	non recorable	6.516	4.163	9.313	7.1
48P	Rod-Cone/Panretinal degeneration	Adulthood	Yes	1/48	6/24	Normal	38 yrs	29.3	73.1	50.8	50.4	22.7	30.1

4.2 CDHR1, GUCY2D and PROM1 patients: Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision (Records continued from previous page, 2/2).

	Electroretinograph (ERG)	Visual fields		Colour vision		
Patient	Any other details/comments on ERG	Peripheral or central field loss? (age of onset)	Any other details/comments on Visual fields	Abnormalities to colour vision (yes/no, age of onset)	Axis of colour dysfunction OD (protan, deutan, tritan, diffuse)	Axis of colour dysfunction OS (protan, deutan, tritan, diffuse)
37C	The cone responses are significantly delayed in response time and also in amplitude. The amplitude of the rod isolated response is only slightly decreased.	Central	Bilateral concentric constriction to smaller targets, more severe in left eye than in right eye. Only slight effects on visual fields	Yes	diffuse	diffuse
40G	The rod responses are moderately reduced in amplitude. The cone responses are more compromised; they are significantly reduced in amplitude.	Central (age 25)	Fields to the largest target are fairly normal. Slightly restricted to I4e, but majorly restricted to the smallest target O4e in both eyes.	Yes, since birth	//	//
41G	Rod and cone responses reduced equally in left eye. In right eye the responses remains fairly normal.	Peripheral (childhood)	Centrally constricted in both eyes	Yes, early childhood	diffuse	//
42G	Cone responses delayed and moderately reduced in amplitude. ERG is not aligned with visual fields results - Inconsistencies with visual fields, unreliable account.	//	Inconsistencies noted with visual fields, ERG gives more reliable results.	Yes, 40s	tritan	//
43G	Not a normal rod response, but remains fairly good. Cone responses delayed and moderately reduced in amplitude. Multifocal ERG; responses attenuated at central targets in both eyes.	Central	Central scotoma in both eyes.	Yes, 34 yrs	protan	diffuse
44G	The cone responses are significantly delayed in response time in both eyes.	Central (since birth)	Central scotoma in both eyes, to the smallest target O4e.	Yes, since birth	//	//
47P	No recordable rod responses. The cone responses are significantly delayed in response time in both eyes. Multifocal ERG; responses attenuated at all 61 segments tested in both eyes	Central / Mid-Peripheral (age 30)	Only has response to the largest target, with patchy loss of fields centrally extending out into mid-periphery.	Yes, 30 yrs	diffuse	diffuse
48P	The cone and 30Hz flicker responses are significantly delayed in response time in both eyes. Multifocal ERG; responses attenuated at all 61 segments tested in both eyes, more pronounced at fovea.	Central (age 38)	Central scotoma in left eye, with only islands of vision left in both eyes centrally. Bilateral concentric constriction. Patchy loss of fields out into periphery	Yes, 38 yrs	diffuse	protan

4.3 *USH2A* patients: Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision (Records divided into 2 tables below, 1/2). (OD = right eye, OS = left eye).

Patient	Clinical phenotype	Age of Onset of Ocular phenotype	Nyctalopia? (yes/no, age of onset)	Visual Acuity (VA)				Electroretinogram (ERG)					
				Visual Acuity OD (right)	Visual Acuity OS (left)	Lens status (phakic/cataract/pseudophakic)	Age of onset	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)	Light adapted 30 Hz - OS (uV)
53U	Classical RP	Mid-teens/Early 20s	//	6/5	6/5	Normal	//	non recordable	non recordable	27.9	17.8	21.6	13.3
55U	Classical RP	Teens	Yes, 14 yrs	6/12	6/12	Normal	//	non recordable	non recordable	11.2	10.4	8.19	8.78
57U	Usher syndrome (simplex)	Early childhood (4 years)	Yes, 4yrs	6/7.5	6/7.5	Bilateral pseudophakic	22 yrs	non recordable	non recordable	non recordable	non recordable	non recordable	non recordable
59U	RP (simplex)	Mid 20s	Yes, 25 yrs	Hand Movement	Hand Movement	Cataract	27 yrs	non recordable	non recordable	non recordable	non recordable	non recordable	non recordable

4.3. (Records continued, 2/2).

Patient	Electroretinograph (ERG)		Visual fields		Colour vision		
	Any other details/comments on ERG		Peripheral or central field loss? (age of onset)	Any other details/comments on Visual fields	Abnormalities to colour vision (yes/no, age of onset)	Axis of colour dysfunction - OD (protan, deutan, tritan, diffuse)	Axis of colour dysfunction - OS (protan, deutan, tritan, diffuse)
53U	Some remaining cones centrally. No recordable rod responses.		Central (age 30) / Peripheral (age 14)	Has islands of remaining visual fields centrally, but very small. Peripheral vision is gone.	Yes, 22 yrs	//	//
55U	Multifocal ERG; responses attenuated at all 61 segments tested in both eyes.		Central / Peripheral	Small island of vision remains centrally. Field are severely constricted to centre. Within 10 degrees fixation	//	//	//
57U	Multifocal ERG; No recordable responses in both eyes, at all 61 targets. No recordable photoreceptor responses, aligning with visual fields.		Peripheral	Small island of peripheral vision at IV4e left in both eyes, displaying a major constriction of visual fields.	Yes	tritan defect	tritan defect
59U	No recordable photoreceptor responses, aligning with visual fields.		Peripheral	Visual fields are within 5 degrees in both eyes.	Yes, 48 yrs	//	//

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records divided into 6 tables below, 1/6). Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Suggested allele frequency cutoff threshold is 0.003 based on most frequent pathogenic variant in *ABCA4*. Where PS4_moderate is applied, the Odds ratio calculated within PMID: 40225145 landscape study was used, allowing PS4 which we will downgrade due to presence in gnomAD.

Gene	Transcript	Variant		Population data		
ABCA4	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	Prevalence in affecteds statistically increased over controls	Total allele frequency is 0.00007992 gnomAD v4.1.0 with no homozygotes. 8 reports on ClinVar of likely pathogenic/pathogenic.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.302+68C>T	p.?	Absent or extremely low frequency in population databases	Total allele frequency is 0.00008219 gnomAD v4.1.0 with no homozygotes. Has 9 submissions to LOVD (PMID: 37296172, 29526278, 26527198- first time reported, 30055151). This variant has not been reported to be seen on an allele alone, it is always found to co-occur with c.4539 + 2028C>T.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.3056C>T	p.Thr1019Met	Absent or extremely low frequency in population databases	Total allele frequency is 0.00005204 gnomAD v4.1.0 with no homozygotes.115 reports on LOVD.nl, with classifications of likely pathogenic/pathogenic and a rare few VUS. There are 9 reports on Clinvar of pathogenic/likely pathogenic. Confirmed in 13 Target 5000 families.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.3113C>T	p.(Ala1038Val)	Absent or extremely low frequency in population databases	Total allele frequency is 0.001848 gnomAD v4.1.0 with 7 homozygotes. Reduced penetrance (17%) was reported in PMID: 32815999. Variant has 1161 reports to LOVD, with varying classes. Most recent LOVD.nl compedium study classes it as Likely pathogenic PMID: 40225145. Odds ratio calculated within PMID: 40225145 landscape study but will not apply PS4 due to prescence of homozygotes in gnomAD (taking reduced penetrance into account).	PM2_supporting
ABCA4	NM_000350.3	c.3322C>T	p.Arg1108Cys	Absent or extremely low frequency in population databases	Total allele frequency is 0.0001939 gnomAD v4.1.0 with no homozygotes. There are 369 entries on LOVD.nl, with classifications of likely pathogenic/pathogenic. 13 reports on ClinVar of pathogenic/likely pathogenic. Confirmed to be causative in at least 12 Target 5000 families.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.3329-1G>A	p.?	Absent or extremely low frequency in population databases	Total allele frequency is 0.0000049580 gnomAD v4.1.0 with no homozygotes.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.4222T>C	p.Trp1408Arg	Absent or extremely low frequency in population databases	Total allele frequency is 0.00003594 gnomAD v4.1.0 with no homozygotes.This variant has been observed to be causative in 11 unrelated probands in the Target 5000 cohort, always occuring in a complex allele with c.4918C>T. There are 106 submissions to LOVD.nl with over 20 papers cited, all with pathogenic/likely pathogenic classes.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.4363T>C	p.Cys1455Arg	Absent or extremely low frequency in population databases	Total allele frequency is 0.00002293 gnomAD v4.1.0 with no homozygotes. 52 reports on LOVD.nl.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.4539+2028C>T	p.[= ,Arg1514Leufs*3 6]	Absent or extremely low frequency in population databases	Total allele frequency is 0.00003943 gnomAD v4.1.0 with no homozygotes. Seen in 4 unrelated individuals in PMID:29526278. We have 18 unrelated pedigrees harbouring this variant, in which only affected family members have this variant occuring along with another severe/mild variant in <i>ABCA4</i> on the other allele.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.4918C>T	p.Arg1640Trp	Absent or extremely low frequency in population databases	Total allele frequency is 0.00005390 gnomAD v4.1.0 with no homozygotes This variant has been observed to be causative in 11 unrelated probands in the T5K cohort, always occuring in a complex allele with c.4222T>C. There are 208 submissions to LOVD.nl with over 50 papers cited, all with pathogenic/likely pathogenic classes.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	Absent or extremely low frequency in population databases	Total allele frequency is 0.0005349 gnomAD v4.1.0 with 1 homozygotes. Has 1148 submissions to LOVD.nl. Multiple studies in which unrelated probands are analysed, as well as full families in which the variant is seen homozygously or in a compound heterozygous manner only in individuals affected with Stargardt, like in PMID: 26976702, 33258285, 27775217, 32467599 among over 40 studies referencing this variant on PubMed. Odds ratio calculated within PMID: 40225145 landscape study but will not apply PS4 due to prescence of homozygotes in gnomAD.	PM2_supporting
ABCA4	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting
ABCA4	NM_000350.3	c.5603A>T	p.Asn1868Ile	MAF is too high for disorder	High allele frequency in gnomad v4.1.0; 0.04219, with 364 homozygotes. This is not surprising due to its low level of penetrance (less than 5%), where this variant has been confirmed to be causative of disease in cases where in trans with severe variant, PMID: 28446513, 29971439, 40225145. Can use PS4 here due to the prevalence of this variant in affected family members heterozygously in the Target 5000 study and in public databases, also due to numerous genotype-phenotype and landscape <i>ABCA4</i> studies as referenced here. Conflicting pathogenicities in LOVD.nl and ClinVar due to hypomorphic nature. Odds ratio calculated within PMID: 40225145 landscape study allowing PS4 application without downgrade due to hypomorphic nature and penetrance less than 5%.	BS1, PS4
ABCA4	NM_000350.3	c.5773A>G	p.Arg1925Gly	Absent or extremely low frequency in population databases	Total allele frequency is 0.000006816 gnomAD v4.1.0 with no homozygotes. 4 reports on LOVD.nl.	PM2_supporting, PS4_moderate
ABCA4	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	MAF is too high for disorder	The frequency for this allele is high in gnomAD v4.1.0; 0.0034. Reduced penetrance (50%) was reported in PMID: 32815999. This is one of the most commonly observed variants in <i>ABCA4</i> patients, with almost 20% of European cases carrying this variant (referenced in "Allelic data" column). Will still apply BS1 criteria	BS1

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records continued from previous page, 2/6).

Gene	Transcript	Variant		Computational and predictive data		
ABCA4	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	Splicing assay data demonstrating that a variant leads to aberrant splicing profile	REVEL- 0.98, Alphasense- 0.977, MetaLR- 0.98, M-CAP- 0.8231. The REVEL score surpasses the threshold for strong which is ≥ 0.932 , as does Alphasense ≥ 0.9784 PMID: 36413997 and 37733863. In vitro splice assay showed strengthening of exon 3 skipping due to this variant PMID: 31397521. The splice defect was classified as moderately severe due to the presence of only 51% remaining product. Both exon skipping and missense mutation thought to contribute severity of this variant PMID: 31397521.	PVS1_moderate
ABCA4	NM_000350.3	c.302+68C>T	p.?	Multiple lines of computational evidence suggests no impact on gene/gene product	Variant is deep-intronic and not located within a canonical splice site. PMID:29526278 confirmed this variant has no impact on the resulting mRNA by performing RT-PCRs on photoreceptor precursor cells (PPCs) generated from fibroblasts obtained from individuals with STGD1, as well as on adult retina mRNA. Variant does not result in the activation of cryptic splice sites and/or exonic splice enhancers. PMID: 40225145 recommend if CADD scores ≤ 17.3 meaning a variant is less likely to be deleterious or disease causing, can use BP4_moderate. CADD-0.591	BP7_strong, BP4_moderate
ABCA4	NM_000350.3	c.3056C>T	p.Thr1019Met	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.96, Alphasense- 0.469, MetaLR- 0.95, M-CAP- 0.6109. The REVEL score surpasses the threshold for strong ≥ 0.932 , and for Alphasense it does not pass the strong cutoff ≥ 0.9784 PMID: 36413997 and 37733863. p.(Thr1019Ala) is a variant at the same amino acid residue with 10 reports on LOVD.nl and classified by PMID: 40225145 as moderate pathogenic with similar REVEL score to our variant differing in 0.02. Additional 7 studies linked on LOVD.nl.	PM5
ABCA4	NM_000350.3	c.3113C>T	p.(Ala1038Val)	N/A	REVEL-0.53, Alphasense- 0.164, MetaLR- 0.48, MCAP- 0.6513. All scores are quite uncertain and do not agree with each other, where REVEL score is not low enough to apply benign score either.	N/A
ABCA4	NM_000350.3	c.3322C>T	p.Arg1108Cys	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.89, Alphasense- 0.302, MetaLR- 0.92, M-CAP- 0.7967. The REVEL score surpasses the threshold for moderate 0.773-0.932, and for Alphasense it does not pass the ≥ 0.9784 cutoff PMID: 36413997 and 37733863. p.(Arg1108Profs*40), p.(Arg1108His), p.(Arg1108Leu), p.(Arg1108Gly) are 4 other variants observed to impact the same amino acid as this variant. The first 3 are classified as pathogenic PMID: 40225145, and the 4th variant p.(Arg1108Gly) has one submission to LOVD.nl PMID: 3774808, where it is classified as pathogenic in a chinese STGD1 cohort.	PM5
ABCA4	NM_000350.3	c.3329-1G>A	p.?	Predicted null variant in a gene where LoF is a known mechanism of disease	This is a canonical acceptor splice site variant in intron 22, where the canonical acceptor site is predicted to be lost (SpliceAI 0.93). Acceptor gain is predicted to occur (SpliceAI 0.47) 80bp into intron 22. This could lead to in-frame elongation.	PVS1_moderate
ABCA4	NM_000350.3	c.4222T>C	p.Trp1408Arg	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.822, Alphasense- 0.9061, MetaLR- 0.7345, M-CAP- 0.5149. The REVEL score surpasses the threshold for moderate which is 0.773-0.932, and for Alphasense 0.8976-0.9784 PMID: 36413997 and 37733863. p.Trp1408* and p.(Trp1408Glyfs*28) are null variants reported in LOVD.nl as pathogenic/likely pathogenic, and p.Trp1408Leu, p.Trp1408Cys are also in LOVD.nl with similar REVEL scores differing in 0.1.	PM5
ABCA4	NM_000350.3	c.4363T>C	p.Cys1455Arg	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	REVEL- 0.93, Alphasense- 0.874, MetaLR- 0.95, M-CAP- 0.6962. The REVEL score surpasses the threshold for moderate which is 0.773-0.932, as does Alphasense 0.7191-0.8976 for support PMID: 36413997 and 37733863, with MetaLR and M-CAP agreeing on moderate.	PP3_moderate
ABCA4	NM_000350.3	c.4539+2028C>T	p.[=,Arg1514Leufs*36]	Splicing assay data demonstrating that a variant leads to aberrant splicing profile	Alamut Visual prediction shows the creation of exonic splicing enhancer sites (PMID: 29526278). c.4539+2028C>T results in a retina-specific inclusion of a 345-nt Pseudo-exon (PE) between exons 30 and 31 in a proportion of ABCA4 transcripts. This PE, which is predicted to lead to protein truncation and introduce a premature stop codon (p.Arg1514Leufs*36), was found as a low-abundance alternative splice form of ABCA4 when performing deep RNA sequencing of human macula RNA (PMID: 29526278), by creating photoreceptor precursor cells (PPCs) from fibroblasts obtained from patients with STGD1.	PVS1_strong
ABCA4	NM_000350.3	c.4918C>T	p.Arg1640Trp	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.797, Alphasense- 0.3808, MetaLR- 0.7873, M-CAP- 0.2794. The REVEL score surpasses the threshold for moderate 0.773-0.932, and Alphasense is too low and does not fall into a pathogenic score bracket PMID: 36413997 and 37733863. p.Arg1640Gln is a variant at the same amino acid residue reported in LOVD.nl (139 subs) as pathogenic/likely pathogenic with similar REVEL score differing in 0.1. It also has a submission to ClinVar and reported in over 10 studies in Europe, linked on Varsome and Franklin.	PM5
ABCA4	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	Splicing assay data demonstrating that a variant leads to aberrant splicing profile	One study showed that fibroblasts from patients harbouring c.5461-10T>C are defective in ABCA4 RNA splicing leading to the reduction of normally spliced full-length ABCA4 mRNA and an accumulation of alternatively spliced mRNAs reducing levels of the ABCA4 protein PMID: 27775217. The effect of the c.5461-10T>C variant on RNA splicing was analysed in another study using photoreceptor progenitor cells (PPCs) mRNA derived from STGD1 patient fibroblasts, and showed skipping of exon 39 or exons 39 and 40 in the mRNA, PMID:26976702. Splicing of the ABCA4 gene was studied in HEK293T, and in human retinal pigment epithelium cells, ARPE-19, using a minigene assay shows the same defect as the previous two studies, PMID:29461686.	PVS1_moderate
ABCA4	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	Predicted null variant in a gene where LoF is a known mechanism of disease	Frameshift variant occurs in exon 40/50 creating premature stop codon 47 amino acid residues downstream, where the resulting mRNA is predicted to undergo NMD. LOF is a known mechanism of disease.	PVS1
ABCA4	NM_000350.3	c.5603A>T	p.Asn1868Ile	N/A	Revel-0.4 (this is uncertain), Alphasense-0.223(benign), MetaLR-0.73(deleterious). Cutoff not reached for BP4 or for any of the other PP3 criteria PMID: 36413997 and 37733863.	N/A
ABCA4	NM_000350.3	c.5773A>G	p.Arg1925Gly	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	Revel-0.945, MetaLR- 0.8079, Alphasense- 0.9102, MCAP- 0.3061. The REVEL score surpasses the threshold for strong which is ≥ 0.932 , and for Alphasense 0.8976-0.9784 for moderate PMID: 36413997 and 37733863. Can use PP3_strong here due to REVEL, MetaLR and M-CAP scores agreeing	PP3_strong
ABCA4	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.76, Alphasense- 0.8529, MetaLR- 0.7001. The REVEL score does not surpass the threshold for moderate which is 0.773, and for Alphasense 0.8976 PMID: 36413997 and 37733863. p.Gly1961Arg is classified as pathogenic on ClinVar, and has 18 submissions to LOVD.nl with likely pathogenic/pathogenic classifications (referenced in over 10 papers). p.Gly1961Ala is a second variant that impacts the same amino acid, is also classified as pathogenic on ClinVar, with 19 submissions as likely pathogenic/pathogenic (referenced in 5 papers). 2 well-classified pathogenic variants occurring at the same amino acid, with similar REVEL scores (differing by less than 0.1) we can use PM5	PM5

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records continued from previous page, 3/6).

Gene	Transcript	Variant		Functional data		
		c.	p.	ACMG guideline	Comment	Score
<i>ABCA4</i>	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	N/A	Functional data listed under 'Computational and predictive data' column	N/A
<i>ABCA4</i>	NM_000350.3	c.302+68C>T	p.?	N/A	Functional data listed under 'Computational and predictive data' column	N/A
<i>ABCA4</i>	NM_000350.3	c.3056C>T	p.Thr1019Met	Well-established functional studies show a deleterious effect	Has F-index of 0.01, reported by PMID:34954332. Lack of significant N-Ret-PE-stimulated ATPase activity results in F-index closer to 0 (below 0.15), meaning the variant is more severe. Due to low F-index and impact on ability of this variant to clear retinoids from photoreceptor. This variant is in region encoding a disulfide bond of the 'ABC transporter 1' domain.	PS3_moderate
<i>ABCA4</i>	NM_000350.3	c.3113C>T	p.(Ala1038Val)	Mutational hotspot or well-studied functional domain without benign variation	Variant is located in the sequence encoding the ABC transporter 1 domain of the protein. Functional studies in mouse models demonstrate that the c.1622T>C/c.3113C>T complex allele as well as the c.1622T>C and c.3113C>T variants independently lead to reduced ATPase activity, where the effect of c.3113C>T is milder compared to that of c.1622T>C alone or the complex allele (PMID: 11017087, 16103129, 25712131). While this variant does have a milder effect, it does result in reduced ATPase activity and is pathogenic.	PS3_supporting
<i>ABCA4</i>	NM_000350.3	c.3322C>T	p.Arg1108Cys	Well-established functional studies show a deleterious effect	Has F-index of 0.11 reported by PMID:34954332 - check F-Index explanation above in c.3056C>T row. PMID: 26092729 report that this variant behaves as a processing mutant, with reduced protein levels, unstable at body temperature or higher, and rapid protein degradation. Less than 11 controls are used in the study so would usually downgrade, but the F-index allows for PS3_moderate.	PS3_moderate
<i>ABCA4</i>	NM_000350.3	c.3329-1G>A	p.?	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.4222T>C	p.Trp1408Arg	Well-established functional studies show a deleterious effect	In vitro analysis using plasmid constructs encoding recombinant ABCA4 proteins with missense mutations c.4222T>C and c.4918C>T, and the complex allele [c.4222T>C; c.4918C>T] was carried out in HEK293 cells PMID: 11687513. RNA and protein analysis via western blot showed the mutant proteins were not efficiently expressed. The mutations individually and together may cause misfolding or the mutant protein is unstable and rapidly degraded. PMID: 11687513 study shows that the 2 variants in this complex allele have a mild impact on the protein function alone but together (which we always observe in our cohort) they have a severe impact with little to no ABCA4 protein being expressed. This variant occurs within a disulfide bond domain, which is flanking a transmembrane region, hence the variants impact on the protein expression.	PS3_supporting
<i>ABCA4</i>	NM_000350.3	c.4363T>C	p.Cys1455Arg	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.4539+2028C>T	p.[=,Arg1514Leufs*36]	N/A	Functional data listed under 'Computational and predictive data' column	N/A
<i>ABCA4</i>	NM_000350.3	c.4918C>T	p.Arg1640Trp	Well-established functional studies show a deleterious effect	In vitro analysis using plasmid constructs encoding recombinant ABCA4 proteins with missense mutations c.4222T>C and c.4918C>T, and the complex allele [c.4222T>C; c.4918C>T] was carried out in HEK293 cells PMID: 11687513. RNA and protein analysis via western blot showed the mutant proteins were not efficiently expressed. The mutations individually and together may cause misfolding or the mutant protein is unstable and rapidly degraded. PMID: 11687513 study shows that the 2 variants in this complex allele have a mild impact on the protein function alone but together (which we always observe in our cohort) they have a severe impact with little to no ABCA4 protein being expressed. This variant occurs within a disulfide bond domain, which is flanking a transmembrane region, hence the variants impact on the protein expression.	PS3_supporting
<i>ABCA4</i>	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	N/A	Functional data listed under 'Computational and predictive data' column	N/A
<i>ABCA4</i>	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.5603A>T	p.Asn1868Ile	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.5773A>G	p.Arg1925Gly	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	Mutational hotspot or well-studied functional domain without benign variation	Functional studies are referenced in PMID: 33909047. They indicate that there is an effect on the ATPase and transport activities of the ABCA4 protein in the presence of this amino acid change. This variant is predicted to lie within a nucleotide binding domain, and in vitro functional assays demonstrated that this variant resulted in decreased ATP-binding capacity and ATP hydrolysis, despite an increase of the total amount of ABCA4 protein (PMID: 11017087). Additionally, the variant protein ATPase activity was inhibited by all-trans retinal, in contrast to the stimulation seen in wild type (PMID: 11017087, 22661473).	PS3_supporting

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records continued from previous page, 4/6).

Gene	Transcript	Variant		Segregation data		
		c.	p.	ACMG guideline	Comment	Score
<i>ABCA4</i>	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	N/A	One family in Target 5000 allowed for N=1/64 probability to be calculated as per PMID: 27236918.	PP1_strong
<i>ABCA4</i>	NM_000350.3	c.302+68C>T	p.?	N/A	Segregates with disease within the full 15A pedigree but is never seen to occur alone and will only occur as a haplotype with c.4539+2028C>T.	N/A
<i>ABCA4</i>	NM_000350.3	c.3056C>T	p.Thr1019Met	Co-segregation with disease in multiple affected family members	2 pedigrees in Target 5000 allow for N=1/64 probability to be calculated when combined as per PMID: 27236918. Additional 13 papers linked on Franklin and LOVD.nl in which the variant is seen to segregate with disease.	PP1_strong
<i>ABCA4</i>	NM_000350.3	c.3113C>T	p.(Ala1038Val)	Co-segregation with disease in multiple affected family members	Segregates in one other family in Target 5000 in a complex allele, and also segregates in the 12A pedigree alone on an allele. Did not upgrade strength to be cautious and take into account penetrance.	PP1
<i>ABCA4</i>	NM_000350.3	c.3322C>T	p.Arg1108Cys	N/A	Within 4A pedigree, only the proband is affected so cannot inform segregation. Only one other family in Target 5000 with enough members to calculate N=1/4 probability as per PMID: 27236918.	N/A
<i>ABCA4</i>	NM_000350.3	c.3329-1G>A	p.?	N/A	Within this the 4A pedigree, only the proband is affected so cannot do calculation with just this info. This is the only patient in Target 5000 where this variant is confirmed to cause disease, and phasing has been completed. There are 3 papers (PMID: 32653833, 37705246, 38602673) where a patient has been reported to have this variant in trans, but no family data is available although they have indicated on LOVD.nl that phasing was completed.	N/A
<i>ABCA4</i>	NM_000350.3	c.4222T>C	p.Trp1408Arg	Co-segregation with disease in multiple affected family members	Segregation data in 2 Irish Target 5000 families allowed N=0.125 probability as per as per PMID: 27236918.	PP1_moderate
<i>ABCA4</i>	NM_000350.3	c.4363T>C	p.Cys1455Arg	N/A	Within 9A pedigree, only the proband is affected so cannot inform segregation, and no other probands in Target 5000 with confirmed segregation.	N/A
<i>ABCA4</i>	NM_000350.3	c.4539+2028C>T	p.[=,Arg1514Leufs*36]	Co-segregation with disease in multiple affected family members	Segregation data in 4 Irish Target 5000 families allowed N=0.008 probability as per as per PMID: 27236918.	PP1_strong
<i>ABCA4</i>	NM_000350.3	c.4918C>T	p.Arg1640Trp	Co-segregation with disease in multiple affected family members	Segregation data in 2 Irish Target 5000 families allowed N=0.125 probability as per as per PMID: 27236918.	PP1_moderate
<i>ABCA4</i>	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	N/A	Within 35A pedigree, only the proband is affected so cannot inform segregation, and 7 other probands in Target 5000 also do not have enough family to inform segregation.	N/A
<i>ABCA4</i>	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	N/A	More family members required	N/A
<i>ABCA4</i>	NM_000350.3	c.5603A>T	p.Asn1868Ile	N/A	N/A	N/A
<i>ABCA4</i>	NM_000350.3	c.5773A>G	p.Arg1925Gly	N/A	More family members required	N/A
<i>ABCA4</i>	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	N/A	Within 9A pedigree, only the proband is affected so cannot inform segregation, and 13 other probands in Target 5000 also do not have enough family to inform segregation.	N/A

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records continued from previous page, 5/6).

Gene	Transcript	Variant		Allelic data		
		c.	p.	ACMG guideline	Comment	Score
<i>ABCA4</i>	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	For recessive disorders, detected in trans with a pathogenic variant	4 points are required to award PM3 at VeryStrong strength, where one point is awarded for each proband where this variant is confirmed in trans with a pathogenic or likely pathogenic variant (SVI/ClinGen PM3 criteria 2019). Due to the prevalence of this variant in probands heterozygously in the Target 5000 study and in general in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.302+68C>T	p.?	Observed in cis with a pathogenic variant	Variant is always occurring in cis with well-studied splice-altering pathogenic variant	BP2
<i>ABCA4</i>	NM_000350.3	c.3056C>T	p.Thr1019Met	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with pathogenic/severe variants in 1A pedigree. IMD-smMIPs study described in Chapter 1, there are 3 patients in which they carry this variant in trans with a second pathogenic/ likely pathogenic variant. Due to the prevalence of this variant in probands heterozygously in the Target 5000 study and in general in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.3113C>T	p.(Ala1038Val)	For recessive disorders, detected in trans with a pathogenic variant	Variant occurs in trans with a pathogenic complex allele in the 12A pedigree. There are also 2 unrelated patients in the iMD-smMIPs cohort from Chapter 1 with this variant in trans with disease causing variant.	PM3_strong
<i>ABCA4</i>	NM_000350.3	c.3322C>T	p.Arg1108Cys	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with likely pathogenic/severe splicing variant in 4A proband. Within iMD-smMIPs study in Chapter 1 there are 2 patients in this variant is found in trans with a second/third pathogenic/likely pathogenic variant. Due to the prevalence of this variant in probands heterozygously in the Target 5000 study and in general in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.3329-1G>A	p.?	For recessive disorders, detected in trans with a pathogenic variant	Within the 4A proband, this variant is seen to be in trans with a pathogenic/severe variant (c.3322C>T). There are 3 papers (PMID: 32653833, 37705246, 38602673) where a patient has been reported to have this variant in trans with another pathogenic/likely pathogenic variant.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.4222T>C	p.Trp1408Arg	For recessive disorders, detected in trans with a pathogenic variant	This variant has been observed to be causative in 11 unrelated probands in the Target 5000 cohort, always occurring in a complex allele with c.4918C>T. However, due to the prevalence of this variant in probands in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.4363T>C	p.Cys1455Arg	For recessive disorders, detected in trans with a pathogenic variant	Within the 9A proband variant is confirmed in trans with a pathogenic/severe variant (c.5882G>A). This is the only person with family members in Target 5000, where phasing has been confirmed. PMID: 17982420, 22449572, 25312043, 29343940, 30834176, 32581362 are some examples of papers listed in ClinVar where the variant is seen in trans with other pathogenic variants in only affected individuals.	PM3_strong
<i>ABCA4</i>	NM_000350.3	c.4539+2028C>T	p.[=, Arg1514Leufs*36]	For recessive disorders, detected in trans with a pathogenic variant	2 homozygotes (unrelated to the best of our knowledge) present in Target 5000, one of which is patient 15A. 18 additional probands with this variant in trans with a second pathogenic variant reported in Target 5000, along with 4 unrelated individuals in PMID:29526278.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.4918C>T	p.Arg1640Trp	For recessive disorders, detected in trans with a pathogenic variant	This variant has been observed to be causative in 11 unrelated probands in the Target 5000 cohort, always occurring in a complex allele with c.4222T>C. However, due to the prevalence of this variant in probands in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	For recessive disorders, detected in trans with a pathogenic variant	This variant is seen in 35A proband in a homozygous manner, with common variant c.5603A>T in cis. It is also seen in trans with a pathogenic variant in 1 other family in the iMD-smMIPs study in Chapter 1, as well as 7 other families in the Target 5000 study. Similarly, this variant is seen to have a high prevalence in probands in literature PMID: 40225145, can use this strength here.	PM3_VeryStrong
<i>ABCA4</i>	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	For recessive disorders, detected in trans with a pathogenic variant	This variant is seen to be in trans with a pathogenic/severe variant (c.5773A>G) within the 7A proband. As we only have this one proband, I can only award 1 point here, meaning PM3 cannot be modified.	PM3
<i>ABCA4</i>	NM_000350.3	c.5603A>T	p.Asn1868Ile	N/A	In this case it is in trans with a severe pathogenic variant, so it is thought to likely be causative of disease but due to penetrance this is uncertain. Will not use this criteria as PM2 threshold is not met, although this variant may be listed as an exception	N/A
<i>ABCA4</i>	NM_000350.3	c.5773A>G	p.Arg1925Gly	For recessive disorders, detected in trans with a pathogenic variant	Variant is seen to be in trans with a novel pathogenic variant (c.5594dup) in patient 7A. As we only have this one proband and the second variant is novel with no validated classification we cannot use this proband to apply criteria. Only other patient found in literature is reported in PMID: 29925512, with pathogenic complex allele [c.1622T>C;3113C>T] suspected to be in trans, allowing PM3 use at supporting level.	PM3_supporting
<i>ABCA4</i>	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	For recessive disorders, detected in trans with a pathogenic variant	This variant has been reported in LOVD.nl 2868 times. It is implicated in disease and observed in trans with a pathogenic/likely pathogenic alleles in 4 other families in the iMD-smMIPs study in Chapter 1. It is the most prevalent disease-causing <i>ABCA4</i> variant, present in ~20% of cases of European descent (PMID: 33909047). This same study has a cohort of 644 <i>ABCA4</i> patients, where there are 140 compound heterozygous cases and 10 homozygous cases for this allele. For this reason will upgrade to PM3_VeryStrong.	PM3_VeryStrong

4.4 Detailed ACMG/AMP variant classification of *ABCA4* variants (Records continued from previous page, 6/6). Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per guidelines. The classification is listed in the final column. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria).

Gene	Transcript	Variant		PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	
		c.	p.									
<i>ABCA4</i>	NM_000350.3	c.161G>A	p.[Cys54Serfs*14, Cys54Tyr]	8	4	4	1	0	0	0	17	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.302+68C>T	p.?	0	0	2	1	0	-4	-3	-4	Likely benign
<i>ABCA4</i>	NM_000350.3	c.3056C>T	p.Thr1019Met	8	4	6	1	0	0	0	19	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.3113C>T	p.(Ala1038Val)	0	4	0	3	0	0	0	7	Likely pathogenic
<i>ABCA4</i>	NM_000350.3	c.3322C>T	p.Arg1108Cys	8	0	6	1	0	0	0	15	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.3329-1G>A	p.?	8	0	4	1	0	0	0	13	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.4222T>C	p.Trp1408Arg	8	0	6	2	0	0	0	16	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.4363T>C	p.Cys1455Arg	0	4	4	1	0	0	0	9	Likely pathogenic
<i>ABCA4</i>	NM_000350.3	c.4539+2028C>T	p.[= ,Arg1514Leufs*36]	8	8	2	1	0	0	0	19	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.4918C>T	p.Arg1640Trp	8	0	6	2	0	0	0	16	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.5461-10T>C	p.[Thr1821Aspfs*6, Thr1821Valfs*13]	8	0	2	1	0	0	-2	9	Likely pathogenic
<i>ABCA4</i>	NM_000350.3	c.5594dupA	p.His1865Glnfs*47	8	0	2	1	0	0	0	11	Pathogenic
<i>ABCA4</i>	NM_000350.3	c.5603A>T	p.Asn1868Ile	0	4	0	0	0	-4	0	0	VUS
<i>ABCA4</i>	NM_000350.3	c.5773A>G	p.Arg1925Gly	0	4	2	2	0	0	0	8	Likely pathogenic
<i>ABCA4</i>	NM_000350.3	c.5882G>A	p.(Gly1961Glu)	8	0	2	1	0	-4	0	7	Likely pathogenic

4.5 Detailed ACMG/AMP variant classification of *CDHR1*, *GUCY2D*, and *PROM1* variants (Records divided into 3 tables below, 1/3). Each coloured heading represents a different group of data used. Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per guidelines. The classification is listed in the final column. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria).

Gene	Transcript	Variant		Population data			Computational and predictive data		
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score
<i>CDHR1</i>	NM_033100.4	c.562G>A	p.Gly188Ser	Absent or extremely low frequency in population databases	Total allele frequency is 0.0001242 gnomAD v4.1.0 with one homozygote. Suggested cutoff threshold is 0.00133 based on most frequent pathogenic variant. Hypomorphic alleles noted in CDHR1 previously PMID:38540785.	PM2_supporting	N/A	Revel- 0.627, MetaLR- 0.0929, MCAP- 0.1339, Alphasense- 0.5901. The alphasense score does not reach supporting level (0.7191), and neither does the REVEL score (0.644), but the scores are not low enough to apply a benign class either PMID: 36413997 and 37733863.	N/A
<i>CDHR1</i>	NM_033100.4	c.743del	p.Gly248fs	Absent or extremely low frequency in population databases	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Frameshift variant occurs in sequence encoding Cadherin 3 domain of CDHR1, and is predicted to undergo NMD as it is in exon 8/13 where LOF is a known mechanism of disease.	PVS1
<i>CDHR1</i>	NM_033100.4	c.2228G>A	p.Arg743Gln	Absent or extremely low frequency in population databases	Total allele frequency is 0.00002479 gnomAD v4.1.0 with no homozygotes. Suggested cutoff threshold is 0.00133 based on most frequent pathogenic variant. Hypomorphic alleles noted in CDHR1 previously PMID:38540785.	PM2_supporting	Multiple lines of computational evidence suggests no impact on gene/gene product	Revel- 0.16, Alphasense- 0.101. Both scores agree on Benign_moderate strength according to PMID: 36413997 and 37733863.	BP4_moderate
<i>GUCY2D</i>	NM_000180.4	c.2480A>C	p.Tyr827Ser	Absent or extremely low frequency in population databases	Not in gnomAD or LOVD.nl. To the best of our knowledge, this variant has been reported once in literature (PMID: 35260635) and has one ClinVar report from this paper. Variant only found in 40G pedigree in Target 5000	PM2_supporting	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	Revel- 0.819, MetaLR- 0.8621, M-CAP- 0.7491, Alphasense- 0.9721. The REVEL score surpasses the threshold for moderate which is 0.773-0.932 PMID: 36413997, as does the Alphasense score (0.8976-0.9784) PMID:37733863.	PP3_moderate
<i>PROM1</i>	NM_006017.3	c.1354dup	p.Tyr452LeufsTer13	Absent or extremely low frequency in population databases	Total allele frequency is 0.0003312 gnomAD v4.1.0 with no homozygotes. Suggested cutoff threshold is 0.0005 based on most frequent pathogenic variant. There are 53 submissions to LOVD, and 14 submissions to ClinVar that contribute to the classification.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in sequence encoding transmembrane region of PROM1 leading to the creation of a premature stop codon 13 amino acid residues downstream, and is predicted to undergo NMD as it is in exon 12/28. LOF is a known mechanism of disease.	PVS1
<i>PROM1</i>	NM_006017.3	c.2446G>C	p.Ala816Pro	Absent or extremely low frequency in population databases	Total allele frequency is 0.00005461 gnomAD v4.1.0 with no homozygotes. Suggested cutoff threshold is 0.0005 based on most frequent pathogenic variant.	PM2_supporting	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	Revel- 0.41, MetaLR- 0.41, MCAP-0.04184, Alphasense- 0.9736. The score for Alphasense passes threshold 0.8976-0.9784 for moderate PMID:37733863. But the REVEL score is uncertain.	PP3

4.5 Detailed ACMG/AMP variant classification of *CDHR1*, *GUCY2D*, and *PROM1* variants (Records continued from previous page, 2/3).

Gene	Transcript	Variant		Functional data			Segregation data		
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score
<i>CDHR1</i>	NM_033100.4	c.562G>A	p.Gly188Ser	N/A	Variant occurs in sequence encoding cadherin 2 domain and has been described as a 'hypomorphic missense variant' PMID:38540785 where they also classified this variant as a VUS	N/A	N/A	N/A	N/A
<i>CDHR1</i>	NM_033100.4	c.743del	p.Gly248fs	N/A	N/A	N/A	N/A	N/A	N/A
<i>CDHR1</i>	NM_033100.4	c.2228G>A	p.Arg743Gln	N/A	N/A	N/A	N/A	N/A	N/A
<i>GUCY2D</i>	NM_000180.4	c.2480A>C	p.Tyr827Ser	N/A	Variant is located within the HNOBA domain. This is conserved across multiple species. The HNOBA domain works synergistically with HNOB domain which is involved in heme-binding, and is found in the N-terminal extensions of the soluble guanylyl cyclases like GUCY2D PMID: 12590654. This region spans from 820-865 amino acid position. This is not a very well studied domain but has been shown to be conserved in animals and bacteria, with a phyloP100 score of 9.091.	N/A	Co-segregation with disease in multiple affected family members	For the 40G family we have genotype info for 8 affected family members. There are 3 affected individuals that we do not have genotype info for, as well as 7 unaffected informative individuals. According to PMID: 27236918, we will apply PP1_strong here due to presence of 9 informative meioses. Will not add PP4 here due to phenotypic overlap with other genes, but have confirmed with clinicians that GUCY2D is appropriate for this pedigree.	PP1_strong
<i>PROM1</i>	NM_006017.3	c.1354dup	p.Tyr452LeufsTer13	N/A	N/A	N/A	N/A	N/A	N/A
<i>PROM1</i>	NM_006017.3	c.2446G>C	p.Ala816Pro	N/A	N/A	N/A	N/A	N/A	N/A

4.5 Detailed ACMG/AMP variant classification of *CDHR1*, *GUCY2D*, and *PROM1* variants (Records continued from previous page, 3/3).

Gene	Transcript	Variant		Allelic data			PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	
		c.	p.	ACMG guideline	Comment	Score									
<i>CDHR1</i>	NM_033100.4	c.562G>A	p.Gly188Ser	For recessive disorders, detected in trans with a pathogenic variant	Reported in trans with a likely pathogenic/pathogenic variant in patients considered 'genetically solved'; 1 patient in PMID:38540785 (assumed in trans) 0.5pts, 1 family with 1 patient in PMID:37728066 (confirmed in trans with parents segregation) 1 pt. Will apply 1 point for patient 37C.	PM3_Strong	0	4	0	1	0	0	0	5	Hot VUS
<i>CDHR1</i>	NM_033100.4	c.743del	p.Gly248fs	N/A	Found in trans with Hot VUS in one patient 37C	N/A	8	0	0	1	0	0	0	9	Likely pathogenic
<i>CDHR1</i>	NM_033100.4	c.2228G>A	p.Arg743Gln	N/A	Confirmed in trans with novel likely pathogenic variant in patient 37C, where classification of second variant has not been validated. It is also in cis with a VUS acting in a complex allele.	N/A	0	0	0	1	0	0	-2	-1	Likely benign
<i>GUCY2D</i>	NM_000180.4	c.2480A>C	p.Tyr827Ser	N/A	Dominant acting variant	N/A	0	4	2	1	0	0	0	7	Likely pathogenic
<i>PROM1</i>	NM_006017.3	c.1354dup	p.Tyr452LeufsTer13	For recessive disorders, detected in trans with a pathogenic variant	As this variant is seen in trans in 47P pedigree with a VUS variant, the max points that can be awarded is 0.25 for the proband in this family. But this variant is present in 8 families total in Target 5000 including 1 homozygote. 2 homozygotes are also reported in PMID: 38540785.	PM3	8	0	2	1	0	0	0	11	Pathogenic
<i>PROM1</i>	NM_006017.3	c.2446G>C	p.Ala816Pro	For recessive disorders, detected in trans with a pathogenic variant	Only reported once in literature to the best of our knowledge PMID: 36819107. This variant is seen in trans in 47P pedigree with a well-reported pathogenic variant in 2 affected members of the family, but only 1 proband.	PM3	0	0	2	2	0	0	0	4	Warm VUS

4.6 Detailed ACMG/AMP variant classification of *USH2A* variants (Records divided into 2 tables below, 1/2). Each coloured heading represents a different group of data used. Below each data heading, the ACMG guideline applied, comments and name of criterion applied are listed in separate columns. Pathogenic criteria that were applied are highlighted in red, with the final 9 columns depicting the number of points awarded for each criterion applied as per guidelines. The classification is listed in the final column. (NMD= nonsense mediated decay, P=pathogenic criteria, B=benign criteria).

Gene	Transcript	Variant		Population data			Computational and predictive data		
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score
<i>USH2A</i>	NM_206933.2	c.655delC	p.His219Ilefs*24	Absent or extremely low frequency in population	Not in gnomAD. No reports on LOVD.nl, ClinVar or in literature.	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in exon 4/72, meaning that NMD is predicted to occur. This variant deletes one base in exon 4, leading to a premature stop to be created 24 amino acids downstream, where LOF is a known mechanism of disease.	PVS1
<i>USH2A</i>	NM_206933.2	c.2276G>T	p.Cys759Phe	MAF is too high for disorder	Filtering allele freq in gnomad v4.1.0 is 0.001460. (BS1_supporting category cutoff ≥0.0007 (0.07%) PMID:30311386). While BS1 has been met here, the <i>USH2A</i> VCEP expert panel suggested that it is still enriched in UsherII and RP populations, and might just be associated with a lower penetrance. They recommend use of PS4, but we will lower the strength to be cautious.	BS1_supporting, PS4_moderate	Multiple lines of computational evidence support a deleterious effect on the gene/gene product	REVEL- 0.902, MetaLR- 0.9471, MCAP- 0.6951, Alphamissense- 0.9091. The score for Alphamissense passes 0.8976-0.9784 for moderate pathogenicity (Zhuo, Aggarwal and Liao, 2024). REVEL score surpasses the threshold for moderate which is 0.773-0.932 (Pejaver et al., 2022). Will keep at PP3 level as VCEP indicates not to alter strength	PP3
<i>USH2A</i>	NM_206933.2	c.2299delG	p.Glu767Serfs*21	MAF is too high for disorder	Filtering allele freq in gnomad v4.1.0 is 0.0009474, with 2 homozygotes. (BS1_supporting category cutoff ≥0.0007 (0.07%) PMID:30311386). While BS1 has been met here, the <i>USH2A</i> VCEP expert panel suggested that it is still enriched in UsherII and RP populations, and might just be associated with a lower penetrance. They recommend use of PS4, but we will lower the strength to be cautious.	BS1_supporting, PS4_moderate	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in exon 13/72, meaning that NMD is predicted to occur. This variant deletes one base in exon 13, leading to a premature stop to be created 21 amino acids downstream, where LOF is a known mechanism of disease.	PVS1
<i>USH2A</i>	NM_206933.2	c.4714C>T	p.Leu1572Phe	MAF is too high for disorder	Filtering allele frequency in gnomad v4.1.0 is 0.001074, with 2 homozygotes. (BS1_supporting category cutoff ≥0.0007 (0.07%) PMID:30311386).	BS1_supporting	N/A	REVEL- 0.66, MetaLR- 0.7669, MCAP- 0.3484, Alphamissense- 0.188. MCAP and MetaLR are both uncertain. Alphamissense does not pass any score for pathogenic criteria but does classify as benign_supporting under PMID: 37733863. The VCEP specific guidelines indicate PP3 can only be applied if REVEL>0.7, or BP4 if REVEL<0.15.	N/A
<i>USH2A</i>	NM_206933.2	c.6721C>T	p.Pro2241Ser	Absent or extremely low frequency in population databases	Not in gnomAD, LOVD.nl or in the literature. 3 reports on clinvar- 2 VUS, 1 likely pathogenic.	PM2_supporting	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	REVEL- 0.48, MCAP- 0.3209, MetaLR- 0.5691, Alphamissense- 6935, all scores inconclusive. Sequence change replaces proline, which is neutral and non-polar, with serine, which is neutral and polar, at codon 2241 of the <i>USH2A</i> protein (p.Pro2241Ser). 3 other reported variants at 2241 amino acid location, all likely pathogenic/pathogenic (Pro2241Thr), (Pro2241His), (Pro2241Leu) with similar REVEL scores to our variant (0.46, 0.53, 0.46 respectively).	PMS
<i>USH2A</i>	NM_206933.2	c.9391dupG	p.Val3131Glyfs*14	Absent or extremely low frequency in population databases	Not in gnomAD or LOVD.nl. 5 pathogenic/likely pathogenic reports on ClinVar. Variant reported only once before in PMID: 34906470	PM2_supporting	Predicted null variant in a gene where LoF is a known mechanism of disease	Variant occurs in exon 48/72, meaning that NMD is predicted to occur. This variant duplicates one base in exon 48, leading to a premature stop to be created 14 amino acids downstream, where LOF is a known mechanism of disease.	PVS1

4. 6 Detailed ACMG/AMP variant classification of *USH2A* variants (Records continued from previous page, 2/2).

Gene	Transcript	Variant		Segregation data			Allelic data			PVS points	PS points	PM points	PP points	BA points	BS points	BP points	Points sum	
		c.	p.	ACMG guideline	Comment	Score	ACMG guideline	Comment	Score									
<i>USH2A</i>	NM_206933.2	c.655delC	p.His219Ilefs*24	N/A	More family members required	N/A	For recessive disorders, detected in trans with a pathogenic variant	Confirmed in trans with a pathogenic variant in a single patient (57U) -1pt.	PM3									
										8	0	2	1	0	0	0	11	Pathogenic
<i>USH2A</i>	NM_206933.2	c.2276G>T	p.Cys759Phe	Co-segregation with disease in multiple affected family members	2 members of 53U family are affected. A second family in Target 5000 have 1 affected sister with 2 unaffected sisters. Will not add PP4 here due to phenotypic overlap with other genes, but have confirmed with clinicians that <i>USH2A</i> is appropriate for this pedigree.	PP1_moderate	For recessive disorders, detected in trans with a pathogenic variant	Reported in 10 individuals with Usher II who were all confirmed compound heterozygous with a second pathogenic variant (PMIDs: 16098008, 24944099, 29912909). 53U proband confirmed variant in trans with a pathogenic complex allele, and 59U proband is homozygous for this variant as part of a complex allele.	PM3_Strong	0	4	4	1	0	0	-1	8	Likely pathogenic
<i>USH2A</i>	NM_206933.2	c.2299delG	p.Glu767Serfs*21	N/A	More family members required	N/A	For recessive disorders, detected in trans with a pathogenic variant	31 reports outside of 53U pedigree of this variant in Target 5000, with 2 homozygotes for this variant (1pt each). In Panneman et al. 2023, this variant was observed in 17 cases where phase was assigned, therefore scoring is slightly altered.	PM3_VeryStrong	16	0	2	0	0	0	-1	17	Pathogenic
<i>USH2A</i>	NM_206933.2	c.4714C>T	p.Leu1572Phe	N/A	Always in cis with c.2299delG in Target 5000 and literature	N/A	Observed in cis with a pathogenic variant	3 homozygotes in Target 5000, where it is always in cis with c.2299delG. 31 instances total in Target 5000 of this allele, all reported with 2299delG.	BP2	0	0	0	0	0	0	-2	-2	Likely benign
<i>USH2A</i>	NM_206933.2	c.6721C>T	p.Pro2241Ser	N/A	N/A	N/A	Observed in cis with a pathogenic variant	We have 59U homozygote but is in cis with another variant c.2276G>T homozygously, as part of a complex allele. This variant has not been reported previously. 1 report to ClinVar where this variant has been identified in the same pattern, in cis with c.2276/p.Cys759Phe - Novel Complex allele	BP2	0	0	2	1	0	0	-1	2	Cool VUS
<i>USH2A</i>	NM_206933.2	c.9391dupG	p.Val3131Glyfs*14	N/A	N/A	N/A	N/A	Confirmed in trans with novel variant where classification has not been validated	N/A	8	0	0	1	0	0	0	9	Likely pathogenic

4.7 ABCA4 variant severities Severities awarded to the ABCA4 variants in previous large-scale studies, for the variants that have been identified in 7 pedigrees analysed within Chapter 2 of this thesis (Cornelis *et al.*, 2022, 2023). The single variant highlighted in red is a novel variant.

Gene	Allele cDNA	Allele protein	In-house Classification	Severity category from (Cornelis et al. 2022, 2023)
ABCA4	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	Pathogenic	Severe
ABCA4	c.302+68C>T	p.?	VUS (Cool)	//
ABCA4	c.3056C>T	p.Thr1019Met	Pathogenic	Severe
ABCA4	c.3113C>T	p.Ala1038Val	Likely pathogenic	Mild
ABCA4	c.3322C>T	p.Arg1108Cys	Pathogenic	Moderate
ABCA4	c.3329-1G>A	p.?	Likely Pathogenic	Severe
ABCA4	c.4222T>C	p.Trp1408Arg	Pathogenic	Severe
ABCA4	c.4363T>C	p.Cys1455Arg	Pathogenic	Severe
ABCA4	c.4539+2028C>T	p.[=,Arg1514Leufs*36]	Pathogenic	Moderate
ABCA4	c.4918C>T	p.Arg1640Trp	Pathogenic	Severe
ABCA4	c.5461-10T>C	p.[Thr1821Aspfs*6,Thr1821Valfs*13]	Pathogenic	Severe
ABCA4	c.5594dupA	p.His1865Glnfs*47	Pathogenic	//
ABCA4	c.5603A>T	p.Asn1868Ile	VUS	Mild
ABCA4	c.5773A>G	p.Arg1925Gly	Likely Pathogenic	//
ABCA4	c.5882G>A	p.Gly1961Glu	Pathogenic	Mild

4.8 List of all primers used to confirm candidate variants

Gene	c.notation for variant covered	forward	reverse	product size
ABCA4	c.161G>A	TAAGAGGTTAGGGGCTCAGC	GAGCTTGAGAGAAGAGGCCA	473bp
ABCA4	c.302+68C>T	TGATTCACTCCACCCAAGCA	AAGGCCATTTCCCAACAAG	474bp
ABCA4	c.3056C>T	CACCCTTAGAAGCTCTCCTG	CAGTGAGCATCTTGATTGC	360bp
ABCA4	c.3113C>T	CACCCTTAGAAGCTCTCCTG	CAGTGAGCATCTTGATTGC	360bp
ABCA4	c.3322C>T	GTCACGTATAAACCCCTTCTG	GCACCAAACTGCTGG	385bp
ABCA4	c.3329-1G>A	GAGCCTGTGTGAGTAGCCAT	ACTGTGTGTGGCAATGCTTT	509bp
ABCA4	c.4222T>C	AGTGAAGTGGGAAGGTCAGG	CTGATTAGGAGGCTGCAGGA	329bp
ABCA4	c.4363T>C	TGAAGCTTTTGGTGAGTGCC	CTTGAGCAGAAACAGCCTC	544bp
ABCA4	c.4539+2028C>T	GTTTTGCTCCAATGGAAGGC	TGAAGCAAAACAGAGGGCAG	702bp
ABCA4	c.4918C>T	TCCAGCTACCAACCCCTTCTGCT	CCAAATCAGCACTTCGCGGTGG	373bp
ABCA4	c.5461-10T>C	CCCTAATCCTCTCCAGCTGG	ACCTCCATTGCAAAACCATG	461bp
ABCA4	c.5594dupA	GCCAGGCTTTGAGAATGTAGA	CTGGAGAGGATTAGGGTGCC	456bp
ABCA4	c.5603A>T	GCCAGGCTTTGAGAATGTAGA	CTGGAGAGGATTAGGGTGCC	456bp
ABCA4	c.5773A>G	GATTCTCACTGAGGCCCTGT	ACATGAGCACCTGACCTCTC	541bp
ABCA4	c.5882G>A	TCTCATGTGGCTAGTGGAAGA	TGTGCCTGCCATGTCAATA	352bp
CDHR1	c.562G>A	TGTAATGAGGGGCAGTGAGG	TGGCCCATAGTTTCGAGGA	442bp
CDHR1	c.743del	GCAAAGGAATGGTGGCACTT	AGCTCATGGTCTAGTTGGGG	536bp
CDHR1	c.2228G>A	CTCTTTCAGACCCCTCTCCCG	GACTIONAGCCCTGTCAGAGC	742bp
GUCY2D	c.2480A>C	GCATAAAGAGGGCATGGCAA	ATGGCAGAGATGGTGGTGAA	428bp
PROM1	c.1354dupT	GATCTCTCCTCTCGCGAC	GAAGGCAAGGGGTGTGAAGG	474bp
PROM1	c.2446G>C	TCTTTGAAGACAGCACACC	AAATGAATCCTAACAATGTGCGT	375bp
USH2A	c.655delC	AGCCCTAGAAGATGAATACACGT	ACACATGGAGATATCACTGAGCT	406bp
USH2A	c.2276G>T	GCAAGGCAGACAGAGGAAA	AGCATTGTTTGTGTCTCGTC	450bp
USH2A	c.2299delG	GCAAGGCAGACAGAGGAAA	AGCATTGTTTGTGTCTCGTC	450bp
USH2A	c.4714C>T	CTGAGGGCAAGTCACATTACT	GGCCTAGACCATGCGAGTAT	458bp
USH2A	c.6721C>T	GGCTCTGACTTAAGATGC	AAGGGAGCACTTTTGAGC	613bp
USH2A	c.9391dupG	CCTTGCAAGCTCATCACTC	TAAACCACCTCACGCCATGT	336bp

Chapter 5

5.1 List of all primers used to carry out midigene splice assays for *RPGR* and *ABCA4* variants

Gene	exon(s) covered	purpose of primer set design	forward	reverse	product size
ABCA4	38-41	long range PCR primer to attach tags for BP step onto ends of fragment of interest	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCG GGGTTACATCAGCAGAGTT	GGGGACCACTTTGTACAAGAAAGCTGGGTGG TGACAGAGGTGTGGTGAGTACT	6859bps
ABCA4	39	segregation analysis and to confirm wildtype/mutant insert in midigene construct	CCCTAATCCTCTCCAGCTGG	ACCTCCATTGCAAACCCATG	461bp
RPGR	10-13	long range PCR primer to attach tags for BP step onto ends of fragment of interest	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCG GACCTCATTATTGAGGAC	GGGGACCACTTTGTACAAGAAAGCTGGGTGC CTACCCATGAACAGTCTCT	9497bp
RPGR	11	segregation analysis and to confirm wildtype/mutant insert in midigene construct	GGCACATTTATCCTGAGAGCA	AATGTTGTGGAGTGTGGCA	336bps
pCI-NEO-RHO backbone	//	RT-PCR primers that bind in the pCI-NEO-RHO backbone	CGGAGGTCAACAACGAGTCT	AGGTGTAGGGGATGGGAGAC	//
RHO	5	RHO exon 5 control primers	ATCTGCTGCGGCAAGAAC	AGGTGTAGGGGATGGGAGAC	140bp
β -actin	4	β -actin exon 4 control primers	ACTGGGACGACATGGAGAAG	TCTCAGCTGTGGTGGTGAAG	309 bp

5.2 Ocular phenotyping data describing Visual acuity (VA), Electroretinogram (ERG) readings, Visual fields and Colour vision for patients carrying *RPGR* and *ABCA4* splice-altering variants (Records divided into 2 tables below, 1/2). (OD = right eye, OS = left eye).

Patient	Male/Female	Clinical phenotype	Visual Acuity (VA)			Electroretinogram (ERG)				
			Visual Acuity OD (right)	Visual Acuity OS (left)	Lens status (phakic/cataract/pseudophakic)	Dark adapted rod isolated b wave amplitude - OD (uV)	Dark adapted rod isolated b wave amplitude - OS (uV)	Light adapted cone b wave amplitude - OD (uV)	Light adapted cone b wave amplitude - OS (uV)	Light adapted 30 Hz - OD (uV)
1.5	M	XLRP	hand movements	hand movements	cataract	//	//	//	//	//
7.5	M	XLRP	6/24	6/15	//	non-recordable	non-recordable	7.51	4.17	non-recordable
5.5	M	XLRP	6/38	6/38	cataract	non-recordable	non-recordable	3.71	2.92	5.4
9.5	M	XLRP	6/15	6/9.5	cataract	non-recordable	non-recordable	non-recordable	non-recordable	non-recordable
11.5	M	Classical STGD1/Fundus flavimaculatus	6/48	6/12	normal	311	211	148	134	78.3
12.5	F	Classical STGD1/Fundus flavimaculatus	6.7.5	6/7.5	normal	366	268	227	173	108

5.2 (Records continued, 2/2).

Patient	Electroretinogram (ERG)		Visual fields		Colour vision	
	Light adapted 30 Hz - OS (uV)	Any other details/comments on ERG	Peripheral or central field loss? (age of onset)	Any other details/comments on Visual fields	Abnormalities to colour vision (yes/no, axis of dysfunction)	Lanthonys Desaturated 15 hue test error score
1.5	//	Non-recordable ERG	Both	No response recorded even to the largest v4e targets	//	//
7.5	non-recordable	Results indicate advanced panretinal photoreceptor dysfunction	Peripheral, 7yrs	Marked concentric visual field constriction, within 10 degrees of fixation in both eyes.	//	//
5.5	3.13	Results indicate advanced panretinal photoreceptor dysfunction	Peripheral in teens and central in mid 30s	Marked concentric visual field constriction, within 5 degrees of fixation in both eyes. Small island of peripheral vision in both eyes at IV4e target.	Yes, diffuse in left eye and tritan in right eye	302 OD and 338 OS
9.5	non-recordable	Results indicate advanced panretinal photoreceptor dysfunction	Peripheral, mid 20s	Marked concentric visual field constriction, within 10 degrees of fixation in both eyes.	//	//
11.5	80.6	Multifocal ERG indicates foveal and parafoveal segments attenuated in both eyes.	Central, mid 30s	Central scotoma noted in left eye. Otherwise fields look alright.	Yes, diffuse in left eye only	Normal OD and 228 OS
12.5	101	Multifocal ERG indicates foveal and parafoveal segments attenuated in both eyes.	Central, mid 40s	//	//	//

5.3 Whole plasmid sequencing of expression clones analysed in *RPGR* constructs.

SpliceAI score was not reported if it was zero. Our variant of interest c.1307A>G is highlighted in purple. (AG = acceptor gain, y = SNP noted in this construct, n = SNP not noted in this construct).

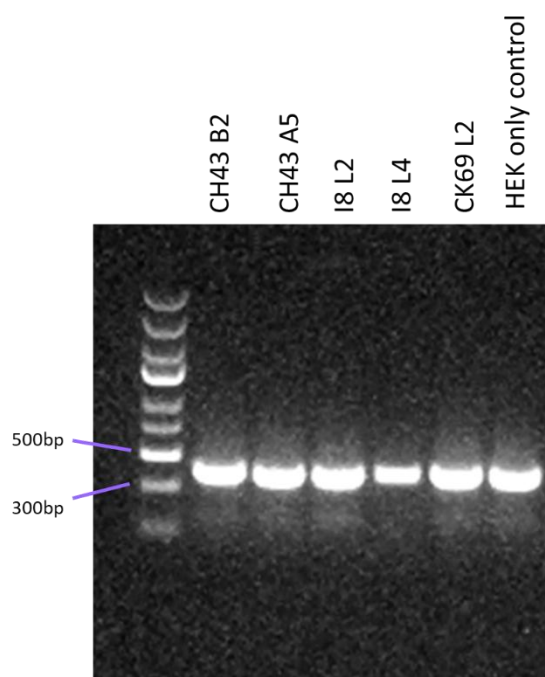
chromosome	genomic location	c.notation	SpliceAI score	Franklin Classification	I8 L4mut	CK69 L2wt
chrX	38,159,002insT	c.1060-599dup		Benign	y	n
chrX	38,158,875A>G	c.1060-481T>C		Likely benign	n	y
chrX	38,158,025T>C	c.1060-408A>G		Likely benign	y	n
chrX	38,158,148insA	c.1245+67dup	AG, 0.01	Likely benign	y	n
chrX	38,157,799A>G	c.1245+410T>C		Likely benign	y	n
chrX	38,157,530insT	c.1245+686dup		Likely benign	y	y
chrX	38,157,491C>A	c.1245+718G>T		Likely benign	n	y
chrX	38,157,444A>G	c.1246-739T>C		Likely benign	n	y
chrX	38,156,866T>C	c.1246-161A>G		Likely benign	y	n
chrX	38,156,732T>C	c.1246-27A>G		Likely benign	n	y
chrX	38,156,644C>T	c.1307G>A	AG, 0.41	VUS	y	n
chrX	38,156,305T>C	c.1414+232A>G		Likely benign	n	y
chrX	38,155,225T>C	c.1414+1312A>G		Likely benign	y	n
chrX	38,155,169A>G	c.1414+1368T>C		Likely benign	n	y
chrX	38,154,975T>C	c.1414+1562A>G		Likely benign	n	y
chrX	38,154,428T>C	c.1414_2109A>G		Likely benign	n	y
chrX	38,152,787A>G	c.1415-2050T>C	AG, 0.01	Likely benign	y	n
chrX	38,151,639A>G	c.1415-902T>C		Likely benign	y	n
chrX	38,151,476T>C	c.1415-739A>G		Likely benign	n	y
chrX	38,151,254A>G	c.1415-517T>C	AG, 0.02	Benign	y	n
chrX	38,151,239delCT	c.1415-492_1415-491del		Likely benign	y	n
chrX	38,151,150A>G	c.1415-413T>C		Likely benign	y	n
chrX	38,150,412A>G	c.1507-135T>C		Likely benign	y	n
chrX	38,150,399delTA	c.1507-107_1507-106del		Likely benign	n	y

5.4 Whole plasmid sequencing of expression clones analysed in *ABCA4* constructs.

SpliceAI score was not reported if it was zero. Our variant of interest c.1307A>G is highlighted in purple. (AG = acceptor gain, DG = donor gain, y = SNP noted in this construct, n = SNP not noted in this construct).

chromosome	genomic location	c.notation	SpliceAI score	Franklin Classification	CH43 A5mut	CH43 B2wt
chr1	94,480,846del	c.5312+452del		Benign	n	y
chr1	94,480,734T>C	c.5313-488A>G		Benign	n	y
chr1	94,480,681A>G	c.5313-435T>C		Benign	n	y
chr1	94,480,529A>C	c.5313-283T>G		Benign	y	n
chr1	94,480,439T>C	c.5313-193A>G		Benign	y	n
chr1	94,480,037C>T	c.5460+62G>A		Benign	y	n
chr1	94,479,751_94,479,762del	c.5460+338_5460+348del		Likely benign	y	n
chr1	94,479,725T>A	c.5460+374A>T		Benign	n	y
chr1	94,479,667T>C	c.5460+432A>G		Likely benign	y	n
chr1	94,479,617A>G	c.5460+482T>C		Likely benign	n	y
chr1	94,479,468C>T	c.5460+631G>A		Benign	y	n
chr1	94,479,338C>T	c.5460+761G>A		Benign	y	n
chr1	94,478,891delAG	c.5460+1205_5460+1208del		Benign	y	y
chr1	94,478,847G>A	c.5460+1252C>T		Benign	y	y
chr1	94,478,562G>C	c.5460+1537C>G		Benign	y	y
chr1	94,478,425C>T	c.5461-1484G>A	DG, 0.04	Benign	y	y
chr1	94,478,348A>G	c.5461-140T>C		Benign	n	y
chr1	94,478,203C>T	c.5461-1262G>A		Benign	n	y
chr1	94,477,893T>C	c.5461-952A>G		Benign	y	y
chr1	94,477,728C>T	c.5461-787G>A		Benign	n	y
chr1	94,477,649C>G	c.5461-708G>C		Benign	y	y
chr1	94,477,634G>A	c.5461-693C>T	AG, 0.01	Benign	y	y
chr1	94,477,577A>G	c.5461-636T>C		Likely benign	y	n
chr1	94,477,295delCG	c.5461-355_5461-354del		Likely benign	y	y
chr1	94,477,278C>T	c.5461-337G>A		Benign	y	y
chr1	94,477,232T>G	c.5461-291A>C		Benign	y	y
chr1	94,476,992del	c.5461-51del		Benign	y	y
chr1	94,476,812del	c.5584+5del		VUS	y	n
chr1	94,476,695C>T	c.5584+123G>A		Benign	y	y
chr1	94,476,467T>A	c.5603A>T		VUS	n	y
chr1	94,476,432A>G	c.5638T>C		VUS	y	n
chr1	94,476,388C>G	c.5682G>C		Benign	y	y
chr1	94,476,204del	c.5714+151del		Likely benign	y	y
chr1	94,476,035C>T	c.5714+321G>A		Benign	y	y
chr1	94,475,901del	c.5714+460del		Benign	y	y
chr1	94,474,727A>G	c.5715-300T>C		Benign	y	n
chr1	94,474,647G>C	c.5715-220C>G		Benign	y	n
chr1	94,474,452T>G	c.5715-25A>C		Benign	y	n
chr1	94,474,328T>C	c.5814A>G		Benign	y	n
chr1	94,474,270C>T	c.5835+37G>A		Likely benign	y	y
chr1	94,474,185A>C	c.5835+122T>G		Benign	y	y
chr1	94,474,020G>A	c.5836-167C>T	AG, 0.05	Benign	y	y

5.5 Gel images of control RT-PCRs with *Beta Actin* primers for all cDNA



RT-PCRs were run as per Materials and Methods 6.3. Expected band is 309 bps in size, with ladder sizes indicated on the left. GeneRuler Express DNA ladder was used for band sizing.

9. References

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