

# On the genotype and phenotype of inherited retinal degenerations in an Irish population

Thesis for the Degree of Doctor in Medicine (M.D.)

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Dr. Emma Duignan, August 2019

# **Publications and Presentations Arising From This Work**

## **Peer-reviewed publications**

### **Panel-Based Population Next-Generation Sequencing for Inherited Retinal Degenerations.**

Carrigan M, Duignan E, Malone CP, Stephenson K, Saad T, McDermott C, Green A, Keegan D, Humphries P, Kenna PF, Farrar GJ. Scientific Reports 2016 Sep 14;6:33248.

### **A novel homozygous truncating GNAT1 mutation implicated in retinal degeneration.**

Carrigan M, Duignan E, Humphries P, Palfi A, Kenna PF, Farrar GJ. British Journal of Ophthalmology 2016 Apr;100(4):495-500.

## **Presentations**

### **Genotype-phenotype correlations of Usher syndrome patients in an Irish cohort**

Duignan E, Carrigan M, Malone C, Farrar GJ, Kenna P  
BIPOSA (British and Irish Paediatric Ophthalmology and Strabismus Association) meeting, Dublin 2016

### **Further evidence suggesting pathogenicity of the PROM1 mutation (S649L) in cone-rod dystrophy**

Duignan E, Carrigan M, Farrar GJ, Kenna P  
ARVO (Association for Research in Vision and Ophthalmology) meeting, Denver, United States, May 2015

### **Genotype-phenotype correlations of Usher syndrome patients attending the RVEEH Research Foundation.**

Duignan E, Farrar GJ, Kenna P

Eithne Walls Research Meeting, Royal Victoria Eye and Ear Hospital Dublin, July  
2015

**Discovery of a homozygous recessive RPE65 mutation in an Irish  
choroideraemia pedigree.**

Duignan E, Carrigan M, Farrar GJ, Kenna P

Irish College of Ophthalmologists meeting, May 2015

**A novel mutation of the GNAT1 gene identified in a patient with late-onset  
retinitis pigmentosa.**

Duignan E, Carrigan M, Farrar GJ, Kenna P

Royal Academy of Medicine in Ireland, Ophthalmology section, meeting March  
2015

**Next Generation Sequencing: PROM1 mutations identified in a cohort of  
retinopathy patients**

Duignan E, Carrigan M, Farrar GJ, Kenna P

Retina meeting, Dublin November 2014

## Summary of Thesis

This thesis describes my contribution to the task of carrying out genotyping and deep phenotyping of all patients with inherited retinal degenerations in Ireland. Huge leaps in technology have allowed us to characterise these patients in great detail and to sequence their genetic data quickly and sift through it precisely. The name given to this project is Target 5000, a collaborative effort to collect data on all affected patients on the island of Ireland. My contribution, which consisted of the deep phenotyping of over 150 patients, was carried out in the research foundation of the Royal Victoria Eye and Ear Hospital from July 2014 to July 2015. Deep phenotyping describes the process of history taking, physical examination, performance of psychophysical testing and performance of electrophysiological tests and retinal imaging.

The introduction will address relevant concepts to give context to the results that follow. The materials and methods chapter describes the ways in which patients are assessed both clinically and molecularly. The third chapter will provide an overview of the Target 5000 project to date and how this data contributes to the international dataset of patients with inherited retinal degeneration. Following this, three further chapters will highlight individual pedigrees from this project which showcase the findings arising from my work.

The fourth chapter describes the case of a patient with a late-onset retinitis pigmentosa phenotype who was found to have a novel GNAT1 mutation. This case is interesting as GNAT1 mutations have only been described to cause

congenital stationary night blindness in the past and this is both a novel mutation to the literature and a novel phenotype for this gene.

The fifth chapter describes the case of a female patient with a biallelic PROM1 mutation, p.S649L, which we feel is likely to be the cause of her cone-rod dystrophy. This variant has been observed before, homozygously, in one patient in a series from The Netherlands, and one further case has been observed in Germany. The variant was also observed homozygously in a control subject. This raises the issue of difficulties in identifying pathogenic variants and managing variants of uncertain significance.

The sixth chapter describes a choroideraemia pedigree which was found to have three members with a biallelic RPE65-mediated retinal dystrophy, which came to light only after next generation sequencing was carried out. This pedigree highlights the value of molecular diagnosis and the difficulty of diagnosing based on phenotype.

The final chapter of this thesis discusses future directions of this work. A gene-based therapy for RPE65-mediated retinal dystrophy has now been approved for use in both the U.S. and the E.U., and a plethora of clinical trials of therapies are underway for the treatment of these conditions. We hope that we can look forward to a future where there is a treatment available for every patient with an inherited retinal degeneration. Until that time comes, providing patients with as much information about their condition as possible by phenotyping and molecular

diagnosis can inform genetic counselling, family planning and the planning of their life.

## **Dedication**

This thesis is dedicated to my husband Max, my parents Éilis and Joe, my magnificent daughter Freya and all the courageous patients with inherited retinal degenerations in Ireland, including those who were kind enough to take part in this work.

## **Acknowledgements**

I would like to acknowledge my supervisor Dr. Paul Kenna who I had the privilege of working with while I carried out this research and observed how he treated patients and colleagues with respect, kindness and empathy. His encyclopaedic knowledge and humility in the level of his involvement in advancing the cause of his patients and the science of their conditions have been an inspiration to me. He has a true legacy in both this field and in the hearts of the patients that he has generously given his time to throughout his and their lives.

I would like to acknowledge my supervisor Professor Lorraine Cassidy who I have had the great pleasure of knowing for almost a decade, since I first started working in the Eye and Ear Hospital. I'm grateful for her continued support and caring nature and I, along with all of her patients, have been a lucky beneficiary of her kindness and wisdom through the years and look forward to many more years of friendship.

I would like to acknowledge the fantastic work of Dr. Matthew Carrigan, Dr. Adrian Dockery and Professor G. Jane Farrar in the Trinity lab whose continued efforts to elucidate the basis of these conditions and develop novel therapies to treat them are bringing patients ever closer to having access to effective treatments for their disease. Thank you all for your help and support in preparing this thesis.

I would like to acknowledge all of my colleagues and friends in the Eye and Ear Hospital who made this work possible by teaching me skills in electrophysiology



and retinal imaging including Ms. Hilary Dempsey, Ms. Karen Collins, Mr. Hugh Nolan and Mr. Stephen Comiskey.

Finally, I would like to thank the charity Fighting Blindness for supporting my work and for their determined pursuit of their aim to cure, support and empower people living with visual impairment.

## Abbreviations encountered in this thesis

AAV	Adeno-associated Virus
AMD	Age-related Macular Degeneration
ATP	Adenosine Triphosphate
BCVA	Best Corrected Visual Acuity
CÉ	Conformité Européenne
cGMP	Cyclic Guanosine Monophosphate
CSNB	Congenital Stationary Night Blindness
DA	Dark-adapted
DAT	Dark Adaptation Threshold
DNA	Deoxyribonucleic Acid
DTL	Dawson, Trick and Litzkow
EDTA	Ethylenediamine Tetraacetic Acid
EOG	electrooculogram
ERG	Electroretinogram
ETDRS	Early Treatment Diabetic Retinopathy Study
ExAC	Exome Aggregation Consortium
GATK	Genome Analysis Toolkit
GCAP	Guanylate-cyclase Activating Protein
GDP	Guanosine diphosphate
gnomAD	Genome Aggregation Database
GTP	Guanosine Triphosphate
HGMD	Human Gene Mutation Database
ILM	Inner Limiting Membrane
	International Society for Clinical
ISCEV	Electrophysiology of Vision
LA	Light-adapted
LCA	Leber Congenital Amaurosis
LHON	Leber Hereditary Optic Neuropathy

logMAR	Logarithm of the Minimum Angle of Resolution
mfERG	Multifocal Electroretinogram
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NGS	Next Generation Sequencing
NPL	No Perception of Light
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PERG	Pattern Electroretinogram
PL	Perception of Light
RNA	Ribonucleic Acid
RP	Retinitis Pigmentosa
UTR	Untranslated Region
VEP	Visual Evoked Potential
VUS	Variant of Uncertain Significance
WES	Whole Exome Screening
WGS	Whole Genome Screening

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# Chapter 1: Introduction

## 1.1 Introduction

Inherited retinopathies are a genetically diverse range of diseases of the light sensitive tissue in the eye, the retina, and represent a significant cause of visual impairment, worldwide. The term ‘inherited retinopathy’ includes a broad spectrum of inherited retinal diseases, the most common of which is retinitis pigmentosa, which has an incidence of approximately 1 in 4000 worldwide (Hartong, Berson et al. 2006). The group also includes less prevalent diseases such as Stargardt disease, cone-rod dystrophy, choroideraemia, achromatopsia, congenital stationary night blindness, X-linked retinoschisis, and Leber congenital amaurosis, amongst others. Inherited retinal disease has now surpassed diabetic eye disease as the most common cause for blind registration amongst those of working age in England and Wales (Liew et al. 2014). Although similar data for Ireland is currently unavailable, it is likely that this will be the case in the near future as screening and treatment of diabetic eye disease becomes more successful.

As more is being learned about the genetic mutations underlying these diseases, it is increasingly acknowledged that some older, descriptive disease terms such as “retinitis pigmentosa” are inadequate in portraying the aetiology of retinal dystrophies. Whilst (non-syndromic) retinitis pigmentosa as a phenotype can be caused by over 80 genes, many of the same genes can cause other retinal dystrophy phenotypes, e.g. cone-rod dystrophy or Leber congenital amaurosis, and it may be more accurate then to diagnose patients in the future based on their affected gene and mutation.

In the late 1980s, the first gene causing an inherited retinal dystrophy was identified in Trinity College Dublin. This gene was the rhodopsin gene, identified in a pedigree of patients with autosomal dominant retinitis pigmentosa and located on chromosome 3 (Farrar, McWilliam et al. 1990). Since that time,

inherited retinal degenerations, and especially retinitis pigmentosa, have been revealed to have vast genetic heterogeneity (Ferrari, Di Iorio et al. 2011). To date, over 140 distinct mutations of this gene have been described and a further 80 genes are known to cause retinitis pigmentosa alone (Daiger 2019). Although some diseases, such as choroideraemia, are monogenic in nature, over 140 mutations have been identified in the underlying CHM gene (Retina International). Improvements in gene sequencing technology, most importantly next generation sequencing, have made identifying mutations faster, cheaper, and available on a larger scale in recent years, making the ability to diagnose the majority of patients' retinal degenerations at a molecular level possible.

Investigative pathways for treatments for retinal dystrophies are now growing in number. In the past, limited knowledge of the genetic mutations underlying these diseases, along with the biochemistry of how these mutations affected functional vision, precluded our abilities to propose and trial treatments. Today, treatment trials abound. Trials of therapy include gene-based therapies, which allow the missing or poorly functioning gene to be replaced in the retina; stem cell therapies, which allow poorly functioning cells to be replaced by fully differentiated cells of the same type; retinal prostheses, which bypass the light-sensing cells of the retina to stimulate the downstream cells in the retina, helping to stimulate the visual cortex of the brain; optogenetics, where the surviving non-light sensing cells of the retina are given the ability to sense light using light-activated channels and many pharmacological trials of increasing diversity e.g. vitamin A derivatives and neurotrophic factors (Athanasίου, Aguila et al. 2018). The approval of the first gene therapy, Luxturna, has proven that gene therapy can work and has boosted confidence in the field. The area is expanding rapidly, with many new trials commencing frequently worldwide. The application of different treatment modalities in the future will likely differ according to the type of degeneration and the severity of the disease in the affected patient, thus making accurate clinical assessment and precise phenotyping essential.

## 1.2 Aim and Research Objectives

The overarching aim of this thesis is to characterise the genotype and phenotype of Irish patients with inherited retinal degenerations. This will address several key aims. Firstly, it will allow us to provide an accurate molecular diagnosis to patients. In a rapidly evolving field, this is increasingly becoming the international standard of care for patients with retinal dystrophies. Secondly, it will allow us to provide higher quality genetic counselling to patients, giving them and relevant family members important information about what to expect with regard to inheritance patterns and, in certain circumstances, prognostic information based on recognised genotype-phenotype correlations. Finally, the information will enable Irish patients to access national and international trials of genetic therapies for inherited retinal degenerations, as a known molecular diagnosis with accurate phenotyping will be critical prior to treatment. Accurate phenotyping will be essential for current and future trials because a therapeutic window is increasingly recognised as an important factor in predicting whether or not a treatment will benefit a particular patient. It is estimated that there are now over 50 ocular gene therapies at the stage of preclinical animal studies ([clinicaltrials.gov](http://clinicaltrials.gov)). Recently in 2017, the first retinal gene therapy was approved by the FDA (United States Food and Drug Administration), Luxturna (voretigene neparvovec), which is administered via subretinal injection, to treat biallelic RPE65-related retinal dystrophy (U.S. Food and Drug Administration 2018). Luxturna has since gained approval from the European Medicines Agency and is licenced in Europe since November 2018 (European Medicines Agency 2019). Furthermore, multiple other human clinical trials of genetic therapies have commenced, the results of which have been positive, to date (Testa, Maguire et al. 2013, MacLaren, Groppe et al. 2014). Data from this current study will allow patients to be assessed so that they may be eligible to be included in such trials, allowing careful selection of those patients who are most likely to benefit from the intended therapies.

In contributing to the bank of data available to international researchers in this area, the work described in this thesis involves furthering worldwide knowledge of

inherited eye disease. Every mutation identified as disease-causing adds to the bank of evidence which is used to diagnose future patients. Large repositories of genetic data now exist, as international collaborative efforts increase with the pace of scientific advancement. The information will also help to steer genetic therapy research toward the mutations that are prevalent in our populations.

This work was undertaken as part of the Target 5000 project, an initiative to identify and record the genotype and phenotype of all patients with inherited retinal degenerations in Ireland. There are three clinical sites where patients are assessed as part of this project: the Royal Victoria Eye and Ear Hospital, Dublin; the Mater Misericordiae University Hospital, Dublin; and the Royal Victoria Hospital, Belfast. I collected data and tested patients at the Royal Victoria Eye and Ear Hospital in Dublin. I performed clinical assessment and phenotype data collection for over 150 patients and took blood samples for genetic analysis. From the two Dublin hospitals, blood samples for genetic analysis were transferred to the Smurfit Institute of Genetics, Trinity College Dublin, for next generation sequencing and further analysis. Targeted sequencing of 254 genes with known involvement in cases of retinal degeneration was then performed on patient samples. In the case that targeted sequencing did not identify a mutation, whole exome sequencing was then carried out.

Target 5000 is funded by grants from Fighting Blindness Ireland, the Health Research Board and the Medical Research Charities Group. My Clinical Research Fellow position was funded by Fighting Blindness Ireland.

In the following sections, an explanation of the anatomy of the retina and the physiological mechanisms underlying vision will help highlight the basis for understanding the pathology involved in retinal degenerations. A brief summary of methods of proposed treatments for these diseases will then follow.

## 1.3 Anatomy of the retina

### 1.3.1 Introduction

The human retina is a layer of tissue approximately 0.5mm in thickness that represents the innermost layer of the eyeball (Figure 1). The retina is the layer within the eye that converts light into neural impulses, which can be interpreted by the brain as images. This occurs in the visual cortex, an area of the posterior brain in the occipital lobe.

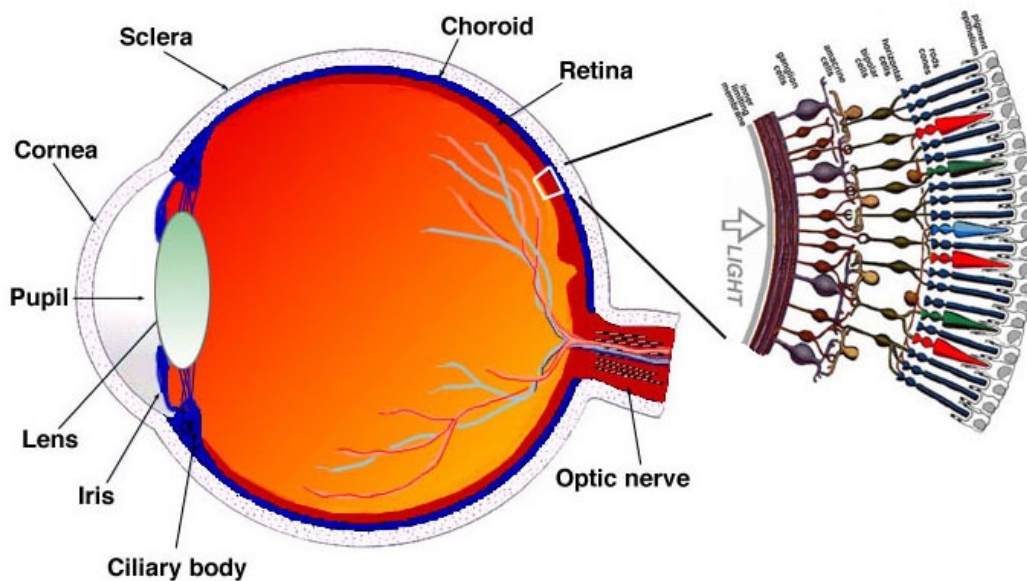
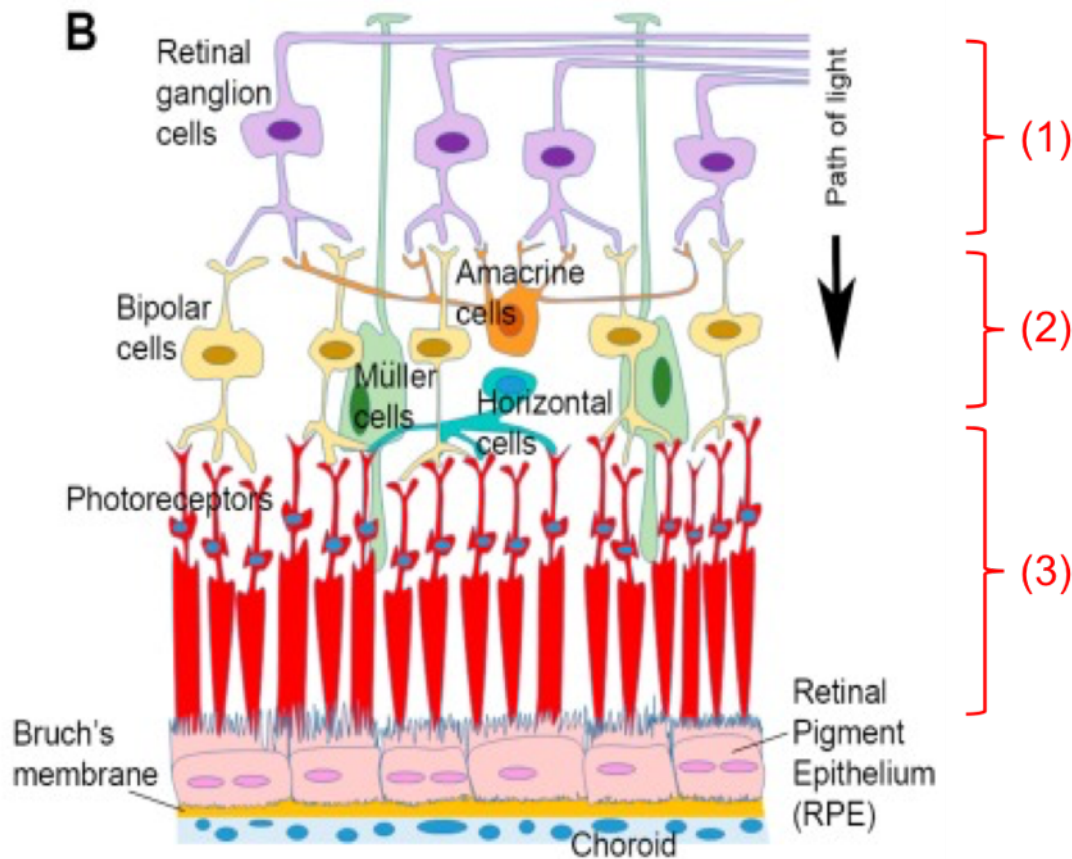


Figure 1. Layers of the retina from (Kolb 2013)

*This illustration depicts the eye and the location of the retina, a magnified section of which is shown on the right, indicating its complex layered structure. Light passes from the left of the figure to the right, passing through the cornea and lens of the eye through to the retina.*

Although the retina consists of ten structural layers, it can be divided into three functional layers to explain how the retina works (Figure 2) – the photoreceptors, interneurons (bipolar cells, horizontal cells, and amacrine cells), and the ganglion

cells. The light first hits the photoreceptor layer, which is supported nutritionally by the retinal pigment epithelium and choroid. The electrical impulse created by the photons of light hitting the photoreceptors is carried to a layer of interneurons (bipolar cells and amacrine cells). These cells synapse with the ganglion cells, which carry the information to the lateral geniculate nucleus and, ultimately, the visual cortex in the occipital lobe.



**Figure 2. Functional layers of the retina, adapted from (Keeling et al. 2018).**

Our understanding of the structural pathology of the retina in disease states has been transformed tremendously in the past 25 years, with the advent of optical coherence tomography (OCT); however, surprisingly detailed anatomical drawings were prepared as far back as 1891 by the Spanish neuro-anatomist, Santiago Ramon y Cajal, despite the technological limitations of this time. OCT



technology, which uses light reflected back to a sensor to create an image, similar to the concept of ultrasound, allows us to image the retinal layers in detail in a clinical setting. The international nomenclature used for the layers of the retina on OCT is shown in Figure 3. In research settings, it is possible to view the photoreceptor cells in even more detail, with the advent of adaptive optics imaging which uses a wavefront sensor to measure ocular aberrations and then a deformable mirror to correct for these aberrations, allowing for high-resolution imaging at an individual cellular level (Liang, Williams et al. 1997). This technology has become important in the clinical trial setting to identify the therapeutic window where patients will benefit from treatment because they still have photoreceptor cells in situ.

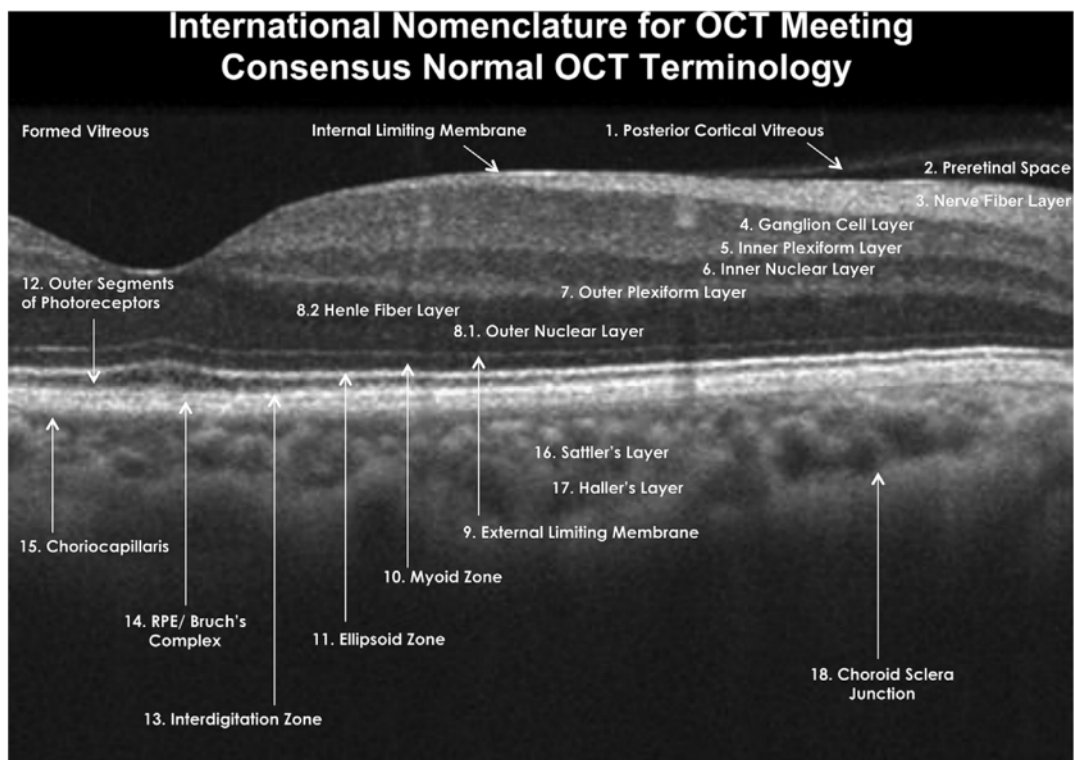


Figure 3. Layers of the retina on OCT (Staurenghi et al. 2014)

***The most common abnormality of the macular OCT observed in retinitis pigmentosa is the gradual loss of the ellipsoid zone (the most easily identifiable part of the photoreceptors) laterally, moving toward the centre of the fovea. Once the central, subfoveal ellipsoid zone is seen to be lost or disrupted, visual acuity is usually affected by the RP.***

### **1.3.2 Retinal Pigment Epithelium (RPE)**

The retinal pigment epithelium (RPE) is a layer of cells joined by tight junctions, which lie external to the neurosensory retina. These tight junctions form a barrier between the retina and the systemic circulation (the “blood-retinal barrier”). Microvilli protrude from their apical walls, which face the photoreceptors. The outer segments of the photoreceptors are continually shed in the process of the visual cycle and the RPE cells collect the debris. The other main function of the RPE cells is to store and recycle the vitamin A derivatives that are involved in the phototransduction cascade; the process whereby light is transformed into electrical impulses in the eye. The restoration of vitamin A through a process with a number of intermediate forms is called the visual cycle. The pigment cells are so called because they contain melanin, which lends this layer a dark colour.

### **1.3.3 Photoreceptors**

There are two types of photoreceptor cells: the rods and the cones. Their names are derived from the shape of the end of the cells, which points towards the pigment epithelium. The rods give rise to grey-scale, undetailed images. The cones are responsible for colour images with fine detail. Both the rods and the cones are composed of several hundred discs or lamellae in their outer segments (the end closer to the RPE), which contain the visual pigments necessary for phototransduction, the ability to transform light energy into an action potential in the cell. There are three subtypes of cone photoreceptors, according to the visual pigment expressed in their outer segments; these pigments absorb different

wavelengths of light and the cones are named after the wavelength: S (short), M (medium), and L (long). Rhodopsin is the visual pigment of the rods and the three opsins of the cone photoreceptors are known as photopsins. There are approximately 110 million rods in the human retina, and 7 million cones. The cones are concentrated most densely at the fovea, a rod-free area found centrally at the posterior pole, and they decrease in density moving toward the periphery, although they are still present in small numbers. The majority of cones are located outside of the macular area, although the rods outnumber them in the periphery of the retina. The cell bodies of the photoreceptors are found on the inner side of the outer segments, in a distinct layer, visible histologically, and on OCT as the outer nuclear layer (Kolb 2011).

#### ***1.3.4 Interneurons***

Although discussion of retinal cells in IRDs is often dominated by the pigment epithelial cells, the photoreceptors, and the ganglion cells, there exist three types of interneuron between the photoreceptors and the ganglion cells. These are the bipolar, amacrine and horizontal cells. These cells are essential in the processing of detailed images and allow for precise vision by modulating the signals from the photoreceptors before activating action potentials in the ganglion cells. Complex processing of visual potentials by these cells, along with higher order processing in the cortex, allows us to achieve our precise level of vision. The attempt to produce light-sensing abilities using gene-based therapies within these layers for patients whose photoreceptors are no longer viable is known as optogenetics.

#### ***1.3.5 Bipolar cells***

The bipolar cells synapse with the photoreceptors and act as an intermediary interneuron to carry messages to the ganglion cells. There are two main types of bipolar cell in the retina. ON bipolar cells are activated (depolarise) in response to a decrease in glutamate release from photoreceptor cells, i.e. the light response to light, and OFF bipolar cells become deactivated (hyperpolarise) in response to the same stimulus. These form the basis for the centre-surround antagonistic receptive field (CSARF), which allows for high contrast vision by polarising cells

surrounding an activated cell in the opposite direction (Levin, et al. 2011). Bipolar cells can be further subdivided by several categories, e.g. molecular, morphological, functional and based on connectivity, and 14 types of mouse bipolar cell have been described to date (Vlasits, Euler et al. 2018).

### **1.3.6 Horizontal and Amacrine cells**

Horizontal cells and amacrine cells are interneurons which facilitate the processing of visual information within the retina. Horizontal cells release the neurotransmitter GABA and are responsible for lateral inhibition of photoreceptor cells. Amacrine cells release the neurotransmitters GABA and glycine and perform a similar inhibitory role to horizontal cells, which modulate visual potentials as part of visual processing.

### **1.3.7 Ganglion cells**

The ganglion cells synapse with the bipolar and amacrine cells. Their axons then converge and travel to the optic disc. The axons of the ganglion cells make up the nerve fibre layer of the retina. Having reached the optic disc, they turn into the disc and pass toward the back of the orbit in the form of the optic nerve. At this stage, they become myelinated. Leber Hereditary Optic Neuropathy and Dominant Optic Atrophy are two inherited disorders that selectively affect the mitochondria of the retinal ganglion cells, causing cell death and visual loss.

### **1.3.8 Layers of the retina**

Having described the cells involved, it is now pertinent to describe the layered structure they come together to form. The retina is described as having ten layers, mainly based upon what was visualised when these layers were first seen histologically, and corresponds to the layers as seen on OCT (figure 4). The names of the layers from inner to the outer retina are: the internal limiting membrane, the nerve fibre layer, the ganglion cell layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, the outer nuclear layer, the external limiting membrane, the photoreceptor layer and the retinal pigment epithelium. The nuclear layers contain the cell bodies of the neurons, with the

plexiform layers containing their synapses. The ganglion cell layer and the nerve fibre layer contain the cell bodies and the axons of the ganglion cells, respectively. The internal and external limiting membrane layers are composed of the end feet and apical processes of the glial Müller cells. The external limiting membrane is found between the inner and outer segments of the photoreceptor cells. Müller cells provide structural support to the retina as well as maintaining metabolic homeostasis of neurotransmitters, pH and metabolites. They also secrete neurotrophic factors and cytokines (Kelly, Wang et al. 2018).

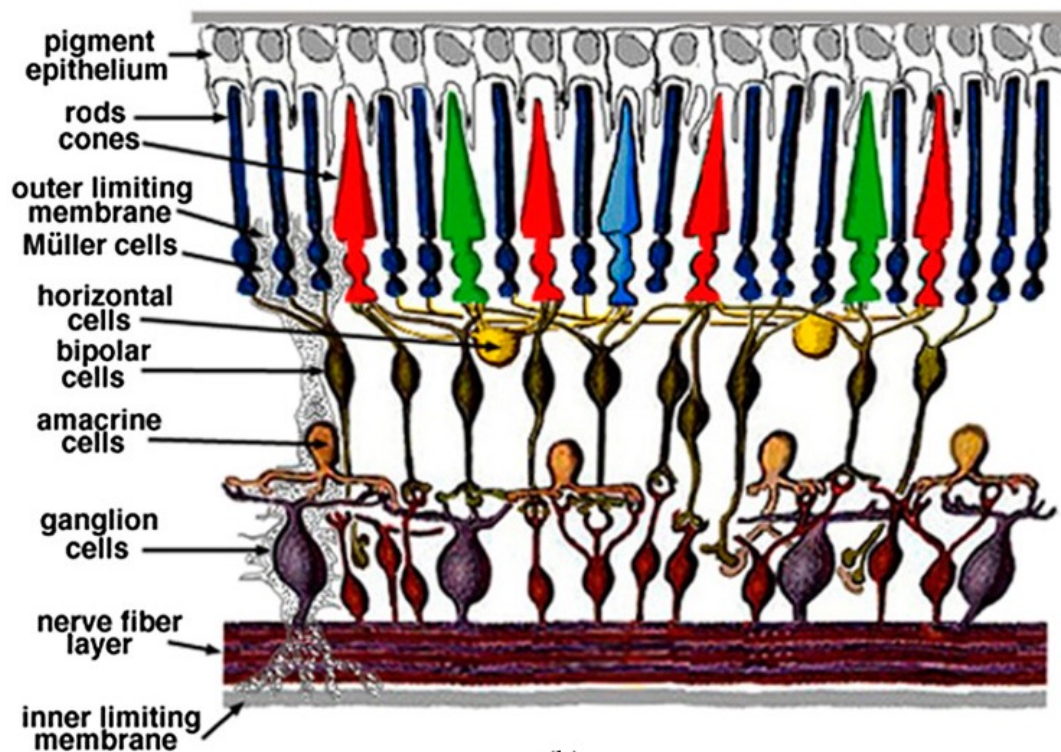


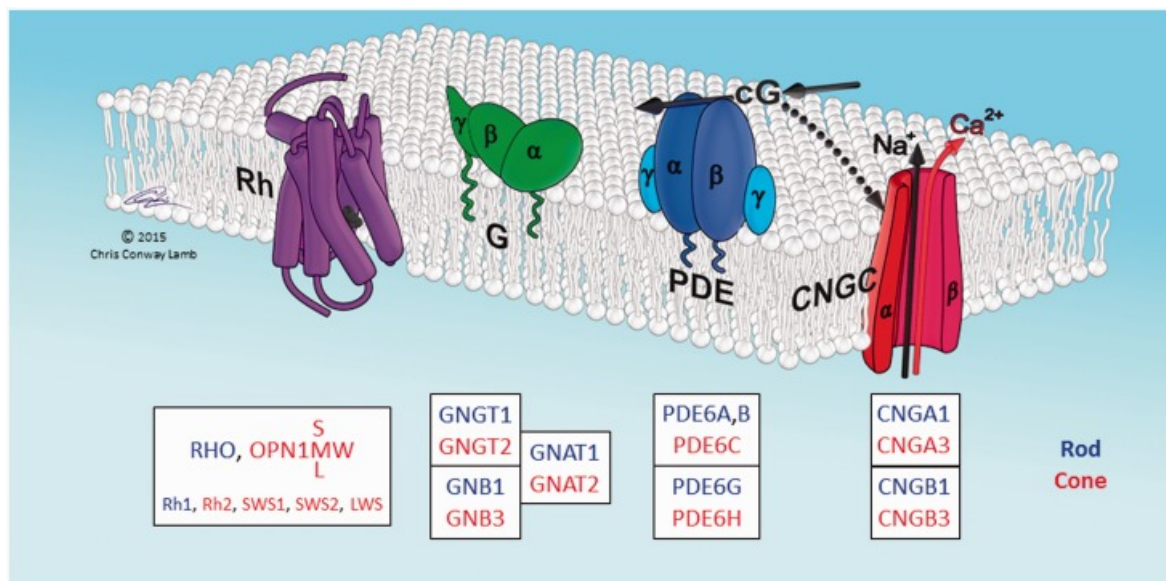
Figure 4. Anatomical retinal layers from (MacGillivray, Trucco et al. 2014)

*This image also depicts the position of the Müller cells.*

#### 1.4 Physiology of the retina

The retina has the ability to turn electromagnetic radiation, in the form of light, into a biological signal interpreted by the visual cortex. This is accomplished

starting with a complex biochemical process that occurs in the photoreceptors. The first step in this complex process is the detection of light by the photoreceptor cell outer segments. The rod photoreceptors are extremely sensitive and are capable of detecting single photons of light (Baylor, Lamb et al. 1979). The cone photoreceptors are less sensitive to light but have the ability to discern light without becoming saturated under much brighter conditions. The main mechanism underlying the high threshold for saturation is the brevity of the cone photoresponse (Astakhova, Firsov et al. 2015). The molecular basis of the phototransduction cascade, the process by which the light response is converted to an action potential within the photoreceptor, has been the subject of intensive research for the past forty years. Significantly, mutations in most of the genes coding for elements in the phototransduction cascade have been shown to be involved in a variety of inherited retinal degenerations (Figure 5).



**Figure 5. Genetic mutations affecting key molecules involved in the process of phototransduction from (Lamb, Patel et al. 2016)**

*This figure depicts the principal molecules in the phototransduction cascade embedded within a phospholipid bilayer. In the rod photoreceptor*

*outer segment, the three molecules to the left would be found within the plasma membrane of the disc and the CNGC molecule would be found within the outer plasma membrane of the outer segment. These molecules include Rh= rhodopsin, G= transducin, PDE= cGMP phosphodiesterase, CNGC= cyclic nucleotide-gated ion channel. The relevant genes that code for these proteins are listed below the diagram, with rod genes denoted by blue font and cone genes denoted by red font.*

In the process of phototransduction, light is absorbed in the eye by visual pigments present on the outer segments of the photoreceptors. In rod outer segments, the discs have a complete plasma membrane that is separate from that of the outer segment and, in cones, the discs exist as evaginations of the outer segment plasma membrane and remain in continuity with it. The rods contain the light-sensitive rhodopsin. Rhodopsin is a transmembrane G-coupled protein receptor composed of a protein, an opsin, and a chromophore, 11-*cis*-retinal (Hargrave 2001). The photoreceptor maintains a semi-depolarised state in the absence of light. This is achieved by the movement of ions in a current known as the dark current (figure 6). The dark current is composed of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  ions, which pass through cGMP-gated channels into the cell. A steady-state is maintained by the outward movement of  $\text{Ca}^{2+}$  through a  $\text{Na}^+/\text{K}^+, \text{Ca}^{2+}$  exchanger, which requires the presence of  $\text{K}^+$  external to the cell, and is present in the membrane of the outer segment of the rod cells (Cervetto, Lagnado et al. 1989). This current maintains the cell in a depolarised state, during which it releases glutamate from its synaptic terminal.



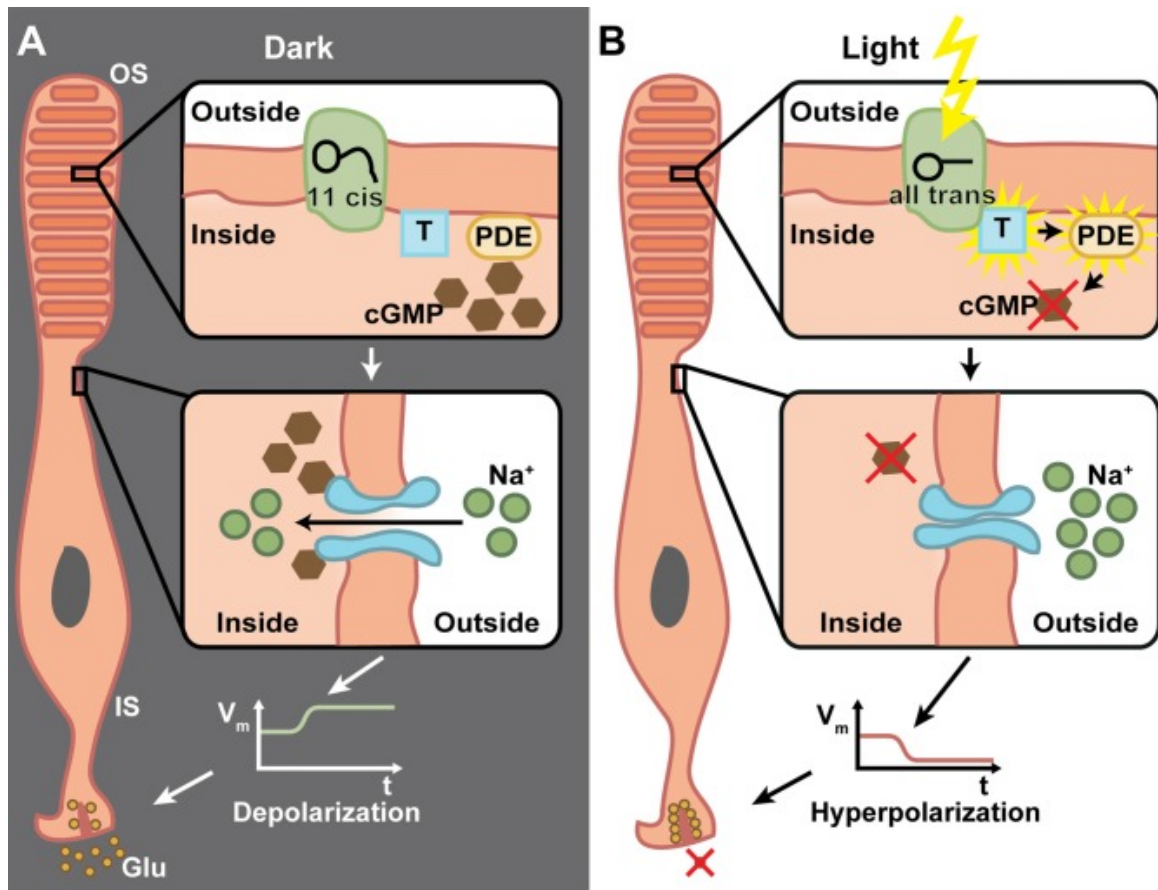


Figure 6. Rod Photoreceptor (Klapper, Swiersy et al. 2016)

*A depicts the rod photoreceptor cell in the dark. The chromophore is maintained as the 11-cis retinal isomer, the cGMP-gated Ca<sup>2+</sup> channels remain closed, the cell is depolarised and continuously releases glutamate. In contrast, B depicts the cell in its light-activated state. 11-cis-retinal has been photoisomerised to all-trans retinal. The cGMP-gated Ca<sup>2+</sup> channels have shut, the cell has become hyperpolarised and glutamate release has ceased. OS=outer segment, IS=inner segment, Glu=glutamate, T=transducin, PDE= cGMP phosphodiesterase.*

Rhodopsin is stimulated by photons of light that are absorbed by the chromophore, 11-*cis*-retinal, and causes its isomerisation to produce all-*trans*-retinal. These changes lead to the production of several rhodopsin intermediaries, including Metarhodopsin II (R<sup>\*</sup>). Metarhodopsin II is formed when the protonated Schiff base that attaches the chromophore to the protein opsin is deprotonated, opening up binding sites for the cytoplasmic molecule, transducin (Hargrave, Hamm et al. 1993). Metarhodopsin II's ability to activate transducin occurs within a short timeframe, estimated at 40ms in wild-type mouse rods (Gross and Burns 2010). It remains active until it is phosphorylated by rhodopsin kinase. This step does not completely eradicate Metarhodopsin II's ability to activate transducin, which is finally ceased by the binding of arrestin to the phosphorylated molecule (Hargrave 2001). Rhodopsin kinase and arrestin are thus the rate-limiting steps in this process. Before a rhodopsin molecule can once again engage in the commencement of the phototransduction cascade, it must be returned to its initial state. The protein part of the molecule must be dephosphorylated and the chromophore must return to its original conformation. For this to happen, it is initially reduced to all-*trans*-retinol by an all-*trans*-retinol dehydrogenase within the outer segment membrane. It is subsequently transported to the retinal pigment epithelium, where it is returned to its alternative isomer, 11-*cis*-retinal, and ultimately returned to the photoreceptor (Fain, Matthews et al. 2001).

Rhodopsin, in the form of metarhodopsin II, stimulates transducin, a heterotrimeric G-protein composed of alpha, beta, and gamma subunits. Rod transducin is coded for by the GNAT1 gene, whereas cone transducin is encoded by the GNAT2 gene. In its inactive form, T $\alpha$  is bound to the other two subunits of transducin. When activated by metarhodopsin II, which exchanges the GDP bound to T $\alpha$  for GTP, T $\alpha$  dissociates from the T $\beta\gamma$ . One R<sup>\*</sup> molecule (metarhodopsin II) activates transducin at a rate of approximately 1 per ms (Jindrova 1998). T $\alpha$ , when activated, binds to phosphodiesterase (PDE) present at the rod outer segment membrane. The T $\alpha$  molecule's activity is terminated due to its intrinsic GTP-GDP hydrolysis activity, which inactivates the molecule.

PDE is composed of four subunits, two catalytic subunits,  $\alpha$  and  $\beta$ , and two identical inhibitory subunits,  $\gamma$ . The activated  $T\alpha$  molecule binds to the  $\gamma$  subunits of PDE, deactivating their inhibitory action and allowing PDE\*, the activated form of PDE, to go on to hydrolyse many molecules of cyclic GMP. In turn, this causes the cGMP-gated channels in the outer segment membrane to close. Closure of these channels causes hyperpolarization of the photoreceptor cell membrane, which causes cessation of glutamate release. Glutamate is the neurotransmitter of the dark-adapted state and interruption of glutamate production allows the propagation of an action potential to interneurons, bipolar and horizontal cells, in the neurosensory retina (Fu 2018).

Once the GTP bound to transducin is hydrolysed to GDP, the transducin molecule dissociated from PDE\* rejoins  $T\beta\gamma$ . The two inhibitory  $\gamma$  subunits of PDE\* are now reactivated and deactivate the PDE molecule\* (Farber 1995).

Calcium ions play an important role in the regulation of the phototransduction cascade in two ways. Firstly, the concentration of calcium affects the amount of cGMP in the cell by its link to the activity of guanylyl cyclases. These enzymes are responsible for the continual replenishment of cGMP and are located within the membranes of the rod outer segments. The relationship of these substances is managed by guanylyl cyclase activating g-proteins (GCAPs). These molecules have  $Ca^{2+}$  binding sites which, when available due to low calcium concentrations, stimulate guanylyl cyclase activity (Lolley and Racz 1982). The second regulatory role of  $Ca^{2+}$  in the cascade is its ability to affect the affinity of cGMP for the cyclic-nucleotide-gated channels that are responsible for maintaining the dark current, where decreased levels of  $Ca^{2+}$  result in an increased affinity of the molecule for the channels (Hsu and Molday 1993).

There are two further processes which allow for vision to occur in a range of light conditions. Firstly, the ability of the photoreceptors to respond to stimuli at an increasing level of background illumination is referred to as light adaptation. This is largely controlled by the cytoplasmic concentration of  $Ca^{2+}$  (Fain, Matthews et al. 2001). This relationship has been studied by observing responses of

photoreceptors that have been placed in solutions with a low concentration of  $\text{Ca}^{2+}$ , which are found to have a reduced adaptive response to light (Torre, Matthews et al. 1986).

The second phenomenon, which is important in the ability of the retina to respond to light of different intensities, is the concept of bleaching. This is when the photoreceptors are exposed to a bright light stimulus, after which the retinal sensitivity decreases dramatically for a period of time after the stimulus is removed. The recovery from bleaching is known as dark adaptation. During dark adaptation, the retina acts as if it were being exposed to a steady light of decreasing intensity. The rate of recovery from bleaching in cones is more swift than in rod cells due to differing pathways of visual pigment restoration (Jones, Crouch et al. 1989). It has been shown that bleaching is due in large part to the activation of the phototransduction cascade by bleached photopigments in both rods and cones. When the opsin separates from the chromophore (11-cis-retinal), it continues to activate the phototransduction cascade. This occurs in a more ineffective manner than activation by light-activated rhodopsin. This ability is terminated when recycled 11-cis-retinal binds once again to the opsin. Proof of this mechanism was shown experimentally by measuring the level of PDE activity in bleached photoreceptors and observing that this was maintained at an increased level for a delayed period after the light stimulus was removed (Cornwall and Fain 1994, Cornwall, Matthews et al. 1995). The rate of dark adaptation possible in the eye is limited by the fact that this process requires 11-cis-retinaldehyde, which is toxic to the retina in high concentrations. Furthermore, in evolutionary terms, the time course of dark adaptation is similar to that of the fading of the light at dusk on our planet and could have provided a survival advantage (Lamb 2016).

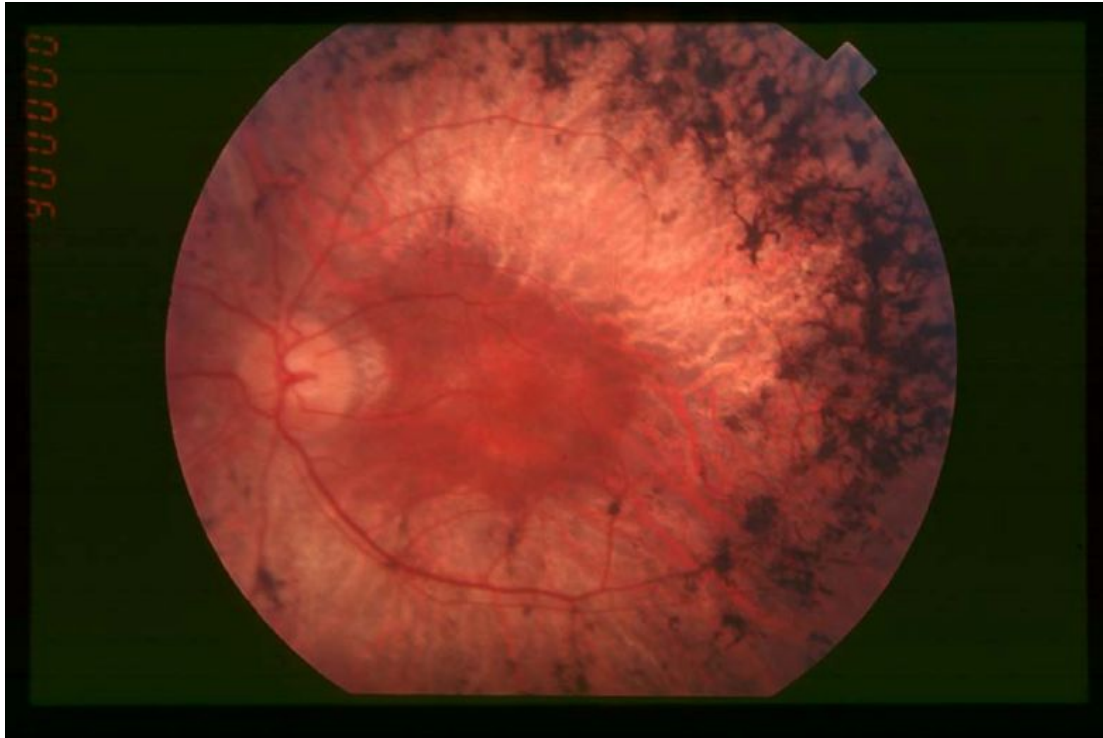
## **1.5 Inherited retinal degenerations**

Although many different diseases exist under this umbrella term, I will give an overview of the most common conditions and those which arise in the body of this thesis.

### **1.5.1 Retinitis pigmentosa**

Retinitis pigmentosa is a group of inherited diseases that result in the degeneration of the rod and cone photoreceptor cells in the retina. Its prevalence is approximately 1 in 4000 people (Pagon 1988) and up to 30% of cases will have systemic manifestations (Verbakel, van Huet et al. 2018). Also known as rod-cone dystrophy, it is characterised by the progressive loss of the rod and then the cone photoreceptor function, leading to characteristic night blindness and peripheral visual field defects which coalesce to become tunnel vision. The progressive visual field loss may culminate in the loss of central vision later in life. The characteristic fundus findings are those of bony spicule retinal pigmentation, retinal vessel attenuation, and pale, waxy optic discs (Figure 7). The explanation for the classical bone-spicule degenerative fundal lesions seen in retinitis pigmentosa and related retinal dystrophies is the migration of RPE cells into the neurosensory retina in response to photoreceptor cell death (Li, Possin et al. 1995).

The conditions have vast genetic heterogeneity and the age at which symptoms develop, along with their speed of progression, vary widely. Mutations in over 80 genes have now been described to cause retinitis pigmentosa and the inheritance pattern can be autosomal dominant, autosomal recessive, X-linked, or, very rarely, digenic or mitochondrial (Daiger 2019).



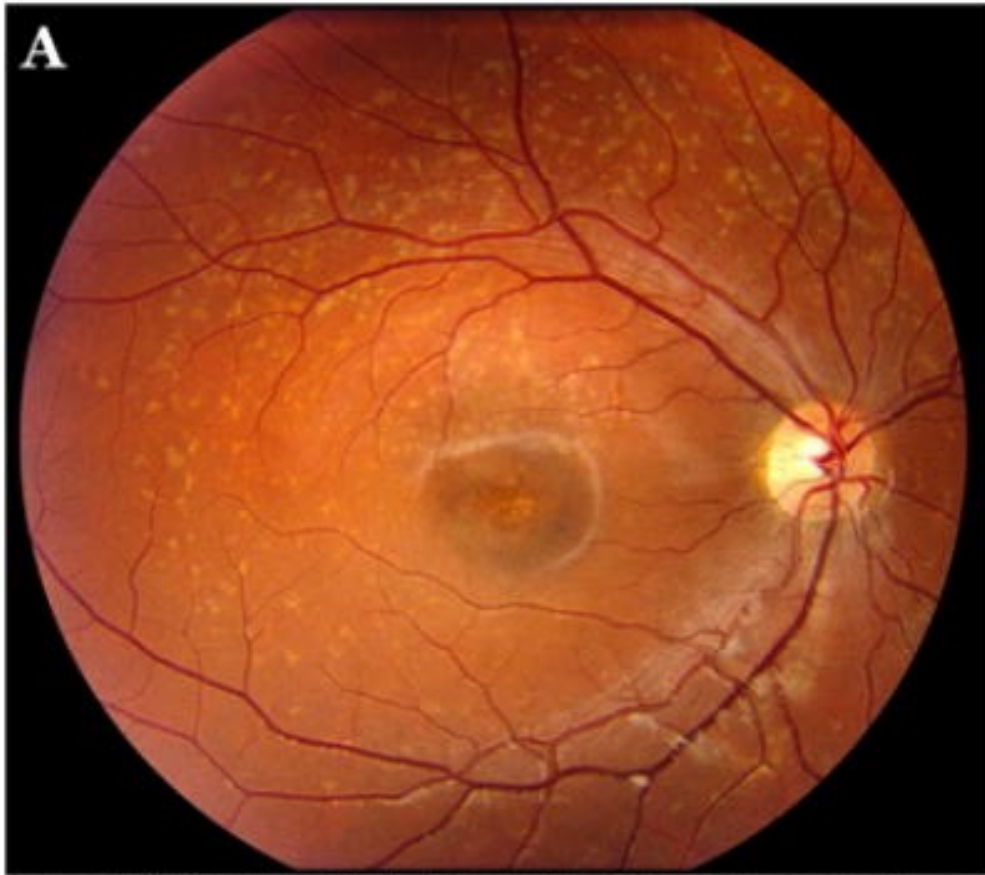
**Figure 7. Classical fundus changes of retinitis pigmentosa (Hamel 2006)**

***These include peripheral bone spicule pigment migration, peripheral retinal pigment epithelium atrophy, attenuated retinal vessels, and a pale optic disc.***

### **1.5.2 Stargardt disease**

Although retinitis pigmentosa is the most common inherited retinal condition, the single most common gene responsible for inherited retinal degenerations is ABCA4, which causes the condition known as Stargardt disease. Stargardt disease is a macular dystrophy with a prevalence of between 1 in 8000 and 10,000 (Tanna, Strauss et al. 2017). It is characterised by progressive central visual loss and is typically inherited in an autosomal recessive manner. The characteristic fundus appearance is that of central foveal atrophy with surrounding yellow pisciform (fish-like) flecks (Figure 8). The clinical course and age of onset are variable with some patients maintaining only mild disease limited to the macula and some patients developing a severe cone-rod dystrophy with

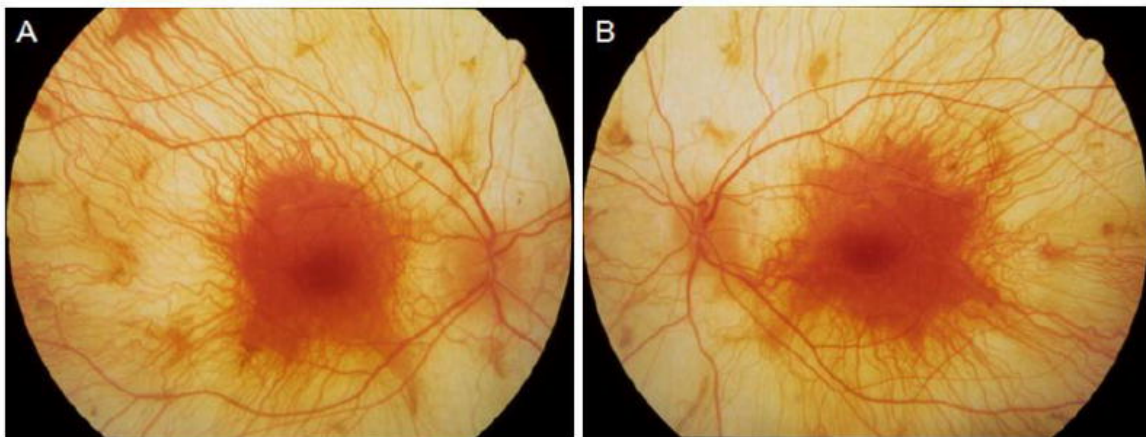
night blindness and losses in the peripheral visual field (Fujinami, Lois et al. 2013). In some cases, the fundus changes are similar to those found in retinitis pigmentosa.



**Figure 8. The characteristic retinal changes of Stargardt disease including central macular retinal pigment epithelial atrophy with surrounding yellow retinal flecks (Stone, Andorf et al. 2017).**

### 1.5.3 Choroideraemia

Choroideraemia is a monogenic condition caused by mutations of the CHM gene, which codes for the protein Rab Escort Protein 1 (REP1). It is characterised by the progressive atrophy of the photoreceptor layer, the retinal pigment epithelium and the choroid and has an X-linked mode of inheritance (MacDonald, Hume et al. 1993). Similar to retinitis pigmentosa, the retinal changes start peripherally and move concentrically toward the macula. Patients experience initial night blindness and visual field loss culminating in loss of central vision in later life. There is accelerated visual acuity loss after the age of 50 but the majority maintain good central vision until the age of 60 (Roberts, Fishman et al. 2002, Coussa, Kim et al. 2012). Fundus changes that are characteristically seen are lobular areas of atrophy encroaching slowly on the fovea (Figure 9).



**Figure 9. The typical appearance of the retina in choroideraemia (MacDonald, Russell et al. 2009).**

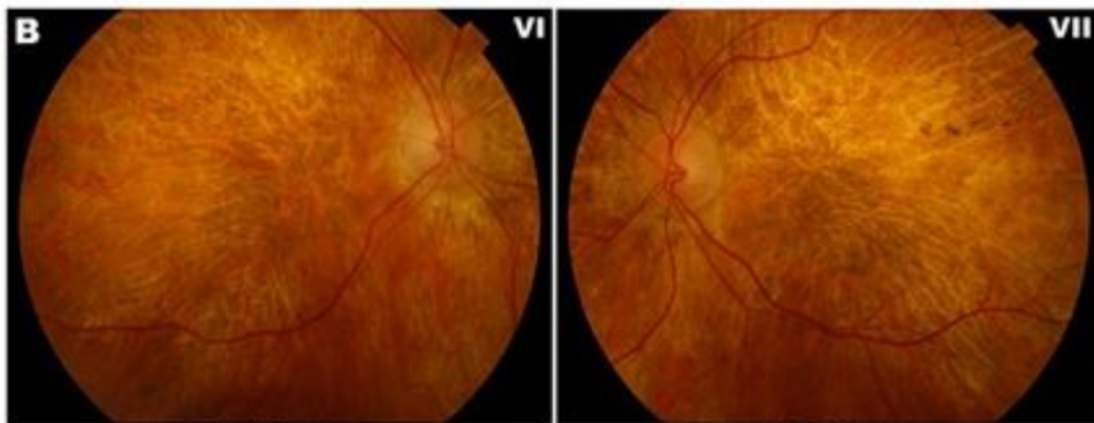
***There is retinochoroidal atrophy creeping toward the fovea with the sclera visible beyond the border of the relatively more normal area of central retina. This is accompanied by retinal vessel attenuation and pale optic discs.***





### **1.5.4 Cone-rod dystrophy**

Whereas retinitis pigmentosa can be described as a rod-cone dystrophy because it is associated with rod photoreceptor malfunction followed by cone photoreceptor malfunction, cone-rod dystrophy involves the initial loss of cone function followed by that of the rod photoreceptors. It is a genetically heterogeneous progressive inherited condition. The inheritance can be autosomal dominant, autosomal recessive or X-linked (Gill, Georgiou et al. 2019). The initial symptoms are that of decreased visual acuity and colour vision, which progress to varying degrees to loss of peripheral visual field and night blindness (Hamel 2007). The fundus changes seen in these patients include initial macular atrophy followed by varying degrees of peripheral retinal atrophy and pigment migration (Figure 10).



**Figure 10. A patient with a mutation of the PRPH2 gene, p.(Arg172Trp), causing cone-rod dystrophy, from (Gill, Georgiou et al. 2019).**

***There is central macular atrophy extending to the periphery, with areas of subtle pigment migration from the RPE.***

## **1.6 Assessing patients with retinal degenerations**

There are two types of assessment which are carried out to delineate the conditions encountered in this thesis – psychophysical tests and electrophysiological tests. Psychophysical tests involve the study of perceptions in response to stimuli and, in the case of visual testing, involve tests such as visual acuity, visual fields, colour vision testing and dark adaptation.

Electrophysiological tests are objective measurements of the electrical impulses in neuronal cells in the eye measured in response to light stimuli. Many inherited retinal degenerations have characteristic findings when these tests are carried out. For example, in retinitis pigmentosa it is common to see a ring scotoma on visual field testing and generalised rod and cone photoreceptor dysfunction with the rods being more severely affected on the electroretinogram. There are specific cases where the electrophysiological findings are even pathognomonic for certain genes e.g. enhanced S-cone syndrome, caused by a biallelic mutation of the NR2E3 gene, exhibits an undetectable rod-isolated ERG, a light-adapted 30Hz flicker ERG amplitude smaller than the light-adapted 3.0 a- and b-wave amplitudes, and a supernormal S-cone ERG (Vincent, Robson et al. 2013). A discussion on the methods used to ascertain these parameters will ensue in chapter 2, Materials and Methods.

## **1.7 Treatment modalities**

There have long been attempts made to treat inherited retinal degenerations going back in the literature as far as 1932, with ineffective and potentially harmful treatments such as sympathectomy, hot compresses, and subconjunctival injections of hypertonic salt solutions for the treatment of retinitis pigmentosa (Pischel 1932, Macdonald and McKenzie 1934). Without the ability to successfully understand the pathophysiology and genetic aetiology of these diseases, the ability of scientists and physicians to conceptualise and deliver treatments was limited. However, there have been numerous scientific breakthroughs over recent decades, providing increased understanding of the

biology of these conditions, which have allowed us to finally start to successfully treat these disorders, although this ability is still in its relative infancy. The elucidation of the anatomy and biochemical processes of the retina, coupled with the ability to identify the disease-causing genetic mutation in patients with these diseases have allowed us to begin to address these genetic and biochemical errors. Several approaches have been identified which attempt to achieve treatment goals using diverse approaches. It remains to be seen which of these methods will be most beneficial in the future however, and we are at an important juncture during the development of these therapies where we must identify which patients would benefit the most from each modality.

Treatment modalities investigated, to date, have included genetic therapy, stem cell therapy to replace lost retinal tissues, retinal prostheses, optogenetics, neurotrophic factors, antisense oligonucleotides and targeted therapies to effect biological pathways involved in retinal degeneration. While some therapies have been under investigation for a shorter period of time, there have been promising findings in all of these areas, to date. At present, all of these therapies are now being investigated in both animal models and human clinical trials.

### **1.7.1 Genetic therapy**

Gene-based therapies for inherited retinal disorders have now become a reality partly due to our ability to perform gene sequencing in a much faster and efficient manner than previously possible with the advent of next generation sequencing, and the development of effective viral vectors to carry these genes to their target cells. The major impediment after conquering the ability to identify the genetic mutation underlying these disorders was the genetic heterogeneity found to exist amongst people with inherited retinal degenerations. The inheritance pattern of these disease traits also varies and includes autosomal dominant, autosomal recessive, X-linked, and mitochondrial modes of inheritance (Farrar, Millington-Ward et al. 2014). The eye is an ideal anatomical site to attempt to treat with genetic therapies due to its relative immune privilege, whereby the blood-retinal barrier is protective against immune-mediated damage (Streilein 2003). The

presence of an in-built control by comparing to the second, untreated eye also makes clinic trials easier to carry out.

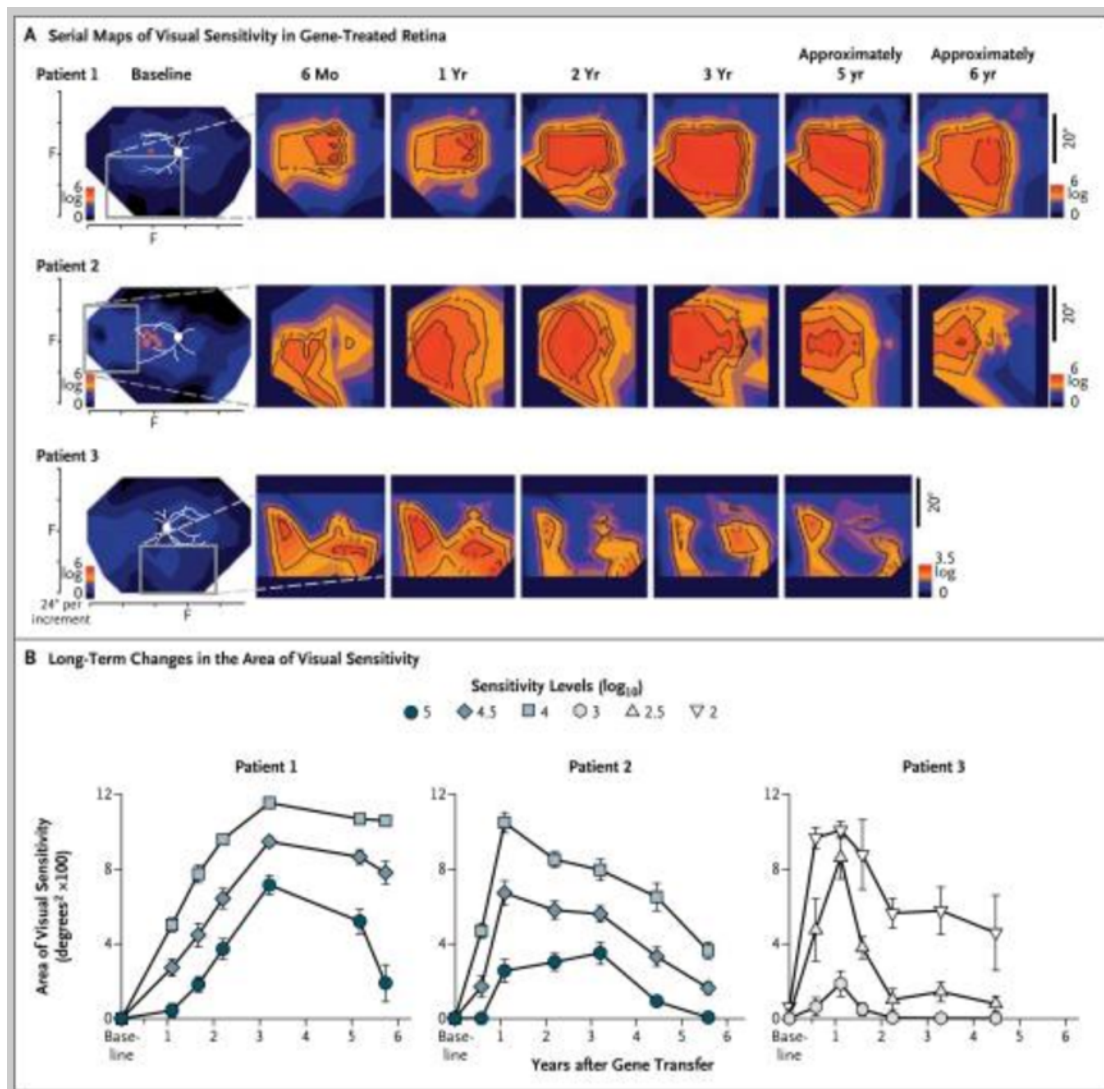
Autosomal recessive disorders and, similarly, X-linked disorders are perhaps more straightforward to treat than autosomal dominant disorders, from a genetic therapy standpoint. This is due to the fact that autosomal dominant disorders, for example Rhodopsin-mediated retinitis pigmentosa, can carry a gain of function mutant protein which must be abolished along with replacement of a wild type protein (Athanasίου, Aguila et al. 2018). Whereas in the case of an autosomal recessive or X-linked disorder, the protein product of a gene is defective or absent and must be replaced.

The most prominent example of replacing an autosomal recessive gene is that of RPE65-mediated retinal dystrophy, most presenting with a phenotype of Leber Congenital Amarois. In this disease, a biallelic RPE65 (retinal pigment epithelium-specific 65-kD protein) mutation causes an inability of the visual cycle to produce 11-cis-retinal from all-trans-retinyl esters, due to the loss of the function of a retinal pigment epithelial protein with isomerase activity (Redmond, Poliakov et al. 2005). Although there is a functional inability of the visual cycle to re-produce 11-cis-retinal, the death of photoreceptors appears to occur later in the disease (Paunescu, Wabbels et al. 2005). Thus, there is a therapeutic window after the loss of function of these cells. The first animal model to show successful photoreceptor cell rescue by any gene therapy was the *rd* mouse (Bennett, Tanabe et al. 1996). Further proof-of-concept work undertaken by subretinal injection of a recombinant adeno-associated virus carrying wild-type RPE65 (rAAV.RPE65) into the eyes of the RPE65 knockout Briard dog led to progressive improvements of ERG recordings and visual functional behaviour up to nine months after treatment (Narfstrom, Katz et al. 2003).

Three phase I human clinical trials commenced in 2008 to evaluate genetic treatment of RPE65 mutations, causing LCA2 in nine patients aged 17-26 years of age (Bainbridge, Smith et al. 2008, Hauswirth, Aleman et al. 2008, Maguire, Simonelli et al. 2008). Between these three studies, nine patients underwent

treatment of one eye. A vitrectomy was performed and the viral vector was injected into a bleb underneath the retina. Delivery of the gene product was undertaken using a recombinant adeno-associated virus (AAV) without the ability to replicate containing RPE65 DNA. The contralateral eye was used as a control in all patients. These early studies showed an increase in dark adapted perimetry in 4 of the 9 patients, an increased ability to navigate an obstacle course in dim light in 2 patients, and an increased subjective visual ability in dim light in 6 patients. One eye developed a macular hole and one developed foveal retinal thinning (Hauswirth, Aleman et al. 2008).

A report with up to six year follow up of one of these cohorts showed how retinal sensitivity peaked and subsided over longer term follow-up (Figure 11). The cause of late contraction of these areas of retinal sensitivity is unknown but may relate to reduced transgene expression in the RPE cells over time, reducing the metabolic support to the overlying photoreceptors (Jacobson, Cideciyan et al. 2015).



**Figure 11. Retinal sensitivity (Jacobson, Cideciyan et al. 2015)**

*Showing A) baseline retinal map with a square overlaid over the area where the bleb containing the treatment was placed and the corresponding retinal sensitivity maps for this area over time in each of the three patients. B) Areas of visual sensitivity plotted over time for each patient. Error bars indicate 1 standard deviation.*

A phase III study was subsequently performed with a carefully designed, novel primary outcome measure, an obstacle course performed at multiple levels of luminance (“multi-luminance mobility testing”), to circumvent previous problems encountered with more traditional outcomes measures such as visual acuity (Russell, Bennett et al. 2017). This confirmed both the efficacy and the safety of the treatment and led to licencing of the drug, voretigene neparvovec (Luxturna) by the U.S. FDA.

These landmark studies have paved the way for gene-based therapy of inherited retinal disease and have established proof-of-concept for the viral vector model. There now exist trials of gene therapy using a viral vector to deliver the wild type gene for both X-linked and autosomal recessive retinitis pigmentosa (genes RPGR, PDE6B, MERTK), choroideraemia (CHM), achromatopsia (CNGA3, CNGB3), X-linked retinoschisis (RS1), and Leber Hereditary Optic Neuropathy (ND4) (U.S. National Library of Medicine 2019). Many further clinical trials are in planning phase and this area is rapidly expanding. Careful phenotyping of patients to match levels of degeneration to the appropriate treatment will be necessary to ensure the best outcomes of these trials. My work, as described in this thesis, will contribute to the founding of this knowledge base.

To date, there have been no reported studies of genetic therapies for autosomal dominant disorders of the eye. Dominant mutations are associated with pathology related to a lack of production of wild-type protein or the production of a toxic mutant protein. Addressing the problem of excess abnormal mutant protein is complicated. There has been some success in animal models, with the use of a “suppress and replace” model, where both the wild-type and mutant native alleles are suppressed and a modified suppression-resistant gene is administered to allow wild-type expression of the protein. This can be achieved by administering two AAV vectors (“dual-vector delivery”) - one encoding a suppressor gene and one encoding a replacement gene. The model has been shown to be successful in the treatment of a murine model of rhodopsin-mediated autosomal dominant retinitis pigmentosa, where improvements in the mouse ERG and retinal



histology, reflective of retinal function and structure, were maintained up to a period of five months after treatment (Millington-Ward, Chadderton et al. 2011). Recent experience of long-term preservation of structure and function in the context of a canine model of autosomal dominant retinitis pigmentosa using a similar method will hopefully pave the way to human clinical trials (Cideciyan, Sudharsan et al. 2018).

The treatment of mitochondrial disorders by genetic therapy again represents a unique challenge. The most common disorder of the eye with mitochondrial inheritance is Leber hereditary optic neuropathy (LHON), a disease characterized by degeneration of the retinal ganglion cells and their axons which comprise the optic nerve, causing acute bilateral, permanent visual loss in the second and third decades of life (Koilkonda, Yu et al. 2014). The genetic defect lies in the mitochondrial DNA and three mutations account for 95% of cases, the most common of which is the G11778A mutation, resulting in an arginine to histidine substitution at the 340<sup>th</sup> amino acid of the ND4 gene (G11778A). The ND4 protein is a subunit of complex I. Mutations of complex I, or mitochondrial respiratory NADH-ubiquinone oxidoreductase complex, cause a decrease in the overall production of mitochondrial ATP (Brown, Voljavec et al. 1992). The method of delivery of the ND4 gene into the mitochondria used by most groups is dubbed “allotropic expression”, whereby the AAV vector delivers a nuclear version of the mitochondrial gene to the cell nucleus first. Then, mRNA translates this in the nucleus, which is then translated by cytoplasmic ribosomes. Finally, the product enters the mitochondrion due to the mitochondrial-targeting sequence, which is attached to the ND4 protein. This is removed after entry and the protein is assembled into complex I (Lam, Feuer et al. 2010). Mouse models treated with this technique have shown evidence of benefit, as assessed by pattern electroretinogram and spectral domain OCT (Koilkonda, Yu et al. 2014), as well as manganese-enhanced MRI and optokinetic responses (Chadderton, Palfi et al. 2013). The surgical delivery for the vector used in these disorders differ from those already mentioned, in that the vector (AAV in the studies mentioned) can be introduced by intravitreal injection rather than subretinal injection and does not

require concurrent vitrectomy. This method has been shown to be a safe way to introduce the ND4-carrying AAV vector in a trial of rodent, non-human primate and ex-vivo human eyes (Koilkonda, Yu et al. 2014). This method allows it to reach the retinal ganglion cells, which lie near the vitreous in the neurosensory retina. It is further postulated that treatment may need to be instigated prior to visual loss, due to the possibility of irreversible apoptosis having already occurred at this time (Qi, Sun et al. 2007). A phase I/II clinical trial with dose escalation established the safety of this intravitreal viral vector-delivered wild type ND4 gene with a trend toward an improvement in visual acuity favouring the treated eye in those with disease duration of  $\leq 2$  years and BCVA  $\geq 20/12000$  at inclusion (Vignal, Uretsky et al. 2018). There are 2 ongoing Phase III studies of this treatment (one studying patients with vision loss of duration of less than 6 months and the other studying patients with a duration of visual loss of between 6 months and 1 year) (Clinicaltrials.gov identifier: NCT03406104). These will further elucidate the characteristics of those patients likely to benefit from future treatments.

While it is not possible to be comprehensive in a description of the constantly emerging variety of methods of genetic therapy approaches to inherited retinal disease, one more which merits mention is that of optogenetics. This term describes the use of gene-based methods to lend second and tertiary neurons of the retina light-sensing capabilities. It has an important advantage over the previous methods mentioned in that it is independent of the disease-causing gene (Simunovic, Shen et al. 2019). Channelrhodopsins are light-gated microbial ion channels which were first discovered in green algae (Holmes 2012). They respond to blue light by the generation of an action potential within the cell. By injecting the gene encoding a channelrhodopsin in a viral vector intravitreally, it is possible to transfect retinal cells with these proteins, giving them the ability to react to light. Animal models of this method have been successful in demonstrating improvement in mouse visually guided behaviour by targeting both ganglion cells and bipolar ON cells (Lin, Koizumi et al. 2008, Doroudchi, Greenberg et al. 2011). Two phase I/II trials of optogenetics by intravitreal

injection, in one case used in conjunction with visual interface stimulating glasses, have now commenced (NCT03326336 and NCT02556736).

### **1.7.2 Stem cell therapy**

Research in the field of stem cell therapy for retinal degeneration, which encompasses inherited retinal degenerations, as well as age-related macular degeneration, has been diverse, to date. The prospect of stem cell therapy for inherited diseases of the retina is made feasible due to the fact that most layers of the retina remain intact despite the death of the photoreceptors and RPE cells (Huang, Enzmann et al. 2011). There have been attempts to graft RPE cells, photoreceptors and the entire neural retina with RPE (M'Barek and Monville 2019). The cell source has varied from fetal cells, to adult cells, both allogeneic (cadaver) and autologous, embryonic stem cells, induced pluripotent stem cells and other cells which can mimic the role of the RPE such as iris pigment epithelial cells. Other types of available stem cells include mesenchymal cells and very small embryonic-like stem cells, both of which can be derived from the bone marrow (Huang, Enzmann et al. 2011). Research using embryonic stem cells and retinal progenitor cells, which are derived from the foetus or neonate retina, have proved promising but are limited in scope due to ethical and logistic concerns (Lamba, Karl et al. 2006, MacLaren, Pearson et al. 2006). There is also a risk of teratoma formation with the use of embryonic stem cells (Cowan, Klimanskaya et al. 2004). The discovery of induced pluripotent stem cells, which can be derived from mature somatic cells was a breakthrough, which allowed the increased use of stem cells unhindered by procurement issues (Takahashi and Yamanaka 2006). Delivery of the cells can be as a suspension or a monolayer, with the injection of an intact monolayer having been shown to be more likely to be successful in restoring vision (Diniz, Thomas et al. 2013). Those cells delivered as a monolayer require support, and there have been varied attempts to find the ideal scaffold material, examples of which include coated polyester, amniotic membrane, and Descemet's membrane (M'Barek and Monville 2019).

Pearson et al., in 2012, showed how transplanted photoreceptor stem cells in the *GNAT1* knockout mouse could successfully integrate with second order neurons in the host retina and demonstrated an improvement in vision using optokinetic head tracking and visually-guided water maze tests under scotopic conditions (Pearson, Barber et al. 2012). A further *GNAT1* knockout mouse study with transplanted photoreceptor cells showed histological migration of the precursor photoreceptors into the retina and confirmed that visual function began to improve, as measured by optokinetic head tracking, when the integrated cells began to produce  $\alpha$ -transducin, the protein product of *GNAT1*. This process reached a peak level at five weeks (Warre-Cornish, Barber et al. 2014).

One phase I study which recently produced findings of a human embryonic stem cell RPE monolayer treatment for acute AMD showed that vision in the two operated eyes of two patients improved by at least 15 letters in each case and was maintained at up to one year post-operatively with the use of local immunosuppression only. Adverse events included an exposed suture of a steroid depot implant, one retinal detachment, and poor control of diabetes due to steroids. They were all addressed successfully (da Cruz, Fynes et al. 2018).

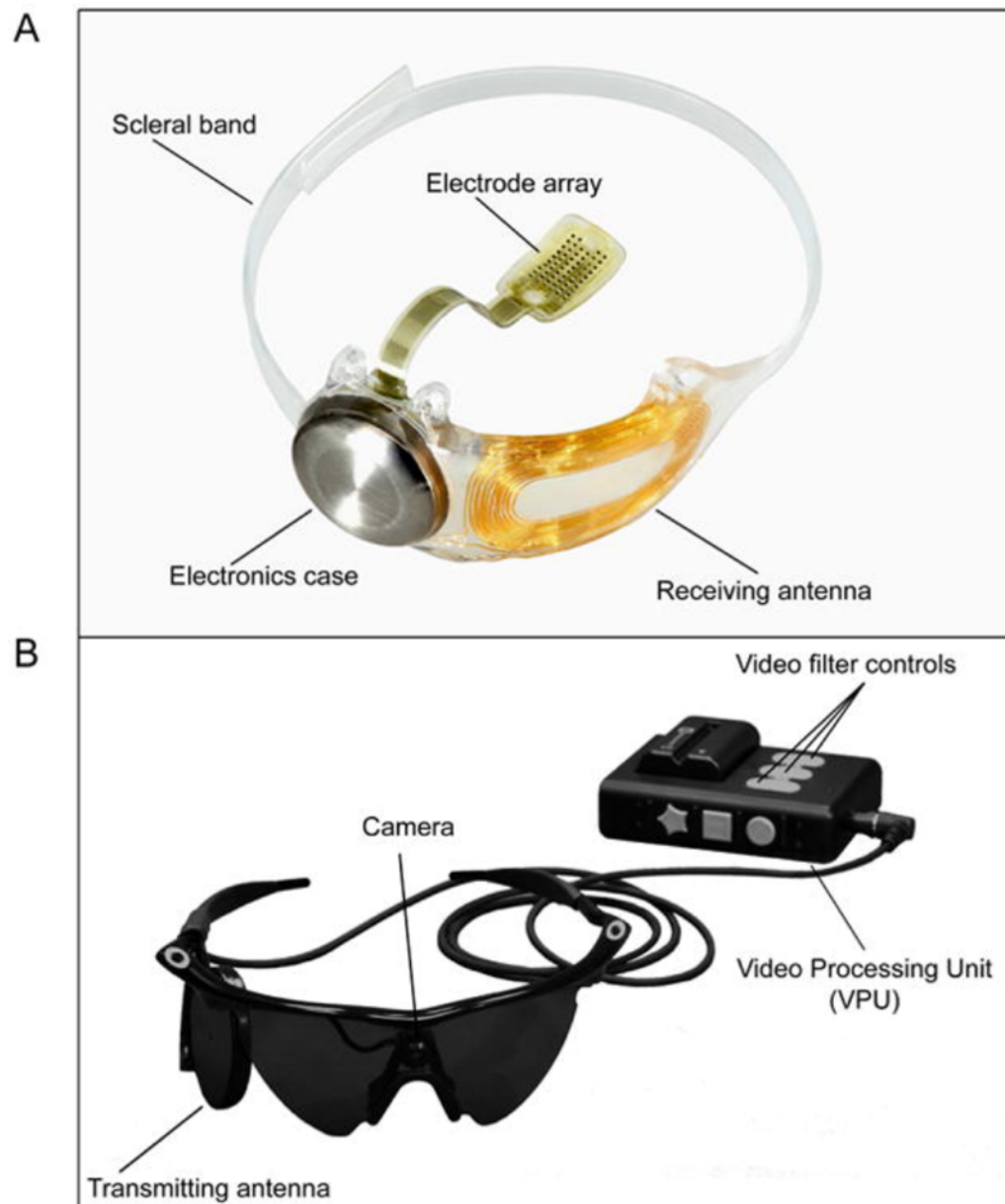
Several early phase trials have now commenced investigating the prospect of stem cell therapy for the treatment of mainly macular disorders such as AMD and Stargardt disease, amongst others. The refinement of these techniques provides an optimistic treatment option for the replacement of damaged retinal cells and for those patients with advanced disease beyond rescue by gene therapy.

### ***1.7.3 Retinal prosthesis***

Retinal prostheses use a similar principle to that of stem cell replacement therapeutic strategies, whereby they interact with the visual processing pathway, which is intact beyond the damaged cells in retinal degeneration. Many research teams across the world have tried to identify the optimal approach to provide stimuli to native retinal cells. There are broadly three types of retinal prosthesis

according to their surgical placement – epiretinal, subretinal, and suprachoroidal (Bloch, Luo et al. 2019).

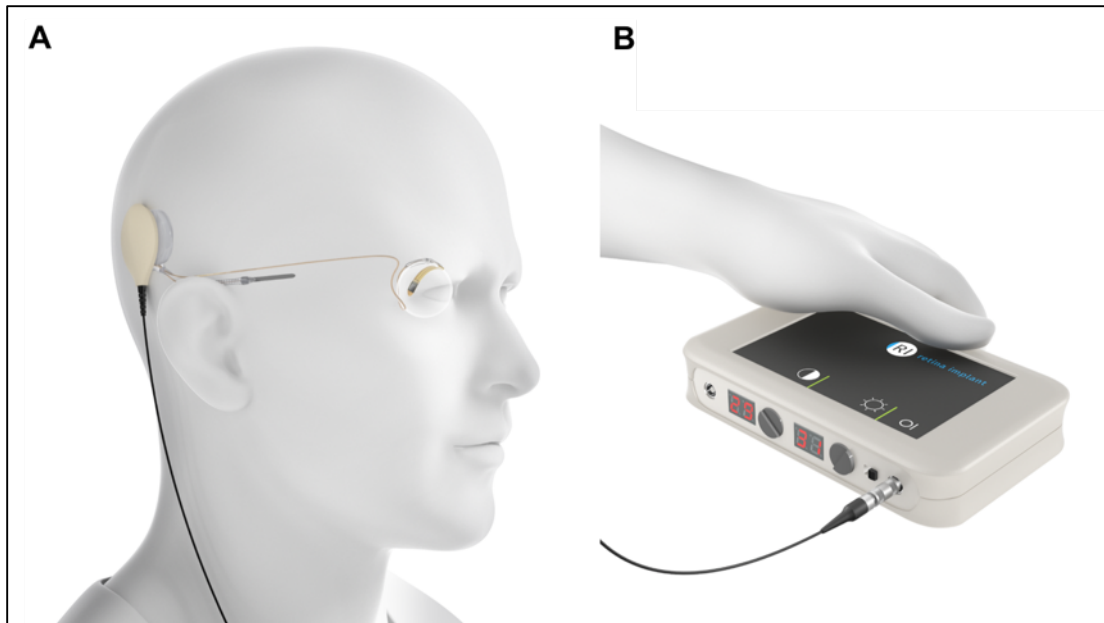
The earliest retinal prosthesis to be licenced for use in Europe and gain the CE mark was the Argus II retinal prosthesis (Second Sight Medical Products Inc., Sylmar, CA, USA) in 2011. This was closely followed by FDA approval in 2013. To date, over 250 patients have had this device implanted and the 10 year results of the phase II multicentre trial of this implant in patients with end-stage retinitis pigmentosa are awaited in 2019 (clinicaltrials.gov identifier NCT00407602). The device consists of a prosthesis with intraocular and extraocular components, which is used in conjunction with a pair of camera-mounted glasses connected to a video processing unit (Figure 12). At five years of follow up, the device remained in situ and functioning in 24 of 30 eyes. One patient had died, two patients' implants became non-functioning, and three were explanted. No eyes were lost in the course of the study and patients have maintained their ability to perform well in unique visual function tests designed to assess the efficacy of the device up to five years. The three tasks designed as primary endpoints included locating a white square on a black background screen, identifying the direction of movement of a high contrast bar on a screen and a visual acuity measurement using square wave gratings of different spatial frequencies. Patients had a lower mean error for the first two tasks with the system on than when it was off and, while all patients had visual acuities of <2.9 logMAR with the system off, all patients had a visual acuity of 2.9 logMAR or greater with the system on (da Cruz, Dorn et al. 2016).



**Figure 12. The Argus II Retinal Prosthesis (da Cruz, Dorn et al. 2016).**

***A) depicts the scleral band which encircles the globe along with the microarray which is passed through a sclerostomy and tacked onto the retinal surface. B) depicts the parts of the device worn externally.***

The Alpha AMS (Retina Implant AG, Reutlingen, Germany) is an example of a subretinal implant and the implant that follows the Argus II as the second most frequently implanted retinal prosthesis with over 60 devices implanted as of 2016 (Retina Implant). The device obtained the CE mark in 2013. Its multiphotodiode array is placed subretinally intraocularly, and its supply cable is tunneled and fixed to the postauricular cranial bone where a removable external coil can be attached magnetically which links to a handset controlling contrast sensitivity and brightness, in a similar fashion to a cochlear implant (Figure 13). Two-year results of this device showed a sustained increase in visual function in 5 out of 6 recipients in a recent clinical trial (Edwards, Cottrill et al. 2018). Comparing these devices, the Alpha AMS retinal prosthesis has the ability to detect light intraocularly, whereas the Argus II retinal prosthesis detects light using an external camera and transmits this information to intraocular electrodes. The Argus II and the Alpha-AMS can provide visual acuity of 20/1260 and 20/546 and a visual field of 20 and 15 degrees, respectively. The coming years will see further refinements of these devices in terms of durability and resolution which will hopefully provide an option for those patients in whom other treatments such as gene-based therapies are no longer possible.



**Figure 13. Retinal Implant (Retina Implant).**

***A) depicts the intraocular and extraocular component of the Alpha AMS device with the silicon supply tubing tunnelled to its attachment postauricularly. An external coil is attached magnetically which is linked to the handheld device in B) which has the ability to control brightness and contrast sensitivity.***



## 1.8 Outline of thesis

In Chapter three, I will outline the Target 5000 project, for which I performed deep phenotyping for over 150 patients during my research post in the Research Foundation of the Royal Victoria Eye and Ear Hospital. I took blood samples which were brought to the Smurfit Genetics laboratory in Trinity College Dublin for gene sequencing in an effort to make a molecular diagnosis in every case. The aim of this ongoing project is to carry out genotyping and deep phenotyping of the estimated 5000 patients in Ireland with an inherited retinal degeneration. This chapter will provide an overview of the genotyping and phenotyping of inherited retinal degenerations and the obstacles we face in identifying the correct aetiology of a patient's condition. This standardised dataset as a whole, to which I contributed, will further the ability to "solve" IRD cases as the international database grows.

Chapters four, five and six will describe individual pedigrees where I performed deep phenotyping that are of particular interest and highlight the importance of this work. Chapter four describes a novel GNAT1 variant which caused a late onset retinitis pigmentosa phenotype, a phenotype which had not been attributed to this gene before as it is generally associated with congenital stationary night blindness. Chapter five describes a patient with a biallelic mutation of the PROM1 gene which we believe to have caused a cone-rod dystrophy. This chapter illustrates the difficulty in assigning disease-causing status to a genetic mutation based on the evidence available in certain cases. Chapter six describes a pedigree of patients where older members of the family presented with a similar advanced phenotype that was found to be caused by two separate underlying genetic mutations in the CHM and RPE65 genes, which meant that there were two separate inherited diseases in the family. As these are inherited in an X-linked and autosomal recessive manner, respectively, this had implications for genetic counselling and illustrates the importance of a molecular diagnosis for these patients. These cases will serve to illustrate the importance of the Target 5000 project on a broad level and to highlight the depth of knowledge that is gleaned about individual patients.

## Chapter 2: Materials and Methods

### 2.1 Patient selection

There are estimated to be approximately 3000 people in the Republic of Ireland, and 5000 people in the island of Ireland, with an inherited retinal degeneration. The initiative “Target 5000” was established to attempt to identify all of these patients and relevant relatives and analyse both their genotype and phenotype. There are three clinical sites involved in this project in Ireland – The Research Foundation at the Royal Victoria Eye and Ear Hospital (Dublin, Ireland), the Mater Misericordiae University Hospital (Dublin, Ireland), and the Royal Victoria Hospital (Belfast, United Kingdom). The work I carried out during my research role was contributing to the Target 5000 project through the Royal Victoria Eye and Ear Hospital in Dublin, under the supervision of Dr. Paul Kenna.

Several avenues of referral exist to patients who sign up to Target 5000:

1. The first is self-referral. Any patient who contacts any of these sites will be given an appointment date;
2. The second is referral by another medical professional, usually an ophthalmologist. This referral pathway has been highlighted at national ophthalmology meetings so that other ophthalmologists are aware of the availability of the service;
3. The third avenue is through referral from the charity, Fighting Blindness, which has contact with many patients with inherited retinal degenerations and which has information about the project available on its website (Fighting Blindness);
4. Finally, many patients refer their affected friends or relatives to the service.

Patients were recruited prospectively and were invited to attend the Royal Victoria Eye and Ear Hospital for ophthalmic assessment including extensive

clinical history with extended pedigree information, diagnostic tests, diagnostic discussion and counselling, where appropriate.

## **2.2 Ethical approval**

Ethical approval for this study was obtained from the Royal Victoria Eye and Ear Hospital ethics committee in 2011. This ethical approval was extended upon acceptance of a Health Research Board grant in 2014. Ethical approval allowed for the collection of genotype and phenotype data of Irish patients who have reached the age of 18 years of age with inherited retinal degenerations, and their relatives. All patient interaction and manipulation of data and biological materials was performed in accordance with the Declaration of Helsinki. Informed consent was obtained prior to obtaining any blood samples for DNA analysis. Information leaflets pertaining to the nature and purpose of the study, along with a copy of the informed consent document, were then provided to patients.

## **2.3 Phenotype Data**

### ***2.3.1 Phenotype data collected***

Phenotype data collected included a detailed history of the patient's condition, other medical conditions and family history, along with detailed testing of their clinical status. The patient's history was obtained by detailed interview. Specific enquiry was made about symptoms common to patients affected by inherited retinal degeneration such as nyctalopia (decreased night vision), constricted visual fields, photopsias, decreased near vision, distance vision and colour vision. The patients underwent tests of visual acuity, colour vision, visual fields, electrophysiological testing as appropriate, and, lastly, ocular coherence tomography (OCT), and colour fundal photography. Relatives of affected individuals underwent some or all of the tests mentioned depending on their carrier status and whether or not their initial tests such as visual acuity and visual fields were within normal limits or suggestive of underlying pathology.

### **2.3.2 Case history**

A proforma “Case Report Form” was produced to reproducibly analyse the history of the patient’s age at initial diagnosis, visual complaints, progression of disease, ocular and systemic co-morbidities, family history and to document the physical examination of the patient. The patient was specifically asked about the symptoms of nyctalopia, decreased visual acuity, peripheral and central visual field defects, and susceptibility to glare. The patient was then asked an “open” question about whether they had any other complaints or problems related to their eyes. For each symptom that was discussed, the age at commencement of symptom was recorded.

The subjective course of the disease was noted - whether the patient felt their condition was stable, progressive, or that the condition was in the initial stages, having been diagnosed within the previous two years.

A question was asked about the historical or current presence of amblyopia, strabismus, or nystagmus and the age at which spectacle-wear commenced. For further information about the patient’s ocular history, cataract development and a history of cataract surgery were specifically enquired about, followed by an open question regarding the existence of any further ophthalmological disorders or previous surgeries.

The patient was questioned about their past medical history, with specific enquiries about their birth history and relevant malformations or disabilities. They were questioned about their past surgical history, current medications, and allergies. They were asked about treatments taken for their eyes and specifically about the use of vitamin A for the treatment of retinitis pigmentosa, along with the dose taken.

A pedigree was drawn to indicate all relatives who were affected, or possibly affected by the retinal degeneration. Other inherited diseases (not necessarily confined to the eyes) were listed. The patient was asked about the geographic origin of their grandparents and any known consanguinity within the family.

A social history with regard to alcohol intake, smoking history, and the use of recreational drugs was noted. Each of these case report forms was kept in the patient's file and subsequently entered into an electronic proforma in fillable PDF format, which is kept in a central, searchable database.

### **2.3.3 Visual acuity**

Visual acuity was tested monocularly using revised 2000 Early Treatment of Diabetic Retinopathy Study (ETDRS) visual acuity charts (Precision Vision, La Salle, Illinois, USA) at a distance of four metres. If the patient was unable to see any letters on the chart from four metres, their visual acuity was tested at one metre. If they were unable to see any letters on the chart at one metre, their vision was assessed by the ability to count fingers held up by the examiner, observe the hand motions made by the examiner, or perceive the presence or absence of light using an indirect ophthalmoscope as a light source. Visual acuity was first measured with the patient's own glasses. They were then refracted the patient's best-corrected visual acuity (BCVA) was measured. When the BCVA was achieved, the eyes were each tested using a new, separate logMAR visual acuity chart for reproducibility of the BCVA. Near visual acuity was tested monocularly using a near vision chart and the appropriate refraction, including a presbyopic near addition, as required.

### **2.3.4 Physical examination**

All patients underwent a thorough examination of the eyes, including the anterior segment (anterior to the crystalline lens) and a dilated pupil examination of the posterior segment (posterior to the crystalline lens). The physical examination was documented in the "Case Report Form" proforma mentioned in the previous section. At the slit lamp examination, any pathology of the lids, conjunctiva, cornea, anterior chamber, or iris was noted. Intraocular pressure testing was performed using a Goldmann tonometer, with topical proxymetacaine hydrochloride 0.5% (Bausch and Lomb), a local anaesthetic which acts by antagonizing voltage-gated sodium channels, and fluorescein sodium 1% dye (Bausch and Lomb). Analysis of light response to pupils was tested using an

indirect, wall-mounted ophthalmoscope as a light source. Both eyes of each patient were dilated with topical drops of tropicamide 1% (a topical antimuscarinic drug that paralyses the sphincter pupillae) and phenylephrine 2.5% (a topical sympathomimetic that causes contraction of the dilator pupillae).

A dilated fundal examination was carried out using a variety of methods to view the fundus. A slit lamp (Haag-Streit Diagnostics) was utilized to assess the degree of cataract formation, amount of cells in the vitreous, and whether there was vitreous body damage. A slit lamp was then used in conjunction with a double-convex condensing lens to view the posterior pole and peripheries of the retina. The retina was also viewed with a wall-mounted indirect ophthalmoscope and a direct ophthalmoscope. Retinal features examined include the disc, macula, retina, and choroid. A note was made of any abnormality of these parameters along with the quality/presence of the ILM reflexes, the foveal reflex, the wall reflex, and the degree and location of retinal pigmentation seen. Finally, a note was made as to whether the patient's visual fixation was central or eccentric.

### ***2.3.5 Colour vision testing***

The patient's colour vision was assessed using Lanthony's Desaturated 15-hue colour vision test. Colour vision was tested monocularly unless visual acuity precluded monocular testing, in which case binocular colour vision was recorded. Habitual lenses were worn. Lighting was controlled using a Daylight Illuminator to provide a consistent interest light source. Results were recorded on a proforma, which was analysed using an online scoring platform (<http://www.torok.info>). This software calculates the total error score in the method set out by Lanthony (Lanthony 1978). The score is also provided in the form of a vector diagram illustrating the subtype of colour deficiency i.e. protan, deutan, tritan, rod monochromacy, or blue cone monochromacy.

### **2.3.6 Goldmann visual field**

The Goldmann visual field is delineated using a bowl-shaped perimeter, which projects a bright light on a white background to map the patient's visual field. The intensity of the background light is standardized. The target light varies in size, luminance, and colour. For the practice of testing these patients, only two target variables were used – that of size and luminance. The Goldmann perimeter is a kinetic test of the visual field, meaning a target is moved from outside the visual field into the visual field. When it enters the visual field, the patient presses a device, which makes a sound. This allows the examiner to annotate the peripheral extent of the patient's field to this target. The most common target sequences (denoting lights of different size or luminance) used in our study included: IV4e, I4e, I2e for patients with normal visual fields, and IV4e, I4e, 04e for patients with partially restricted fields. Other sequences were rarely used in the case that the patient could not see the IV4e target. Visual fields were tested monocularly for the right and then the left eye. A reading addition, pertaining to degree of presbyopia according to age, was added for smaller targets.

Visual fields were mapped for each target and the points were joined together to make the "isopters" which denoted the field of vision for each light of equal size and luminance. Each isopter corresponds to a different target. Scotomata are also mapped out in a reverse fashion, moving the target from the centre of the scotoma into the seeing area and then mapping the boundaries.

### **2.3.7 Electrophysiology**

Electrophysiology testing in our cohort of patients is tailored to the patient's condition. The vast majority of patients with diffuse retinal degenerations underwent an electroretinogram (ERG). However, for those patients whose primary pathology was confined to the macula or the optic nerve, a pattern electroretinogram (PERG), multifocal electroretinogram (mfERG), electrooculogram (EOG), visual evoked potentials (VEP), or a selection of the above tests was performed.

### 2.3.7.1 Dark adaptometry

Dark adaptometry was performed using a Goldmann/Weekers dark-adaptometer after 30 minutes of dark adaptation. The dark-adapted threshold (DAT) was measured using a series of dim light stimuli at 15° inferior to fixation. The result was measured on a log scale.

### 2.3.7.2 Electroretinogram (ERG)

A standard full-field Ganzfeld electroretinogram was performed on the majority of patients using a Roland Consult RETI-port retiscan (Brandenburg an der Havel, Germany). This test quantifies the electrical responses from cells of different types in the retina and represents the most important diagnostic test for the majority of our patients, as the majority are affected by retinitis pigmentosa or other related disorders. This involved a period of dark adaptation of thirty minutes prior to testing. Contact lens electrodes were used as the recording electrode, along with three reference skin electrodes. Where patients were not able to tolerate contact lens electrodes, either gold foil or DTL (Dawson-Trick-Litzkow) electrodes were used. ERGs were carried out to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards with the exception of the strong flash which the Roland Consult system in our institution does not currently support (McCulloch, Marmor et al. 2015). The clinical protocol consisted of:

1. Weak flash (DA (dark-adapted) 0.01 cds/m<sup>2</sup>): The dim flash ERG arises from rod bipolar cells and is an assessment of rod function in isolation of cone cells.
2. Standard flash (DA 3.0 cds/m<sup>2</sup>): This response consists of an a-wave, mediated by inner retinal rod photoreceptor cells with a smaller contribution from cone photoreceptors and a b-wave mediated by rod bipolar cells.
3. Oscillatory potentials (assessing mesopic vision): The standard flash is used but the bandpass filter is reset to a low frequency of 75-100Hz and a high frequency of minimum 300Hz. Oscillatory potentials are thought to arise from the amacrine cells and can be an early indicator of inner retinal



dysfunction. They are found on the upward limb of the b-wave in the 3.0 and 10.0 cds/m<sup>2</sup> DA ERG.

Prior to light-adapted ERG testing, a period of light adaptation with the head remaining in the Ganzfeld bowl with a luminance of 30 cds/m<sup>2</sup> was undertaken for ten minutes.

4. Single flash cone (LA 3.0 cds/m<sup>2</sup>): This wave consists of a negative a-wave deflection and a positive b-wave deflection. The a-wave arises from the cone photoreceptors and OFF bipolar cells whereas the b-wave arises from ON and OFF bipolar cells which synapse with cone photoreceptors.
5. 30Hz Flicker (LA (light-adapted) 30Hz): The 30 Hz test is undertaken with a background luminance of 3 cds/m<sup>2</sup>. These responses arise from the cone bipolar cells.

Electroretinogram results were interpreted using site-specific normative values. A normal suite of electroretinogram and pattern electroretinogram results are shown in Figure 14. In retinitis pigmentosa, a classic finding is early depression of the scotopic ERG followed slowly by involvement of the photopic ERG. In the late stages of the disease, no discernible signal is detectable from either the rod or cone system.

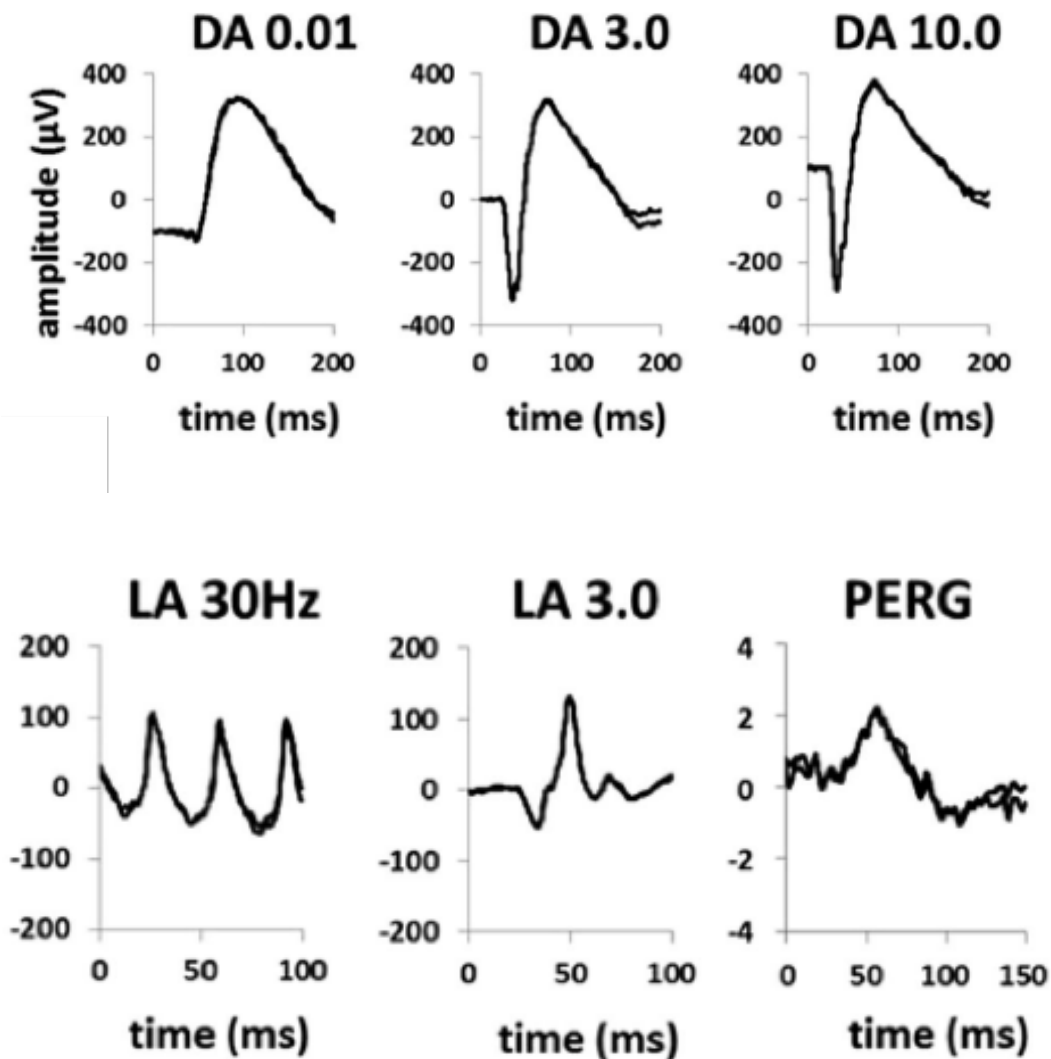


Figure 14. The normal electroretinogram (ERG) and pattern electroretinogram (PERG) trace, adapted from (Robson, Nilsson et al. 2018)

*These graphs illustrate the normal appearance of the ERG and PERG demonstrated after stimulation of the retina in accordance with ISCEV guidelines. The relevant waves elicited are described in terms of their latency or implicit time, measured in milliseconds, and their amplitude, measured in microvolts. The waveform of these responses correlates to the activity of rod and cone photoreceptors, ON and OFF bipolar cells and ganglion cells.*

#### *2.3.7.3 Pattern electroretinogram (PERG)*

The pattern electroretinogram provides an estimation of macular function by stimulating the retina with a series of pattern-reversal checkerboards in both a standard (15 degree) and large (30 degree) field. Corneal electrodes are used. The doubling in size of the stimulus allows for the inspection of the peripheral macula and as a rule of thumb, the amplitude of the positive deflection, or P50, would be expected to double in response to the large field. The negative component, N95, is a reflection of ganglion cell activity. Both the positive P50 component and the negative N95 component arise from the macular ganglion cells. The PERG is often depressed in macular dystrophies, cone dystrophies and cone dysfunction syndromes.

#### *2.3.7.4 Multifocal electroretinogram (mfERG)*

The multifocal electroretinogram performs individual ERGs of small individual areas of retina across the posterior pole and can identify focal retinal pathology which is often not detected by a full field or pattern ERG. It measures cone system function. The stimulus on a screen consists of multiple hexagons which illuminate in a “pseudo-random sequence” and the responses for each separate hexagon are mathematically extracted and visualised as an individual ERG recording for each area. It is used to delineate, for example, a bull’s eye maculopathy and can identify the small focal defect found in cases of occult macular dystrophy.

#### *2.3.7.5 Electrooculogram (EOG)*

The electrooculogram provides an assessment of the function of the retinal pigment epithelium. It measures the electrical polarity of the retinal pigment epithelial cells and assesses the change in the amplitude of this response when elicited in the dark compared to the light (scotopic versus photopic conditions). The ratio of the lowest scotopic amplitude (“dark trough”) to the highest photopic amplitude (“light peak”) is expressed as a percentage as is known as the “Arden ratio”. If it is smaller than 160%, it is considered abnormal. Although a strongly

depressed ERG will cause a reduced Arden ratio regardless of pathology, a reduced Arden ratio in the presence of a robust ERG recording often leads to a diagnosis of Bestrophinopathy, an inherited maculopathy which can be found in an autosomal dominant or recessive form.

#### *2.3.7.6 Visual evoked potentials (VEP)*

The VEP is an important measure of optic nerve, chiasmal and post-chiasmal optic pathway disease. The measurement is taken from scalp electrodes measuring potentials in the visual cortex in response to visual stimuli. Stimuli used include pattern reversal, flash and pattern appearance. Leber's Hereditary Optic Neuropathy often has a severely depressed, if not absent, P100 component of the pattern VEP at presentation, although this is not a pathognomonic sign.

#### **2.3.8 Fundus photography**

Fundus photography was performed using a Topcon CRC50DX fundus camera (Topcon Medical Systems Inc., Oakland, NJ, USA). Colour images were obtained of the posterior pole and with the patient looking in four directions – up, down, left and right to capture peripheral retinal changes.

Autofluorescence imaging of the posterior pole was also obtained using the Topcon system and the contrast was adjusted to obtain a high quality image post-processing. Autofluorescence imaging describes the ability of certain molecules within the eye called fluorophores to become excited when stimulated by certain wavelengths of light, which causes them to emit light (Calvo-Maroto and Cerviño 2018). Lipofuscin is the fluorophore within the RPE cells.

Autofluorescence imaging is extremely useful in the case of inherited retinal degenerations, as it can highlight subtle retinal changes when there is little to see with white-light colour photography and is a reflection of RPE cell health. A characteristic ring of hyperautofluorescence is seen in many patients with retinitis pigmentosa which contracts over time. The area bound by the ring is thought to contain preserved photoreceptors and the radius of the area correlates with PERG P50 function (Robson, Michaelides et al. 2008).

For those patients who were unable to undergo mydriatic photography, wide angle fundus photography was carried out using a scanning laser ophthalmoscope (Optos Inc., Marlborough, MA, USA).

### ***2.3.9 Optical coherence tomography (OCT)***

Spectral-domain optical coherence tomography (OCT) allows for the visualisation of the layers of the retina by way of laser interferometry. We used the Cirrus HD-OCT (Carl Zeiss Meditec, Jena, Germany) to perform macular OCT on all patients and optic disc OCT where applicable (e.g. those patients with Leber Hereditary Optic Neuropathy). Where there was an inability to obtain a high-quality OCT image, a 5 line raster image was taken which requires less time to obtain (e.g. in cases of nystagmus or eccentric fixation). The OCT changes seen most often in patients with retinitis pigmentosa included loss of the ellipsoid zone moving centrally toward the fovea and cystoid macular oedema.

## **2.4 Molecular Genetic Studies**

### ***2.4.1 Blood samples***

Blood samples were taken for genetic analysis using standard phlebotomy techniques. Two 7.5 ml blood bottles with EDTA were obtained for genotyping purposes. One was transferred from the Research Foundation, Royal Victoria Eye and Ear Hospital to the Smurfit Institute of Genetics, Trinity College Dublin for further genetic analysis. The second sample was banked in the Royal Victoria Eye and Ear Hospital for the purposes of future confirmatory molecular testing.

### ***2.4.2 DNA isolation and sequencing***

Following informed consent, blood samples were collected from patients after clinical assessment. DNA was isolated from 2 mL of patient blood using the QIAamp DNA Blood Midi kit (QIAGEN, Valencia, California, USA), according to manufacturer's recommendations.

DNA was fragmented for sequencing by ultrasonication in a Diagenode Bioruptor (Diagenode, Belgium) for 35 cycles, each consisting 30 s of low-intensity sonication followed by a 30s pause. Water was replaced and crushed ice added regularly to maintain a sonication temperature of <math><4^{\circ}\text{C}</math>.

Sequencing libraries were generated and target capture was performed using the Agilent SureSelect XT2 kit (Agilent Technologies, Santa Clara, California, USA), according to manufacturer's recommendations. Later captures used a redesigned panel with the Roche Nimblegen SeqCap EZ kit (Roche), incorporating new genes implicated in retinopathies since the design of the earlier panel. Exons and untranslated regions (UTRs) for all genes previously implicated in retinal degeneration, as listed by Retnet, were included as capture targets (Figure 15).

A	BBS9	CNGB3	GNAT1	K	NEK2	PEX1	RDH12	T	WFS1
ADIPOR1	BEST1	CNNM4	GNAT2	KCNJ13	NEUROD1	PEX2	RDH5	TEAD1	WFS1
ABCA4	C	COL11A1	GNB3	KCNV2	NMNAT1	PEX7	RGR	TMM8A	WHRN
ABCC6	C12orf65	COL2A1	GNPTG	KIAA1549	NPHP1	PGK1	RGS9	TIMP3	Z
ABHD12	C1QTNF5	COL9A1	GPR179	KIF11	NPHP3	PHYH	RGS9BP	TMEM126A	ZNF408
ACBD5	C21orf2	CRB1	GRK1	KIZ	NPHP4	PITPNM3	RHO	TMEM216	ZNF423
ADAM9	C2orf71	CRX	GRM6	KLHL7	NR2E3	PLA2G5	RIMS1	TMEM237	ZNF513
ADAMTS18	C8orf37	CSPP1	GUCA1A	L	NR2F1	PLK4	RLBP1	TOPORS	
ADGRA3	CA4	CTBP2	GUCA1B	LAMA1	NRL	PNPLA6	ROM1	TREX1	
ADGRV1	CABP4	CTNNA1	GUCY2D	LCA5	NYX	POC1B	RP1	TRIM32	
ADIPOR1	CACNA1F	CYP4V2	H	LRAT	O	POMGNT1	RP1L1	TRNT1	
AGBL5	CACNA2D4	D	HARS	LRT3	OAT	PRCD	RP2	TRPM1	
AHI1	CAPN5	DHDDS	HGSNAT	LRP5	OFD1	PRDM13	RP9	TSPAN12	
AIPL1	CC2D2A	DHX38	HK1	LZTFL1	OPA1	PROM1	RPE65	TTC8	
ALMS1	CDH23	DMD	HMCN1	M	OPA3	PRPF3	RPGR	TTL5	
ARL2BP	CDH3	DRAM2	HMX1	MAK	OPN1LW	PRPF31	RPGR	TTPA	
ARL3	CDHR1	DTHD1	I	MAPKAPK3	OPN1MW	PRPF4	RPGRIP1	TUB	
ARL6	CEP164	E	IDH3B	MERTK	OPN1SW	PRPF6	RPGRIP1L	TUBGCP4	
ASRGL1	CEP250	EFEMP1	IFT140	MFN2	OTX2	PRPF8	RS1	TUBGCP6	
ATF6	CEP290	ELOVL4	IFT172	MFRP	P	PRPH2	RTN4IP1	TULP1	
ATXN7	CERKL	EMC1	IFT27	MFS2D8	PANK2	PRPS1	S	U	
B	CFH	EXOSC2	IMPDH1	MIR204	PAX2	R	SAG	UNC119	
BBIP1	CHM	EYS	IMPG1	MKKS	PCDH15	RAB28	SDCCAG8	USH1C	
BBS1	CIB2	F	IMPG2	MKS1	PCYT1A	RAX2	SEMA4A	USH1G	
BBS10	CLN3	FAM161A	INPP5E	MTTP	PDE6A	RB1	SLC24A1	USH2A	
BBS12	CLRN1	FLVCR1	INVS	MVK	PDE6B	RBP3	SLC25A46	V	
BBS2	CLUAP1	FSCN2	IQCB1	MYO7A	PDE6C	RBP4	SLC7A14	VCAN	
BBS4	CNGA1	FZD4	ITM2B	N	PDE6G	RCBTB1	SNRNP200	W	
BBS5	CNGA3	G	J	NBAS	PDE6H	RD3	SPATA7	WDPCP	
BBS7	CNGB1	GDF6	JAG1	NDP	PDZD7	RDH11	SPP2	WDR19	

**Figure 15. Genes included in target capture sequencing for inherited retinal degenerations as part of the Target 5000 project (Dockery, Stephenson et al. 2017)**

Captured patient DNA was multiplexed into either 24- or 96-sample pools and sent for sequencing. Sequencing of 96-sample pools was performed off-site by BGI Tech using an Illumina HiSeq 2000 (Illumina, San Diego, CA). 24-sample pools were sequenced locally using an Illumina MiSeq. Confirmatory single-read sequencing was also performed to verify the presence of candidate mutations.

#### ***2.4.3 PCR and Sanger sequencing***

In order to validate candidate mutations found in NGS experiments, amplicons containing the mutations were generated by polymerase chain reaction (PCR) and analysed by direct sequencing. Oligonucleotides were purchased from Sigma-Aldrich (Gillingham, England). The target DNA products were amplified using Q5 High-Fidelity 2× Master Mix (New England Biolabs Inc., Ipswich, MA, USA). The annealing temperature for reactions was optimised for each mutation; all other details were executed as per the supplier's recommendations. Sanger sequencing was performed by Eurofins Genomics (Ebersberg, Germany).

#### ***2.4.4 Mutational Analysis***

Sequence data were demultiplexed and mapped to the human genome (hg19) using BWA version 0.7.4 (Li and Durbin 2009). Duplicate reads were flagged using Picard version 1.106 (Broad Institute), and downstream analysis and variant calling were performed using the GATK version 2.8 (McKenna, Hanna et al. 2010) according to the protocol specified in the GATK Best Practices Workflow (Broad Institute).

Variants were annotated using SnpEff (Cingolani, Platts et al. 2012), dbNSFP (Liu, Wu et al. 2016), MetaLR (Dong, Wei et al. 2015) and M-CAP (Jagadeesh, Wenger et al. 2016) for the purposes of identifying rare, pathogenic coding changes. Annotations from the SPIDEX database were used to identify variants with predicted splicing impacts. Common variants, as measured by either frequency within our sample pool or frequency in external population databases, were filtered out of the analysis. Synonymous variants were also filtered out of downstream analysis, with the exception of analysis of potential splice site



alterations. 15× coverage was required at a site in order to call variants. This means that the nucleotide sequence had to be reconstructed at least 15 times to ensure the sequencing was accurate and that a sequence variant did not represent an error. This was calculated at the individual base level rather than the exon level. For almost all samples, more than 98% of targeted sites were covered to this depth (interquartile range: 98.3–99.1%).

Identifying structural variants requires separate strategies. Structural variants include insertions, deletions, inversions and translocations > 50 base pairs in length (Alkan, Coe et al. 2011). The term can be used to include copy number variations, which are short sequences of nucleotides that are abnormally repeated. In this study, structural variants were called by a separate pipeline, based on the tools LUMPY (Layer, Chiang et al. 2014), which was used to identify structural variants by detecting split reads or unusual paired read alignments, and CoNIFER (Krumm, Sudmant et al. 2012), which was used to identify structural variants where it identified aberrant read depths. Results from these tools were combined and filtered to output a final, high-quality list of putative structural variants and associated confidence scores for each sample.

Synonymous variants and common polymorphisms were filtered out, and the remaining list of rare variants with the potential to affect protein sequence was output for manual curation. The output for each patient also included a list of coding regions, where coverage was insufficient for reliable variant calling.

#### *2.4.4.1 MetaLR*

Meta Logistic Regression (LR) (Dong, Wei et al. 2015) incorporates pathogenicity prediction scores and maximum minor allele frequency from nine different tools for more accurate and thorough evaluation of deleterious effects of missense mutations. This allows for the more accurate assessment of variants than any of the singular methods alone.

#### 2.4.4.2 M-CAP

Mendelian Clinically Applicable Pathogenicity (M-CAP) Score (Jagadeesh, Wenger et al. 2016) was the first high sensitivity pathogenicity classifier for rare missense variants in the human genome aimed at the clinic. It combines pathogenicity scores from other tools and databases (including SIFT, Polyphen-2 and CADD) with novel features to create a more powerful model.

#### 2.4.4.3 Revel

REVEL (rare exome variant ensemble learner) (Ioannidis, Rothstein et al. 2016), is also an ensemble predictor tool and was trained with recently discovered pathogenic and rare benign missense variants, excluding those previously used to train its constituent tools. REVEL also claims to have the best performance for categorising pathogenic from rare benign variants with allele frequencies < 0.5%.

The American College of Medical Genetics and Genomics (ACMG) criteria for classifying pathogenic variants was utilised (Richards, Aziz et al. 2015). This report advises the use of standardised nomenclature for the reporting of variants in genetic disease i.e. 'pathogenic', 'likely pathogenic', 'uncertain significance', 'likely benign', and 'benign'. Placing a variant into one of these categories is based on a number of weighted categories including population data, computational and predictive data, functional data, segregation data, de novo data and allelic data, amongst others.

#### 2.4.5 Protein modelling

3D models of wildtype and mutant proteins were generated using Iterative Threading ASSEmblY Refinement, I-TASSER (Yang, Yan et al. 2015). Polymer structures of wild type proteins were obtained from the Protein Data Bank (PDB, ID#3JD6,) (RCSB Protein Data Bank). The effect of single point mutations on protein stability was measured using STRUM (Quan, Lv et al. 2016). Protein alignments were generated using Clustal Omega (Sievers, Wilm et al. 2011).



# **Chapter 3: Results of next generation sequencing and deep phenotyping in Target 5000 patients**

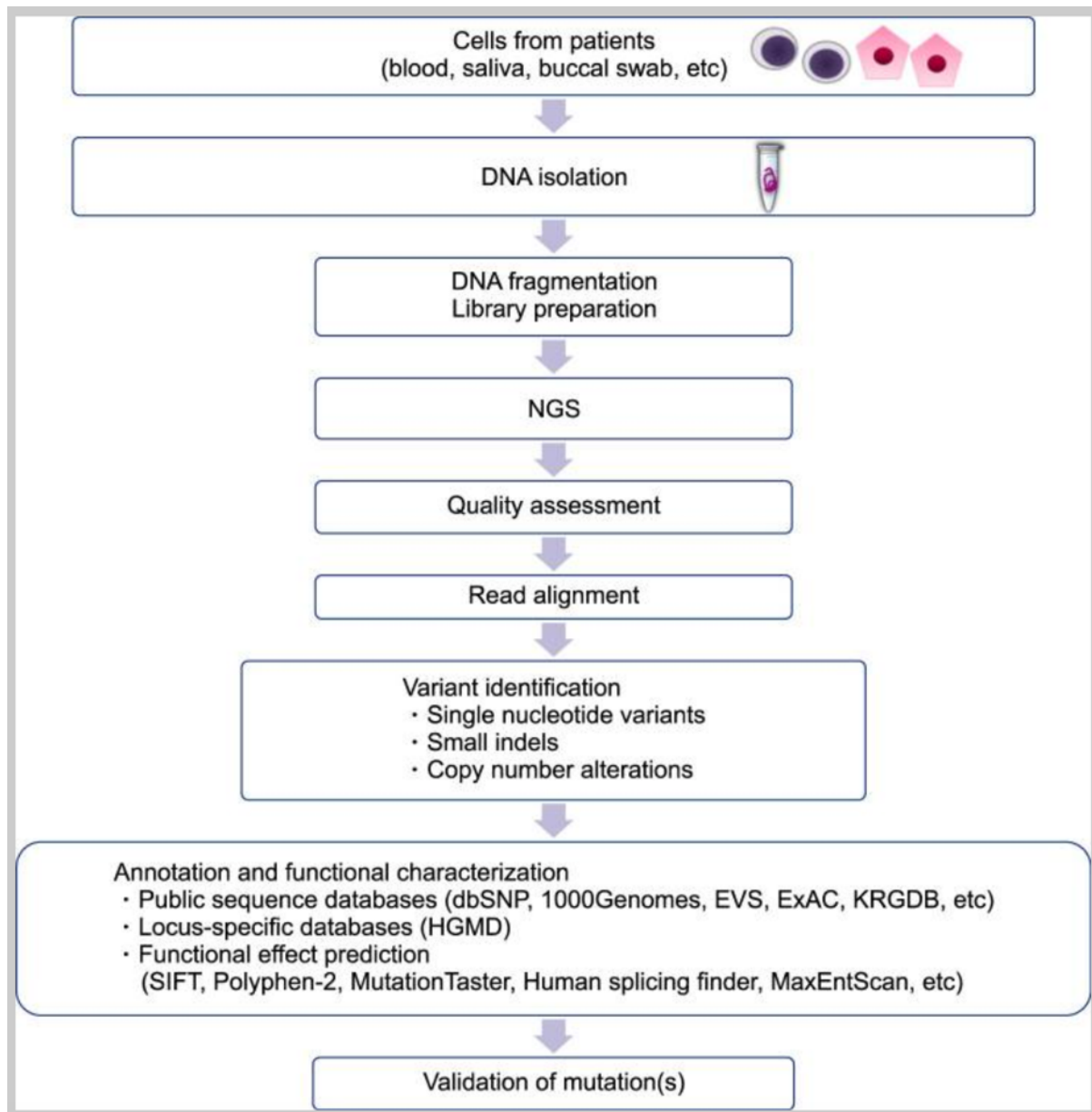
## **3.1 Summary**

The Target 5000 project aims to collect data on the deep phenotyping and molecular diagnoses of all patients in Ireland with an inherited retinal degeneration, the number of which is estimated to be in excess of 5000. In the past decade, the method of collecting both of these sets of data has changed dramatically. Imaging techniques such as autofluorescence and OCT have allowed more detailed phenotype analysis and targeted next generation sequencing (NGS) of related genes has allowed high-volume molecular testing at a relatively lower cost than previously used methods such as single-gene testing with Sanger sequencing which was laborious and expensive. To date, as part of this project, over 750 patients from over 520 pedigrees have been assessed. I carried out deep phenotyping of over 150 of these subjects. This is in addition to the group of Irish patients diagnosed prior to the current study using methods prior to NGS. In this chapter, I will provide an overview of the results of testing in this group and how this knowledge has contributed to our understanding of these conditions. Limitations of this study and future possibilities are considered.

## **3.2 Introduction**

As outlined in chapter 1, inherited retinal diseases have now surpassed diabetic eye disease as the most common cause of blindness in the working age group in England and Wales, which is likely reflected in the Irish population (Liew, Michaelides et al. 2014). The focus on improving the prognosis for these patients has intensified over recent years and many avenues of treatment are being actively investigated in animal models and clinical trials. There has been a momentum amongst international vision scientists to collate these data in large

databases in order to further the diagnostic process into the future. Vast genetic variant databases have been created and many are available freely online, for example the Exome Aggregation Consortium (<http://exac.broadinstitute.org/>), Genome Aggregation Database (<https://gnomad.broadinstitute.org/>) and ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>). Further to this, open access online software allows for in silico analysis of pathogenicity based on factors such as conservation across species and protein structure and function. These prediction software platforms are used in conjunction with protein modelling programmes to give an estimate of pathogenicity. The process of diagnosis using this paradigm is summarized in Figure 16.



**Figure 16. The process of next generation sequencing and mutation analysis (Kim, Park et al. 2017).**

**Abbreviations:** NGS = Next generation sequencing, dbSNP, 1000genomes = 1000 genomes project, EVS = Exome Variant Server, ExAC = Exome Aggregation Consortium, KRGDB = Korean Reference Genome Database, HGMD = Human Gene Mutation Database, SIFT = Sorting Intolerant from Tolerant

### 3.3 Materials and Methods

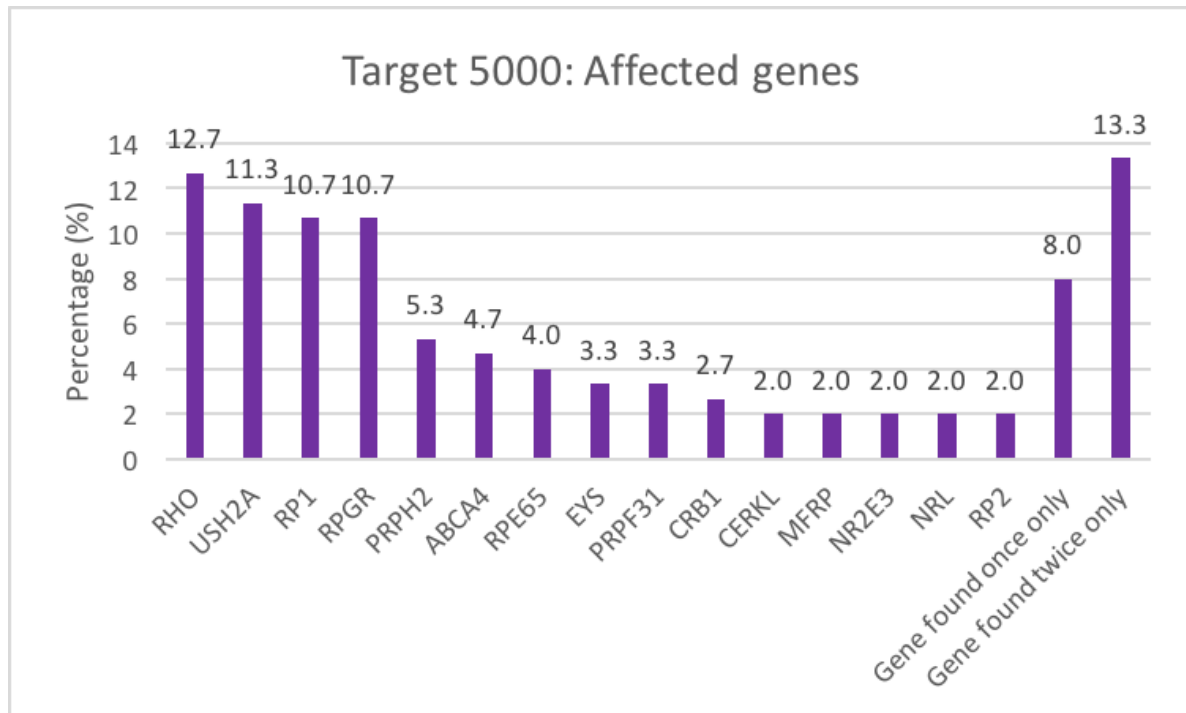
Patients underwent clinical assessment, with DNA isolation and sequencing. The details of these processes, along with data analysis of variants provided by sequencing, are detailed in chapter 2.

### 3.4 Results

As of 2017, there were 523 pedigrees entered into the current study throughout the island of Ireland. This work is remains ongoing. Patients were consecutively enrolled in the study on presentation at each of the three clinical sites and the current analyses do not include patients with historical diagnoses and confirmed molecular diagnoses. A molecular diagnosis was possible in 68% of cases, with a further 8% having a single disease-causing variant identified in a gene which is known to cause a recessive disease fitting with the phenotype. Internationally, the most common inherited retinal disease is retinitis pigmentosa which is a genetically heterogeneous disease with over 80 described causative genes (Daiger 2019). At a molecular level, the most common gene associated with inherited retinal degeneration is ABCA4 due to the genetic homogeneity of Stargardt disease; over 900 mutations have been identified in this single gene (Cornelis, Bax et al. 2017). In keeping with this, the most common condition encountered in our cohort was retinitis pigmentosa and the most common single gene was ABCA4 (80 pedigrees).

Of the retinitis pigmentosa patients sequenced, the most common gene identified was RHO, accounting for 12% of all RP patients and 30% of those with dominant RP (see Figure 17 below). This result provides an underestimation of the proportion of Irish patients carrying pathogenic variants in this gene, as many Irish patients with RHO-mediated RP were included in previous single-gene studies from our group and were thus excluded from this cohort. The most common disease-causing gene identified in those patients with autosomal recessive disease was USH2A, which accounted for 30% of recessive RP. The

more commonly found genes correspond closely to those most commonly identified in large series from the United States, Europe and Asia (Stone, Andorf et al. 2017, Koyanagi, Akiyama et al. 2019, Martin-Merida, Avila-Fernandez et al. 2019).



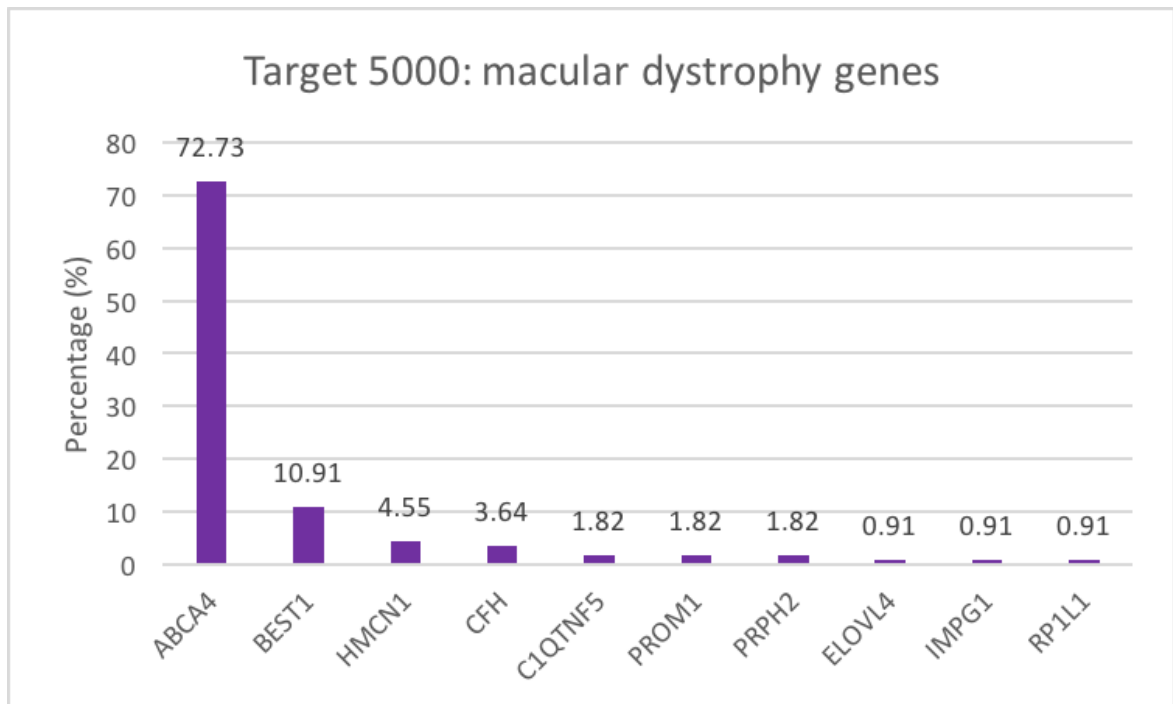
**Figure 17. Proportion of patients diagnosed with retinitis pigmentosa affected by described genes.**

*These relate to the proportion of patients affected by a specific gene. Genes which were observed only once or twice in the group are displayed separately.*

Eighty pedigrees were found to have mutations of ABCA4, causing Stargardt disease. Three novel mutations were identified. A discovery of importance to our cohort was that of the disease-causing nature of the ABCA4 variant p.Asn1868Ile (c.5603A>T), which has been shown to cause disease only when in trans with a loss-of-function allele and, even then, shows incomplete penetrance in a debated



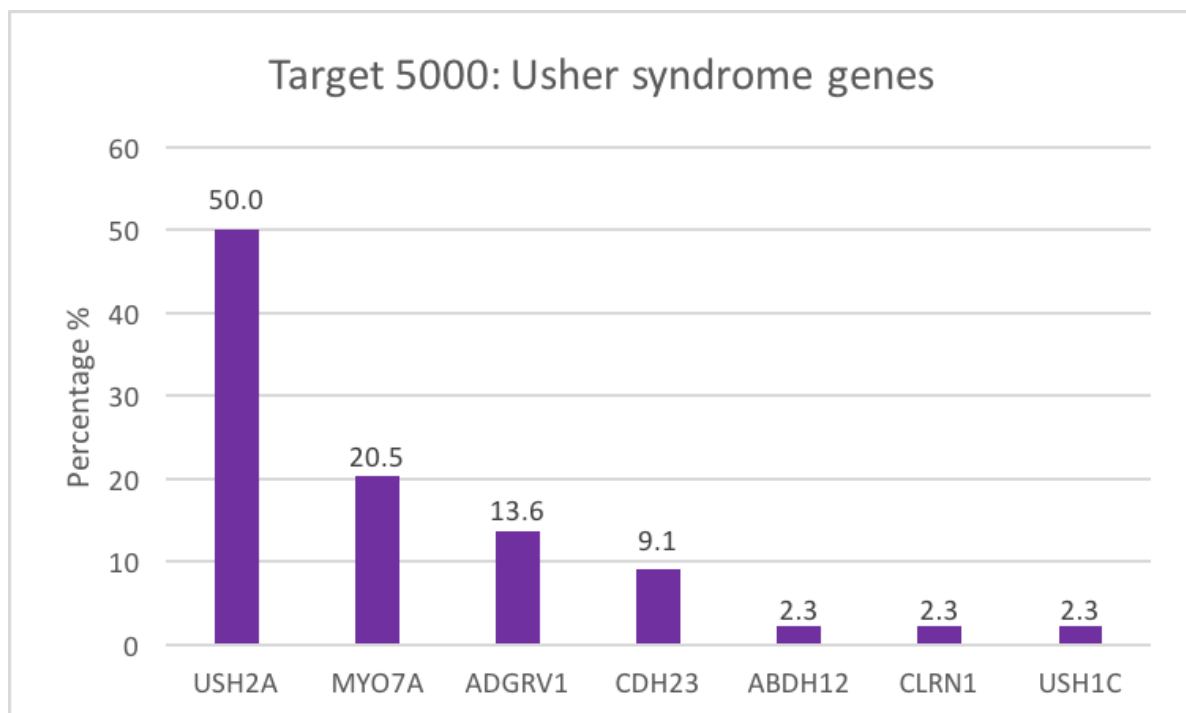
proportion of patients (Cremers, Cornelis et al. 2018, Runhart, Sangermano et al. 2018). Due to this re-classification of the mutation, a further 19 ABCA4 pedigrees were solved. A further 5 Irish cases were solved in collaboration with a group in Nijmegen (Frans Cremers) who identified a previously reported deep intronic mutation c.4539+2028C>T in all five cases. This variant has been shown to strengthen exonic splice enhancer elements and would be likely be amenable to antisense oligonucleotide-based treatment (Sangermano, Garanto et al. 2019). Antisense oligonucleotides are small RNA molecules that bind to a target sequence of DNA and can obstruct aberrant splicing, allowing the translation of the full protein. Other genes associated with maculopathies identified included ELOVL4, PROM1, and BEST1, the latter being the most common cause of dominant maculopathy in this cohort (see Figure 18 below).



**Figure 18. Target 5000 patients diagnosed with macular dystrophy, including Stargardt disease, and their underlying causative gene.**

A surprising finding was the number of patients diagnosed with Bardet-Biedl syndrome upon genotyping. Eight pedigrees were initially found to have Bardet-Biedl syndrome, and a further 19 pedigrees were found to be affected upon discovering their molecular diagnosis. Follow-up clinical examinations revealed signs of previously surgically addressed polydactyly, learning disability and obesity. The most commonly affected gene was BBS1, with the most common mutation being c.1169T>G, p.Met390Arg.

All three types of Usher syndrome were encountered in this group of patients. There were 47 pedigrees clinically identified as having Usher syndrome, and of these a molecular diagnosis was established in 44 (94%). The most common type was Type 2 and the most common gene identified in this group and indeed overall was USH2A (50% total Usher syndrome patients), in keeping with other reports (Stone, Andorf et al. 2017, Koyanagi, Akiyama et al. 2019, Martin-Merida, Avila-Fernandez et al. 2019). The most common gene identified in Type 1 Usher syndrome was MYO7A and, together, these two genes made up 70% of all diagnoses in this cohort (figure 19).



**Figure 19. Target 5000 patients diagnosed with Usher syndrome and respective causative genes.**

***With reference to the Irish dataset described by this table, and not limited to the following genes only; MYO7A, CDH23 and USH1C cause Usher syndrome type 1, USH2A and ADGRV1 cause Usher syndrome Type 2 and CLRN1 causes Usher syndrome type 3.***

### **3.5 Discussion**

To date, approximately 15% of what we believe to be the cohort of patients with inherited retinal degenerations on the island of Ireland have had deep phenotyping and genetic testing. The cohort described in this thesis underwent targeting next generation sequencing using a panel of 254 genes, in its most recent iteration. Notably, 4% of our patients with retinitis pigmentosa have mutations in the RPE65 gene, most commonly the autosomal dominantly inherited c.1430A>G, p.Asp477Gly variant. It is clear that, with the gene therapy Luxturna having been licenced for use in Europe, we are closer than ever to the era of treatment for inherited retinal degenerations being available to Irish patients. It is possible that gene replacement therapy such as Luxturna may be efficacious in the case of haploinsufficiency-related dominant disease, whereas dominant negative models may require further additional therapy to reduce the effect of the mutant protein (O'Reilly, Palfi et al. 2007). Bypassing of the visual cycle defect caused by this mutation with oral supplementation of the photoreceptors with a 9-cis-retinal analogue (QLT091001) has been attempted in these patients with some success and will soon be published. This has already been shown to improve retinal function in human subjects with autosomal recessive retinitis pigmentosa due to mutations of the RPE65 and LRAT genes (Scholl, Moore et al. 2015, Koenekoop, Sui et al 2014).

Seventy novel variants have been identified through the Target 5000 project, along with, in some cases, novel phenotypes as will be described in a case of

late onset retinal dystrophy associated with a novel GNAT1 variant in chapter 4. Some data have contributed to the possibility of pathogenicity of variants of uncertain significance, such as the example of a patient with cone-rod dystrophy with a PROM1 mutation described in chapter 5. Chapter 6 will allow us to explore the possibility of reclassification of patients exhibiting a certain phenotype given new genotype information within a pedigree where initial family members were found to have choroideraemia and others were later found to have retinitis pigmentosa.

The analysis of this large cohort of patients has highlighted common variants amongst unrelated families, likely pointing to a founder effect and the possibility that these families may be more distantly related. This theory will be further probed in future work by this group.

Although the “solve rate” of 68% is similar to those of other groups performing similar analyses internationally, a molecular diagnosis for many patients identified during this study was not established. Although there are undoubtedly further genes to be discovered, it is likely that many of the variants we are trying to identify in these patients will be found in the genes already featured in the panel. One piece of evidence for this is the fact that many patients will have one known disease-causing mutation in a gene known to cause autosomal recessive disease which fits with their phenotype, indicating there is another hidden mutation, which has yet to be identified in the same gene e.g. a patient with a Stargardt-like phenotype with one ABCA4 mutation indicates there is another ABCA4 mutation to be identified, rather than a novel gene. Some genetic variants are more difficult to elucidate than others, specifically because they are elusive when using modern NGS methods. The first of these are intronic mutations, which can be close to the exon and affect the splicing of the intron or they can be hidden deeply within the intronic sequence and may represent, for example, a binding site for certain transcription factors. Even when whole genome sequencing is used to search for these variants, it can be difficult to realise their pathogenicity

when their role may be unknown. Large structural variants and copy number variants can also escape identification by modern biostatistical software.

Further contributions such as the data from this project to international databases will provide more robust platforms on which to base diagnoses in the future.

Natural history studies will allow better prognostication for patients and more detailed phenotyping on which to base diagnoses. This information will lead to an increased solve rate in the future for all patients, which will allow for more specific diagnoses, the ability to plan and perform gene therapy, and to enable family planning.

# Chapter 4: A novel homozygous truncating GNAT1 mutation implicated in retinal degeneration

## 4.1 Summary

The *GNAT1* gene encodes the alpha subunit of the rod transducin protein, a key element in the rod phototransduction cascade. Variants in *GNAT1* have been implicated in congenital stationary night-blindness in the past, but unlike other proteins in the same pathway, *GNAT1* has not previously been implicated in retinitis pigmentosa. We present here a novel homozygous truncating mutation in the *GNAT1* gene in a patient with significant pigmentary disturbance and constriction of visual fields, a presentation consistent with retinitis pigmentosa. This is the first report of a patient homozygous for a complete loss-of-function *GNAT1* mutation. The clinical data from this patient provides definitive evidence of retinitis pigmentosa with late onset, in addition to the lifelong night-blindness that would be expected from a lack of transducin function. These data suggest that truncating *GNAT1* variants can indeed cause a mild, late-onset retinal degeneration in humans, with interesting parallels to the phenotype of the *Gnat1* knockout mouse.

## 4.2 Introduction

Transducin is a heterotrimeric G-protein found on the membrane of photoreceptor cells in the retina and is a key component of the vertebrate phototransduction pathway. Upon exposure of rod photoreceptor cells to light, the light sensitive pigment rhodopsin is photoisomerised into its active form. This form activates the G-protein transducin, which in turn stimulates cGMP-phosphodiesterase (PDE). The degradation of cGMP causes cGMP-gated ion channels to close, resulting in hyperpolarisation of the cell membrane and causing the electrical light response

(Ebrey and Koutalos 2001).

Transducin is composed of three polypeptide chains,  $\alpha$ ,  $\beta$ , and  $\gamma$ . The  $\alpha$  subunit is the active GDP/GTP-binding component. Upon activation by Rhodopsin, GTP/GDP exchange occurs and the  $\alpha$  subunit dissociates from the  $\beta\gamma$  subunits, at which point it becomes capable of binding PDE. The  $\alpha$  subunit proteins in rod and cone cells are encoded by the genes *GNAT1* and *GNAT2*, respectively (Lin, Weadick et al. 2013).

Variants in *GNAT1* have been implicated in congenital stationary night-blindness (CSNB) in the past, although never in retinal degenerations (Dryja, Hahn et al. 1996) reported a missense variant (NM\_000172.3:c.113G>A/p.Gly38Asp) causing the dominant 'Nougaret' form of CSNB, which they hypothesised to be a result of constitutive activation of transducin. A second example of a pathogenic *GNAT1* variant (c.598C>G/p.Gln200Glu) was also reported in a Danish dominant CSNB pedigree (Szabo, Kreienkamp et al. 2007).

*GNAT1* variants have also been associated with recessive forms of CSNB. A missense variant (c.386A>G/p.Asp129Gly) was reported to segregate with recessive CSNB in a large, consanguineous Pakistani family (Naeem, Chavali et al. 2012). The Asp129Gly variant altered a conserved residue of the protein and was predicted to be deleterious to function, although no functional studies on it were carried out.

We report here a retinal degeneration patient homozygous for a premature stop codon in the *GNAT1* gene (c.904C>T/p.Gln302\*). The *GNAT1* mutation was identified during the course of a next-generation sequencing (NGS) study covering the Irish retinal degeneration patient population, Target 5000. This variant causes the elimination of 49 extremely conserved amino acids from the C-terminus of the transducin alpha subunit protein (Figure 20), comprising just under one-sixth of the total protein sequence. This is a much more radical alteration to protein structure than that caused by other *GNAT1* variants previously associated with CSNB (Dryja, Hahn et al. 1996, Szabo, Kreienkamp et

al. 2007, Naeem, Chavali et al. 2012).

Chicken	1	QFLELNMRRDVKEIYSHMTCATDTE	NVKFVFDAVTDIIIKENLKDCGLF
Zebrafish	1	QFLELNMRRDVKEIYSHMTCATDTE	NVKFVFDAVTDIIIKENLKDCGLF
Human	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Chimp	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Macaque	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Dog	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Cow	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Mouse	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Rat	1	QFLELNMRRDVKEIYSHMTCATDTQ	NVKFVFDAVTDIIIKENLKDCGLF
Xenopus	1	QFLDLNLRRDVKEIYSHMTCATDTE	NVKFVFDAVTDIIIKENLKDCGLF

**Figure 20. Conservation of the Q302\* variant across multiple species.**

***Multiple alignment of the amino acids removed by the Q302\* variant against the homologous region in other species, showing extremely high levels of sequence conservation, which implies that it is important to protein function.***

An extensive search of the literature and relevant databases and have not located any other record of a patient with two nonsense *GNAT1* alleles. Hence, it is our view that this represents the first report of a homozygous nonsense *GNAT1* mutation in humans, as well as the first observation of retinal degeneration associated with *GNAT1* mutations.

This clinical presentation is of particular interest, as it strongly resembles the phenotype observed in homozygous knockout (*Gnat1* *-/-*) mice. Mice with targeted deletions of the *Gnat1* gene have few visible changes in gross morphology, but do present with a mild retinal degeneration with age. These mice also show preservation of cone cells, a complete absence of electrical response from rods, as well as a slow loss of rod cells with corresponding thinning of the outer nuclear layer (Calvert, Krasnoperova et al. 2000), features which closely parallel the clinical presentation in the *GNAT1* Gln302\* patient.



### **4.3 Materials and Methods**

The patients were assessed in the Research Foundation, Royal Victoria Eye and Ear Hospital, and underwent clinical assessment with DNA isolation and sequencing. The details of these processes, along with data analysis of variants provided by sequencing, are detailed in chapter 2.

### **4.4 Results**

#### ***4.4.1 Clinical Findings***

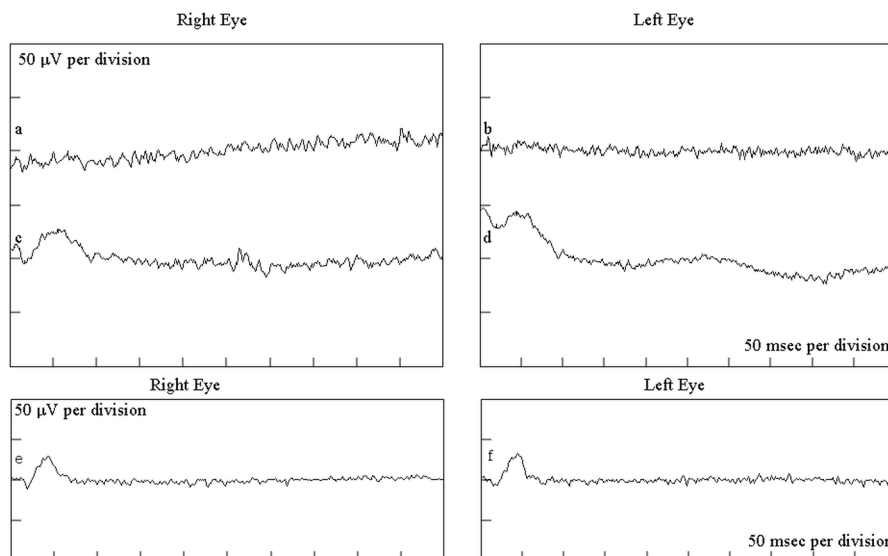
The proband presented at 80 years of age with a six-month history of difficulties when climbing steps. He commented on a lifelong history of night-blindness, which had remained unchanged throughout his life. There was no family history of night-blindness or day vision problems affecting either his parents, 3 siblings (ranging in age from 73 to 75 years of age), or his 3 children (aged 38 to 51 years). There was no history of parental consanguinity. His general health was good. He suffered for 10 years prior to presentation with non-insulin dependent diabetes mellitus, which was well-controlled.

On examination, the proband's unaided visual acuity was 6/7.5 in the right eye, which improved to 6/6 with +0.50 DS / +0.50 CX at 165°, and 6/7.5 in the left, which improved to 6/6 with 0.00 DS / +0.50 CX at 165°. Slit lamp examination revealed healthy corneae, normal anterior segments, and clear lenses, apart from some peripheral cortical opacities. The vitreous was normal, apart from the presence of a grade 1 cellular infiltrate. Intra-ocular pressures were normal, at 14mm Hg in the right eye and 14 in the left.

The proband's dark-adapted threshold to an 11° target was assessed at 15° from fixation in each eye using a Goldmann-Weekers dark-adaptometer. Thresholds were significantly elevated at 4.3 Log Units in the right eye and 4.1 Log Units in

the left; the upper limit of normal for our laboratory being 2.0 Log Units.

A full-field electroretinogram (ERG), performed to ISCEV standard, revealed no convincing rod-isolated responses. Convincing ERG responses were recorded to the maximal intensity flash recorded in the dark-adapted state (Figure 21). These responses are usually mediated by both the rods and cones. Clear cone-mediated responses were recorded to both single flash and 30Hz flicker stimulation, in each case against a rod-suppressing background illumination. These responses were significantly delayed and attenuated in amplitude (Figure 21).

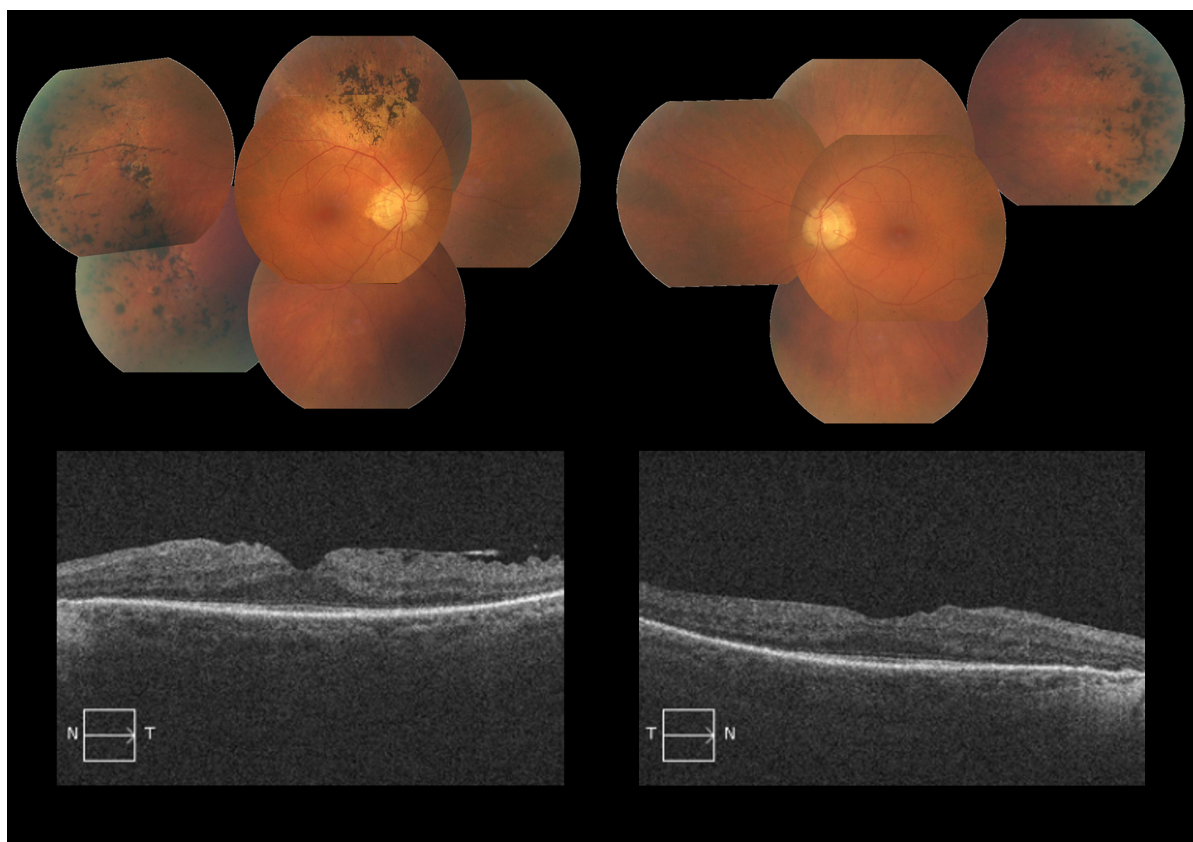


**Figure 21. Full field ERGs in the proband showing a lack of rod phototransduction.**

***Tracings a and b show non-recordable dark-adapted rod-isolated responses in the right and left eyes, respectively. Tracings c and d show significantly attenuated dark-adapted responses to the maximal intensity flash. Ordinarily, these responses reflect rod and cone activity. Owing to the absence of rod-isolated inputs, these responses are most probably***

***cone-driven in our proband. Tracings e and f show significantly-attenuated light-adapted cone-isolated responses.***

Fundus examination showed slight disc pallor and arteriolar attenuation in each eye. Both maculae appeared healthy. Marked peripheral pigmentary deposits, some of which were bone-spicule in appearance and some with a more clumpy appearance, were evident in the retinal peripheries (Figure 22). There was no evidence of diabetic retinopathy in either eye.



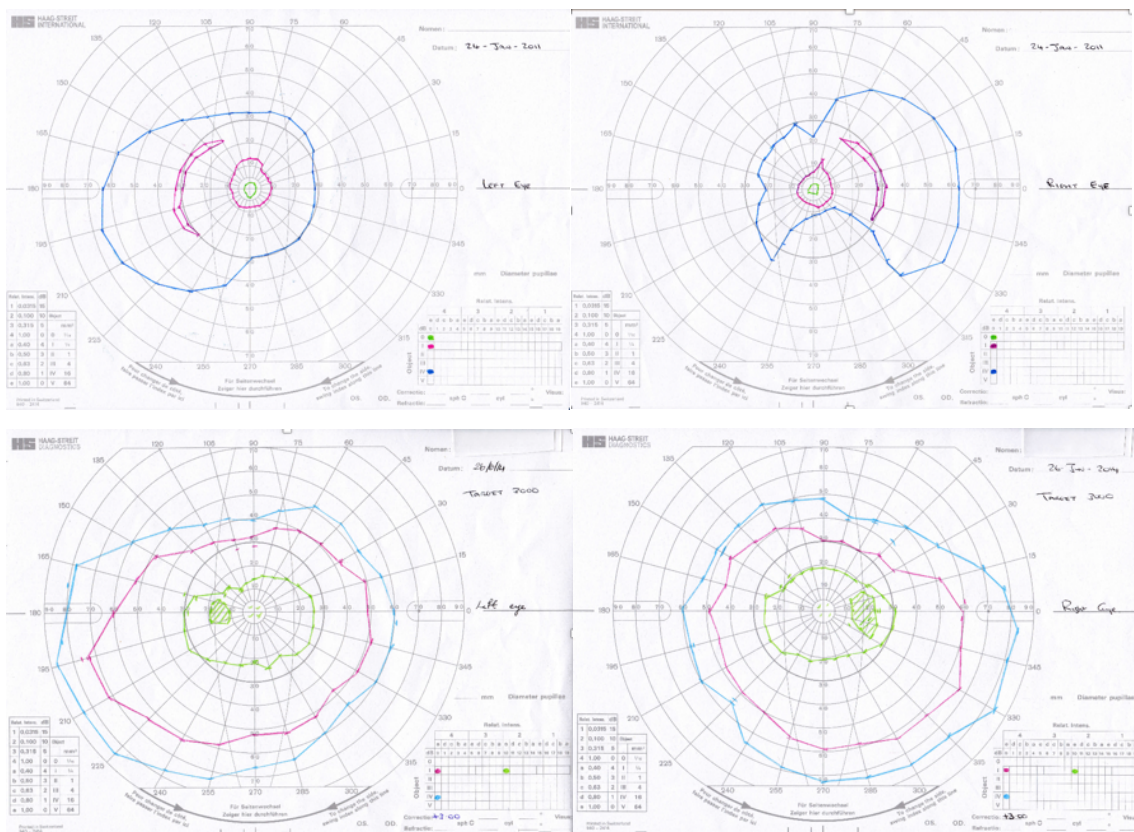
**Figure 22. Fundus Examination**

***Above: Fundus photographs of the proband's right and left eye, showing optic disk pallor, arteriolar attenuation, normal maculae and scattered bone-spicule pigment deposits.***

***Below: OCT images of the proband's right and left eye, showing normal foveal contour with some thickening and epiretinal membrane. There is loss of the ellipsoid zone outside the parafoveal area.***

One of the proband's children was also assessed. As the proband was homozygous for the GNAT1 variant, his daughter was an obligate carrier, and the presence of the heterozygous variant was confirmed by Sanger sequencing.

The proband's daughter was 56 years of age at the time of assessment. She had no complaints of night vision or day vision difficulties. Her dark-adapted thresholds were normal at 1.4 Log Units in each eye. Full field ERGs showed normal rod and cone-isolated responses in both eyes. Detailed fundoscopic examination was entirely normal in each eye, and visual fields did not show the constriction evident in the proband (Figure 23).



**Figure 23. Visual fields for both eyes of the proband (above) and unaffected offspring (below).**

***The proband shows clear constriction of visual fields, consistent with a diagnosis of retinitis pigmentosa.***

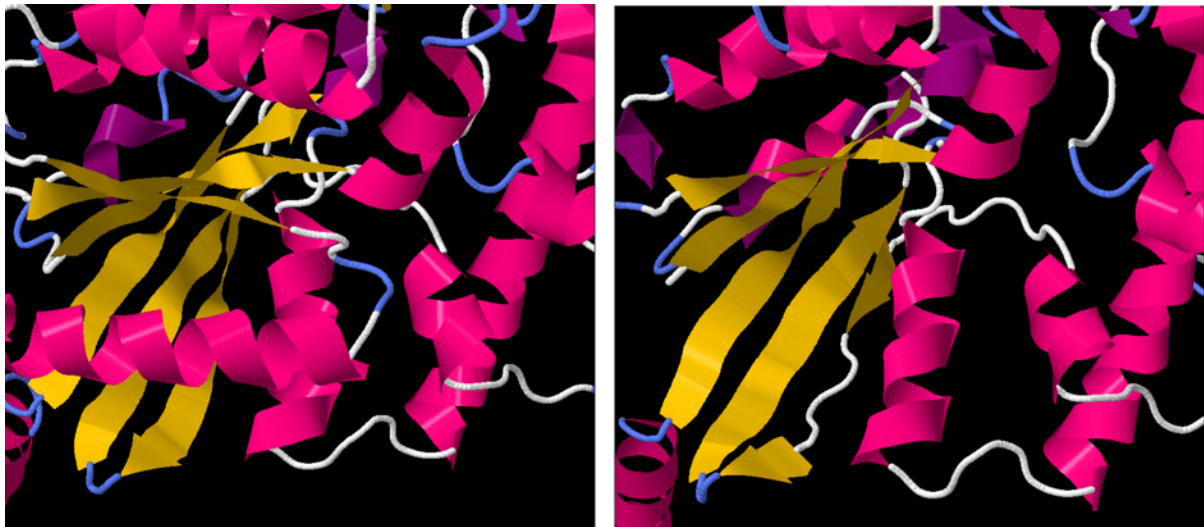
#### **4.4.2 Genetic Studies**

The *GNAT1* Gln302\* variant was first identified in the proband during analysis of next-generation sequencing data. Sequencing quality for the proband's sample was overall very good: 98.3% of bases in coding regions and 94.3% of bases overall were considered "callable", with a coverage of at least 10X. 57.3% of reads were "on-target" (overlapping a capture region). Confirmatory Sanger sequencing confirmed the presence of the homozygous variant. This variant eliminates 49 amino acids from the C-terminus of the *GNAT1* protein, approximately one-sixth of the total protein length (Figure 24).

The variant was not observed, even heterozygously, in 190 other patients involved in this study, four of whom had been diagnosed with CSNB. No other *GNAT1* mutations were observed in any patients involved in this study. The variant was not observed in the 1000 Genomes project (Abecasis, Auton et al. 2012), but was observed at an extremely low allele frequency of 5/66,210 in European populations by the Exome Aggregation Consortium (Exome Aggregation Consortium (ExAC) 2016), with no homozygous cases and no observations of the allele in other ethnic groups. A literature search of Pubmed and HGMD (Stenson, Ball et al. 2003) did not reveal any previously-reported cases of any other homozygous truncating *GNAT1* variant. Sequencing of the exons of 181 other retinopathy-associated genes in this patient did not reveal any other variants that were considered to be plausibly responsible for the observed phenotype. Sequencing of the unaffected daughter detected the variant to be present heterozygously, but without any disease phenotype, suggesting a recessive mode of disease inheritance.

The deleted residues include several crucial to most aspects of  $\alpha$  transducin subunit protein function. Bonds between Thr 323 and Lys 266 are involved in

forming the guanine-binding pocket (Noel, Hamm et al. 1993). Residues 311-329 have been shown to bind photoactivated rhodopsin (Noel, Hamm et al. 1993), while residues 306-311 and 314 are involved in PDE activation (Artemyev, Mills et al. 1993). Simulated folding of the truncated peptide performed using the I-TASSER software suite (Yang, Yan et al. 2015) suggested that the six-stranded beta-sheet GTPase domain would be destabilized by the deletion, with only four of the strands forming correctly and a fifth forming very incompletely; the sixth did not form at all (Figure 24).



**Figure 24. GNAT1 Protein**

***Left: The protein structure of GNAT1, derived from X-ray crystallographic measurements. Alpha-helical regions are coloured in pink, while beta-sheets are coloured in yellow. The six-stranded beta sheet domain, central to GTPase function, is fully-formed. Right: The predicted structure of the truncated GNAT1 protein, showing the malformations in the fifth and sixth strands of the beta-sheet domain.***

GTPase activity is crucial to the function of all G-proteins. The *GNAT1* Gln302\* variant is therefore predicted to very severely affect protein function, affecting the

protein's interactions with both upstream and downstream members of the signalling pathway.

There is a substantial difference in phenotype between the proband and other reported patients with CSNB caused by *GNAT1* variants. In addition to the expected lifelong night blindness, the *GNAT1* Gln302\* patient presented with a late-onset retinitis pigmentosa-like degeneration of the retina, including bone-spicule deposits. In contrast, Naeem et al. (Naeem, Chavali et al. 2012) explicitly stated they did not observe any such RP-like features upon fundus examination of patients homozygous for the Asp129Gly variant. While retinal degeneration is clearly a feature of the *GNAT1* Gln302\* disease, it is slow; at age 80, the patient still has good central and colour vision.

#### **4.5 Discussion**

There are interesting parallels between the observed clinical presentation of the homozygous *GNAT1* Gln302\* mutation and the phenotype of the *Gnat1* knockout mouse. No convincing rod-isolated ERG responses were recorded from the proband to the -25 dB flash stimulus, providing an explanation for the subject's history of night blindness and consistent with the elevated dark-adapted thresholds. Similarly, Calvert et al. (Calvert, Krasnoperova et al. 2000) could not record any rod-isolated ERG responses in mice with a targeted deletion of the *Gnat1* gene. Retinal excitation by flash stimuli, sufficiently bright to elicit mixed rod and cone responses from the healthy retina, only elicited responses compatible with cone stimulation alone, in both the proband and in *Gnat1* *-/-* mice.

The pattern of the dark-adapted electro-retinographic responses in the *GNAT1* Gln302\* patient differ significantly from many CSNB patients, where there is an 'electronegative' conformation to the dark-adapted mixed rod/cone response to the maximal intensity flash stimulus. However, they are similar to those reported in autosomal recessive (Naeem, Chavali et al. 2012) and autosomal dominant

(Sandberg, Pawlyk et al. 1998) forms of CSNB caused by GNAT1 mutations. Notably, however, other GNAT1 CSNB kindreds did not show the clear pigmentary disturbances observed in the Gln302\* patient.

Cone-isolated ERG responses in the *Gnat1* *-/-* mice, when assessed at 8 to 10 weeks, showed no appreciable difference compared to age-matched wild-type controls (Calvert, Krasnoperova et al. 2000). The cone-isolated responses in the GNAT1 Gln302\* patient, at 80 years of age, showed delayed and reduced amplitude responses (Figure 21), indicating significantly compromised cone function. Calvert et al. (Calvert, Krasnoperova et al. 2000) did not present data on cone ERG responses in *Gnat1* *-/-* mice of an age equivalent to that of the proband in the current study.

It is particularly noteworthy that mice homozygous for the *Gnat1* deletion showed a subtle thinning of the retina over time (Calvert, Krasnoperova et al. 2000). By 13 weeks of age, rod-outer-segment length shortens, and the thickness of the outer nuclear layer decreases by about one row of nuclei, indicating a loss of approximately 10% of rod cells, followed by a later mild loss of thickness in the inner nuclear layer (Calvert, Krasnoperova et al. 2000). The rate of decline in the thickness of the outer nuclear layer slowed substantially after the first 13 weeks.

Despite this decline in retinal thickness, the authors predicted that deletion of GNAT1 in humans would cause a recessive stationary night-blindness rather than retinitis pigmentosa. The proband, however, clearly shows cone photoreceptor dysfunction on electroretinographic examination and visible fundoscopic signs indicative of a mild and localised, but definite, retinitis pigmentosa.

Given the above observations, we conclude that the novel Gln302\* mutation in the GNAT1 gene may indeed cause a slow and late-onset form of retinitis pigmentosa in humans, and hypothesise that slow loss of rod cells with age, similar to that observed in the *Gnat1* *-/-* mouse, may be responsible for this.



In all previously-reported cases of *GNAT1*-mediated CSNB, the causative mutations have been single amino acid substitutions, rather than truncating or frameshift mutations. This is the first case of a homozygous variant introducing a premature stop codon into *GNAT1*, eliminating crucial domains from the *GNAT1* protein. In addition, this study represents the first evidence that *GNAT1* mutations can cause retinal degeneration in human patients. Until now, transducin was unusual in the rod phototransduction pathway, in that it had not been associated with retinal degeneration; variants in both rhodopsin, the immediately-upstream component of the pathway, and cGMP-phosphodiesterase, the immediately-downstream component, have been implicated in retinitis pigmentosa (Farrar, McWilliam et al. 1990, Dryja, Finn et al. 1995, Dryja, Rucinski et al. 1999).

It is important to note that the *GNAT1* Gln302\* patient developed retinitis pigmentosa at a late age, older than many of the patients assessed in prior studies of CSNB caused by *GNAT1* variants (Dryja, Hahn et al. 1996, Szabo, Kreienkamp et al. 2007, Naeem, Chavali et al. 2012). We, therefore, cannot exclude the possibility that the observed phenotype is not confined to this or other truncating variants, and may occur in other patients with *GNAT1*-mediated CSNB as they advance in age. We recommend that clinicians monitor older patients with *GNAT1*-mediated CSNB for evidence of degeneration. We also recommend that *GNAT1* should be considered as a candidate for sequencing in patients with recessive or simplex lifelong night-blindness and late-onset retinal degeneration.

# **Chapter 5: Further evidence suggesting pathogenicity of the PROM1 mutation p.S649L in cone-rod dystrophy with comparison to the p.R373C mutation phenotype and mouse model**

## **5.1 Summary**

The PROM1 mutation p.S649L was originally described by Littink et al. in their paper describing a large cohort of patients with cone-rod dystrophies (Littink, Koenekoop et al. 2010). They identified a patient with an autosomal recessive cone-rod dystrophy harbouring this mutation homozygously but ultimately decided to label it as a non-pathogenic variant because it was found in a control group. Here, we describe the case of a 31-year-old patient from a consanguineous cone-rod dystrophy pedigree who was found to have this mutation homozygously. We explore the possibility that this variant is disease-causing and we compare this patient to another in our cohort with a well-known p.R373C PROM1 variant causing a dominant Bull's Eye Maculopathy phenotype and the mouse model harbouring this variant.

## **5.2 Introduction**

PROM1 codes for prominin-1, which is an 865-amino acid protein and a member of the prominin family of 5-transmembrane glycoproteins located on evaginations of the photoreceptor outer segment. In 2008, Yang et al. created transgenic mice with mutant and wild-type PROM1 genes and demonstrated that lack of functioning prominin-1 causes abnormal morphogenesis of the rod photoreceptor disc membranes, leading them to become disorganised rather than the way they are physiologically laid down in highly organised layers, similar to a pancake stack (Yang, Chen et al. 2008). The protein encoded by PROM1 is also known in

the medical literature as CD133 and is used as an antigenic surface marker for human haematopoietic stem cells and cancer stem cells, indicating its ubiquitous nature in human tissue (Glumac and LeBeau 2018). Monoclonal antibodies with affinity for CD133 are being pursued as possible targeted cancer therapies (Qiu, Li et al. 2018).

The PROM1 gene was first described in the case of retinal degeneration in a consanguineous Indian pedigree (Maw, Corbeil et al. 2000). The affected members had extinguished ERGs at presentation but gave a history of rod, followed by cone, photoreceptor dysfunction corresponding to a rod-cone dystrophy. PROM1 was later found to be causative in a previously described Caribbean pedigree with an autosomal dominant macular dystrophy. It bore similarities to the Stargardt phenotype identified in 1909 by Karl Stargardt and was thus named Stargardt-like Macular Dystrophy 4 (STGD4). It had a genetic locus on chromosome 4p, which was later identified as the PROM1 gene (Kniazeva, Chiang et al. 1999).

PROM1 mutations have now been shown to cause rod, rod-cone, cone-rod and macular dystrophies in both autosomal dominant and recessive patterns depending on the mutation involved (Michaelides, Gaillard et al. 2010). It stands to reason that, due to the ubiquitous expression of prominin1, there might be evidence of systemic involvement in some affected patients. In the original pedigree describing a PROM1 mutation, one affected child was found to have polydactyly (Maw, Corbeil et al. 2000). A further study of patients with the PROM1 missense mutation p.R373C speculated that their features of an empty sella turcica on brain imaging, microhaematuria and endothelial cell dysfunction could be familial and related directly to this mutation (Arrigoni, Matarin et al. 2011). An ongoing natural history study of patients with retinal dystrophies caused by PROM1 gene mutations may shed further light on the characteristics of patients bearing these variants in the future (Strauss, Munoz et al. 2018).

Here, we describe two cases of retinal degeneration putatively caused by different mutations in the PROM1 gene. We provide some evidence for the

pathogenicity of the p.S649L mutation and use the second case, which carries the common p.R373C variant, to provide insight into the spectrum of disease represented by mutations in this gene, along with insights from transgenic mice expressing p.R373C mutant human PROM1.

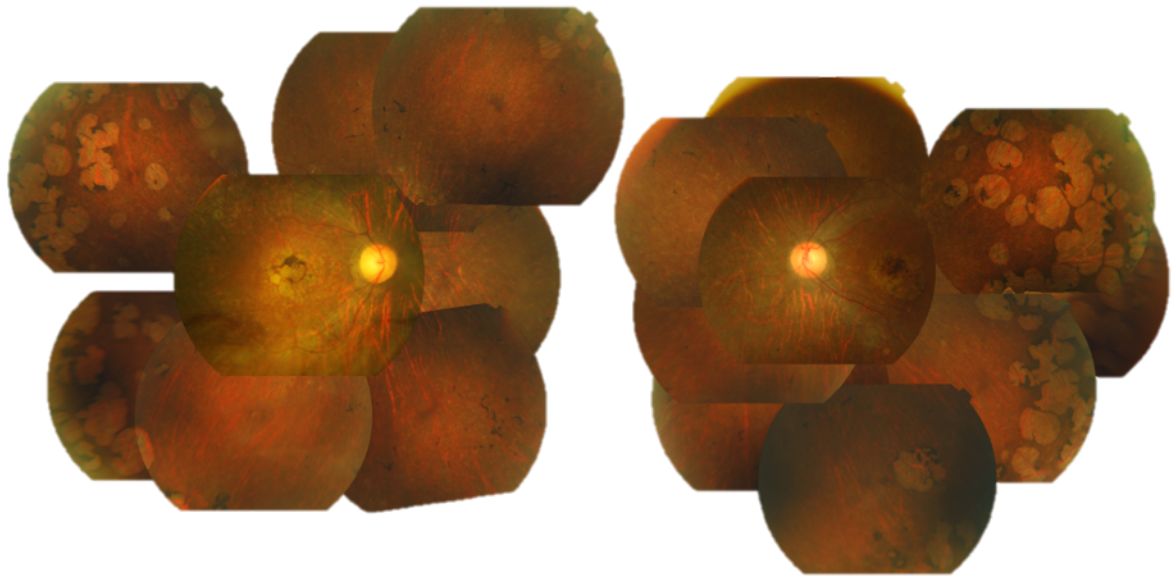
### **5.3 Materials and Methods**

The patients were assessed at the Research Foundation, Royal Victoria Eye and Ear Hospital, and underwent clinical assessment with DNA isolation and sequencing. These processes, along with data analysis of variants provided by sequencing, are detailed in chapter 2.

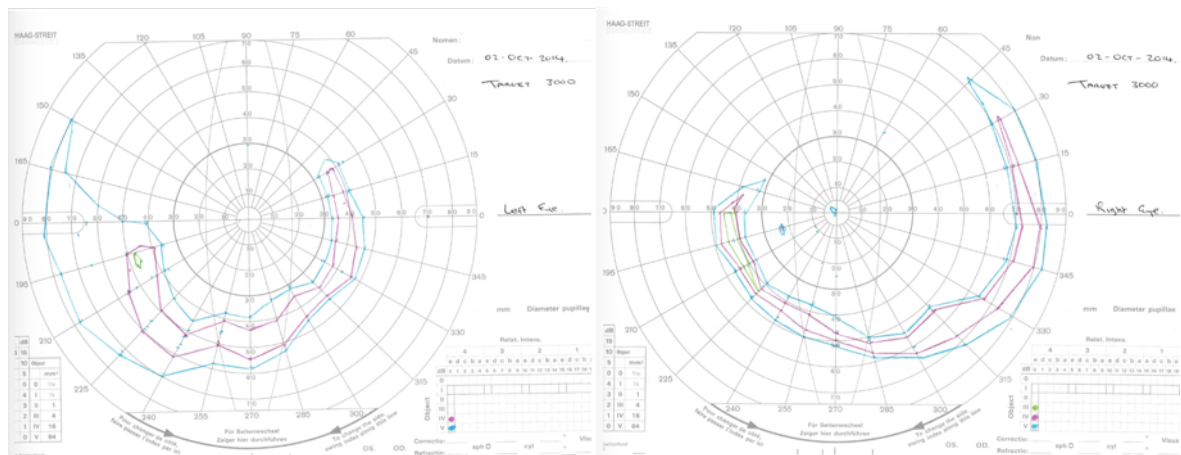
### **5.4 Results**

#### **5.4.1 Clinical Findings**

Patient 1 was a 31-year-old Indian female of Portuguese descent. The patient gave a history of early central visual loss. She was initially diagnosed with Stargardt disease at the age of 5. Her central visual acuity continued to deteriorate followed by her peripheral visual field and she developed nystagmus. Her visual acuity at presentation to our institution was reduced to hand movements in both eyes. Anterior segment examination revealed fine nystagmus and fundal examination showed bilateral marked disc pallor, vessel attenuation and macular atrophic scarring, with punched out chorioretinal atrophy peripherally and bone spicule pigmentary deposits nasally (figure 25). Goldmann visual fields were severely constricted in the left eye and the central visual field was undetectable in the right. There was a small retained arc of visual field inferiorly in both eyes (figure 26). ERG showed extinguished rod and cone responses.



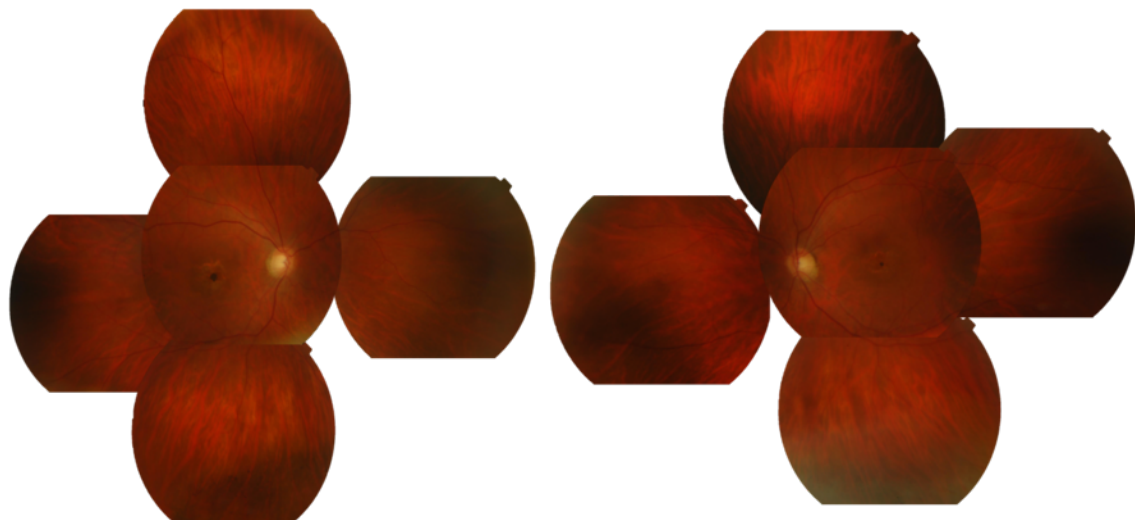
**Figure 25. Fundus photographs of patient 1**



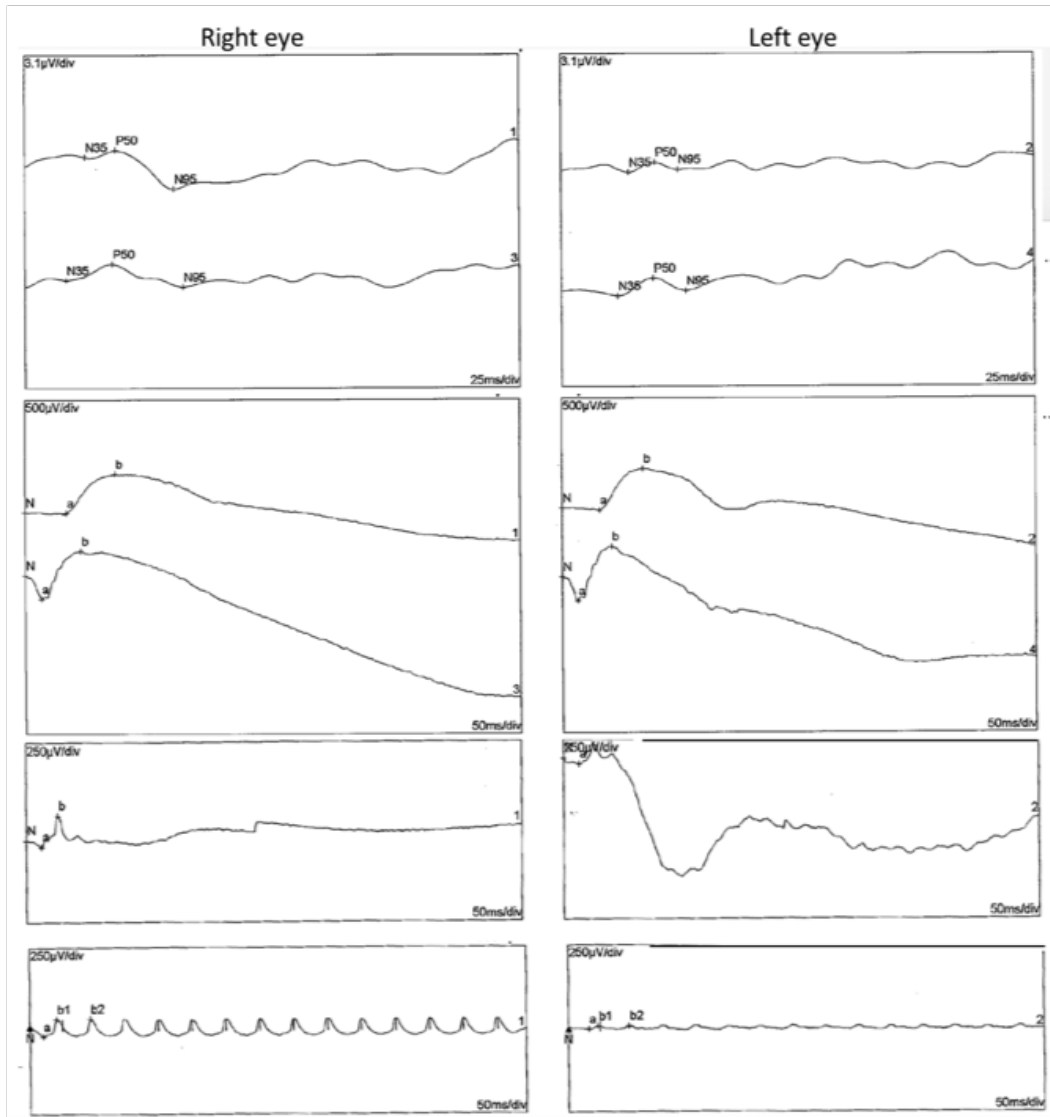
**Figure 26. Visual fields of patient 1**

Patient 2 was a 34-year-old female of Polish origin who presented with a four-year history of decrease in visual acuity. There was a family history of inherited retinal degeneration in an autosomal dominant pattern and her mother and

maternal grandmother were also affected. Visual acuity was 6/12 in the right eye and 6/15 in the left with moderate myopic correction. Fundal examination showed intensely pigmented deposits at both maculae with a bull's eye maculopathy phenotype and sparse mid-peripheral bone spicule pigmentary deposits (figure 27). The optic discs and retinal vessels in both eyes were normal. Lanthony D-15 colour vision testing was within normal limits. Dark adaptation was just above the upper limit of normal, at 2.1 Log units. Her ERG showed decreased rod-isolated amplitudes in the left eye with normal latency. The cone-isolated responses in both eyes were normal in timing but reduced in amplitude. Pattern ERG P50 amplitudes were reduced in both eyes (figure 28). The EOG was normal and the Humphrey visual fields showed bilateral small, central scotomata (figure 29).

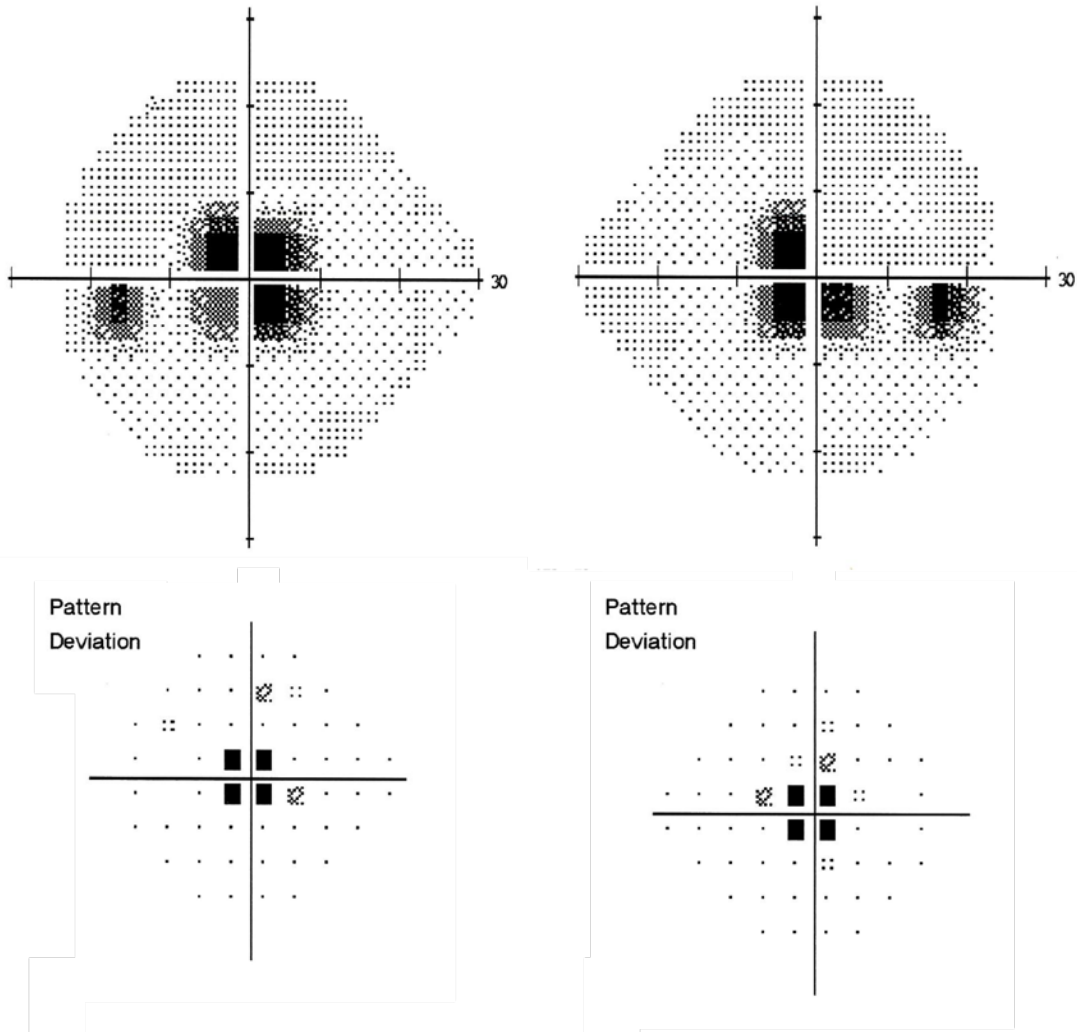


**Figure 27. Fundus photographs of patient 2**



**Figure 28. Patient 2 ERG +PERG**

*The pattern ERG showed reduced P50 amplitudes in both eyes consistent with compromised macular function. The rod-isolated amplitudes were normal in the right eye and slightly reduced in the left eye due to eye closure during testing. The cone-isolated 30Hz flicker and single flash were reduced in amplitude with normal timing. The asymmetry between the eyes is attributed to significant eye closure during testing.*



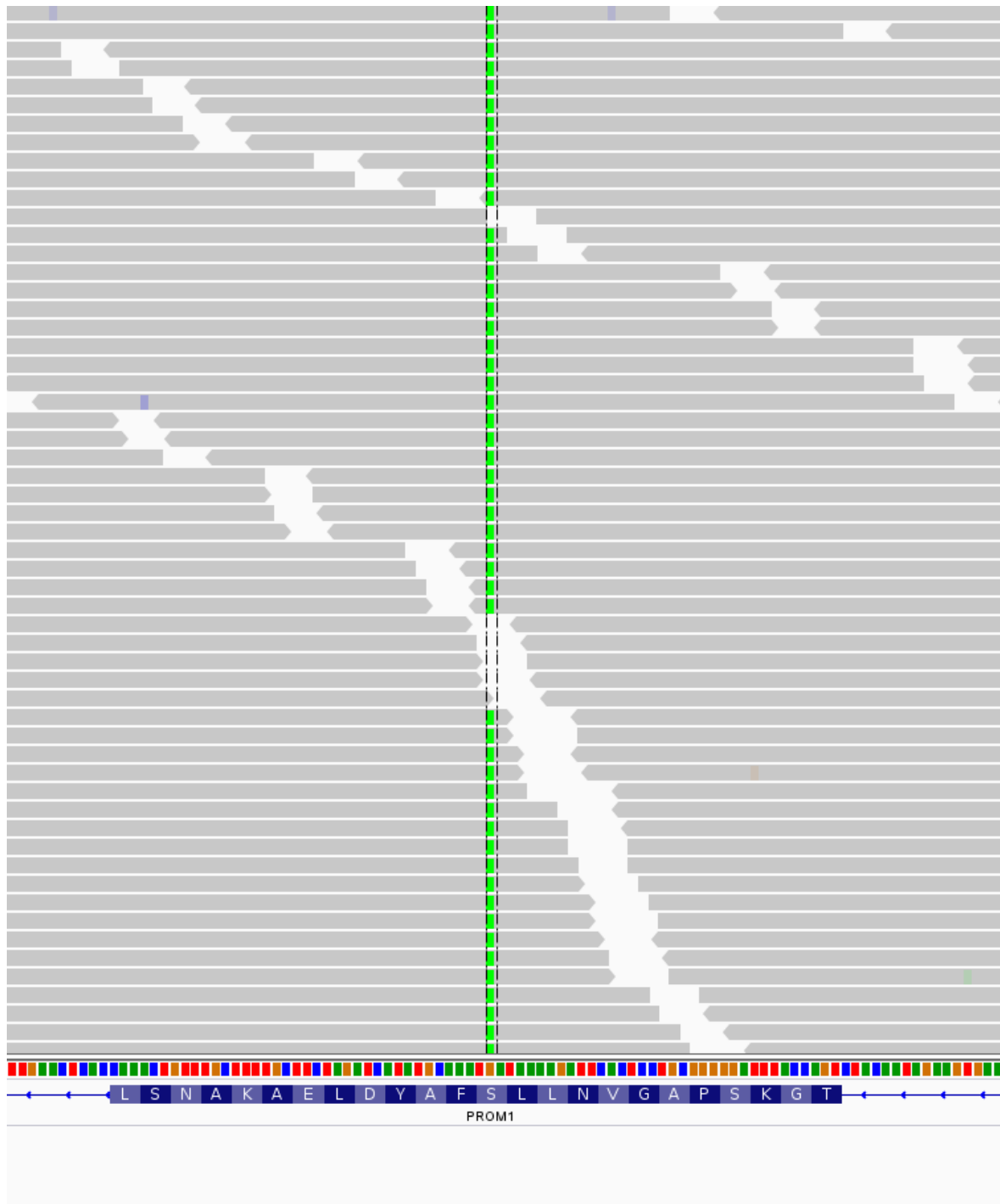
**Figure 29. Patient 2 visual fields showing central scotomata. Humphrey (static) visual fields, SITA-Standard testing protocol.**

#### **5.4.2 Genetic findings**

The first patient was found to have a homozygous missense variant in the PROM1 gene, c.1946C>T; p.S649L (NM\_001145849.1) (depicted in figure 30). No putative sequence changes were observed in any other candidate genes. The patient was homozygous for the mutation and each of her unaffected parents were heterozygous for the same mutation. The variant was not observed homozygously in the genome aggregation database (gnomAD - allele frequency



0.00002898) or the exome aggregation consortium (ExAC - allele frequency 0.00005455835). The second patient's sequencing revealed a heterozygous missense mutation, c.1117C>T, p.R373C (NM\_006017.1). This is a known pathogenic mutation with a mouse model of disease, as described elsewhere in this chapter, which has been described several times in the human literature to cause retinal dystrophies (Yang, Chen et al. 2008, Michaelides, Gaillard et al. 2010, Watson, El-Asrag et al. 2014, Van Cauwenbergh, Coppieters et al. 2017).



**Figure 30. NGS panel sequencing.**

***PROM1* pileup. NM\_001145849.1:c.1946C>T (p.Ser649Leu). The coloured boxes toward the bottom of the figure represent the reference genome. Grey parallel lines represent individual reads from the patient and the green**

***box at the same position on the reference genome in each of the grey reads is indicative of the changed nucleotide, which is consistent across all reads indicating the change has not been identified in error.***

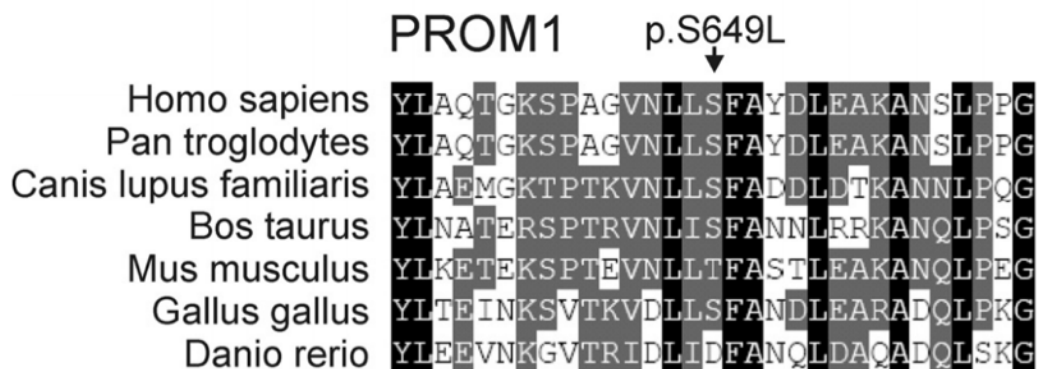
In Silico testing of variant c.1946C>T, p.S649L, Fathmm score was “tolerated”, with a score of 0.82. Mutation Assessor functional impact combined score 0.9 (functional impact estimated low). Polyphen prediction was “benign” – score 0.55. Fathmm and Mutation Assessor make judgements based on evolutionary conservation, while polyphen uses a mixed model evaluating both protein structure and function along with evolutionary conservation.

## **5.5 Discussion**

We present the case of a cone-rod dystrophy phenotype possibly caused by a homozygous missense variant c.1946C>T causing the substitution p.S649L in PROM1. The pathogenicity of this variant has previously been questioned in the literature and there is one known case of cone-rod dystrophy reported which may or may not be related (Littink, Koenekoop et al. 2010). Littink et al. described a pedigree of Greek descent, living in the Netherlands, with a cone-rod dystrophy and found them to have the same homozygous missense variant of the PROM1 gene found in our patient. The affected patient was homozygous for this variant, both parents were heterozygous, and the unaffected brother of the proband was found to have two wild type variants. However, the mutation was identified by the authors to be homozygously present in 1 of 90 ethnically-matched controls, suggesting its lack of pathogenicity. Personal correspondence with a group in Tuebingen, Germany, reveals that they too have seen a pedigree with two affected siblings with a cone-rod dystrophy, reported on the ClinVar database, with biallelic p.S649L mutations in the PROM1 gene. The healthy parents inherited the variants in a heterozygous state. They identified one other mutation, with a similar level of segregation in the PROM1 gene in this pedigree c.1632G>T; p.Gly544=, and cannot assign pathogenicity to either of these

variants with certainty. The latter variant has not been described previously as being disease-causing in the literature.

Further evidence for the pathogenicity of this mutation is its evolutionary conservation throughout the animal kingdom. The S649L mutation affects a highly conserved region of the PROM1 gene (Figure 31). Conservation across a number of species is indicative of preservation over time by a process of natural selection and is an indication of functional importance (Tang and Thomas 2016). Prediction of variant pathogenicity measures using in silico programs provided mixed results; Polyphen score “Possibly Damaging”, BLOSUM 62 score -3, SIFT prediction “Tolerated” and Grantham score 145 (Littink, Koenekoop et al. 2010).



**Figure 31. PROM1 Gene conservation across species (Littink, Koenekoop et al. 2010).**

*This figure depicts the conservation of the missense variant p.S649L of the PROM1 gene identified in both a patient reported by Littink et al and our own patient. The p.S649L residue shows a similar amino acid in 6 of 7 residues across the 7 species depicted in the column on the left. The white letters on a black background indicate full conservation, while white letters on grey indicate well-conserved residues (species and their common names are as follows: Homo sapiens = human, Pan troglodytes = chimpanzee, Canis lupus familiaris = dog,*

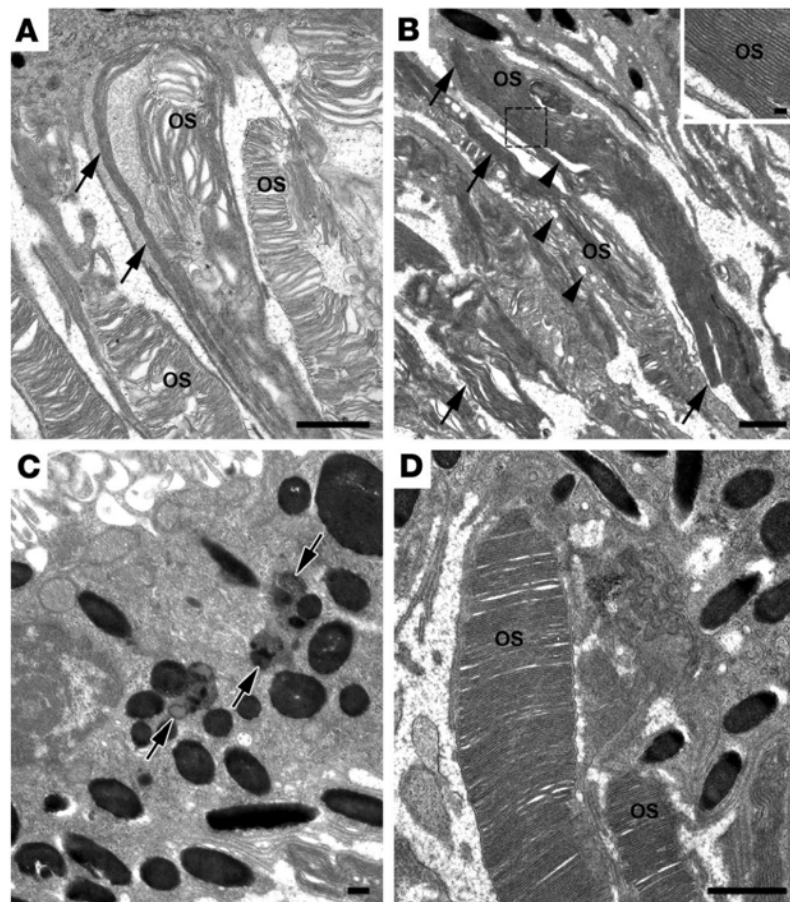
***Bos Taurus = cattle, Mus musculus = mouse, Gallus gallus = chicken, Danio rerio = zebrafish).***

Autosomal recessive disease has been described previously related to a PROM1 variant in an Indian consanguineous family with rod-cone dystrophy. Maw et al. describe their pedigree, along with transfection studies indicating the mutated PROM1 product does not reach the cell surface, becoming trapped in the endoplasmic reticulum, indicating that this pedigree may represent the null phenotype (Maw, Corbeil et al. 2000). They attributed this phenotype to a 1-bp deletion in exon 16 of the PROM1 gene, c.1878delG, causing a frameshift and a prematurely truncated protein product. This caused a severe, early onset rod-cone dystrophy in the four affected family members. Interestingly, one affected member of the cohort exhibited polydactyly and the heterozygous mother exhibited unilateral signs of a retinal degeneration. Another pedigree with a recessive mutation of PROM1, identified as c.1726C>T (exon 15), p.Gln576X, described an early onset rod-cone dystrophy with ERGs extinguished by the teen years in a consanguineous Pakistani pedigree (Zhang, Zulfiqar et al. 2007). The affected members also had early macular atrophy. This mutation results in a prematurely truncated protein which may not reach the cell surface considering previous transfection studies described.

Autosomal dominant pedigrees have previously been described in the literature with a bull's eye maculopathy phenotype similar to our second proband (Michaelides, Gaillard et al. 2010). This pedigree exhibited the common p.R373C single amino acid substitution caused by a missense mutation. Five families described with this substitution were found to have predominantly a bull's eye maculopathy phenotype but an isolated rod dysfunction and rod-cone dystrophy were also described as occurring.

A study of wild type and transgenic mice expressing the p.R373C mutant human PROM1 has revealed information about the location and function of PROM1 within the photoreceptor cell (Yang, Chen et al. 2008). Wild type PROM1 was found densely at the base of the photoreceptor outer segments, whereas the

mutant protein was found richly in the myoid region of the photoreceptors, scattered as far as the synaptic terminal of the cell. The endogenous protein from this heterozygote mouse was also found to be mislocated throughout the photoreceptor. The activity of PROM1 was found to be associated with that of photoreceptor cadherin PCDH21 (*Pcdh21*<sup>-/-</sup> mice also exhibit photoreceptor disc disorganisation) and actin filaments (components of the cytoskeleton) within the cell. The resulting photoreceptor disc disorganisation and thickening which occurred in mutated mice led the authors to believe that PROM1 has an essential role in disc morphogenesis (figure 32). Accumulation of lipofuscin in the mutant mouse RPE was also noted.



**Figure 32. PROM1 and disc morphogenesis from (Yang et al. 2008)**

***This shows that transgenic mice with the R373C mutant PROM1 gene produced defectively shaped photoreceptor outer segments on electron microscopy. A and B depict the transgenic mouse photoreceptor outer segments (OS), with “overgrown and abnormally oriented” disk membranes at age 4 and 16 months, respectively. C depicts the transgenic mouse RPE with pathological lipofuscin deposits, compared to D, which shows wild type mouse photoreceptors on the left and RPE on the right.***

Further work providing information about the location and function of the gene product was provided by Zacchigna et al., who showed that PROM1 <sup>-/-</sup> (knockout) mice showed post-developmental progressive thinning of the outer nuclear layer, which contains photoreceptor cell bodies (Zacchigna, Oh et al. 2009). They confirmed that the abnormalities of disc morphogenesis extended to cones as well as rods and that the defect caused a retinal degeneration in an autosomal recessive manner i.e. heterozygotes were not affected. Interestingly, in frog retinae (*Xenopus Laevis*), Han et al. showed that prominin-1 and peripherin-2/RDS localized to mutually exclusive zones in both cone and rod outer segment lamellae and proposed that peripherin-2/RDS, likely acts as a fusogenic molecule, allowing for cleavage of discs away from the outer plasma membrane, and prominin-1 acts as an anti-fusogenic stabiliser, which in cone outer segments could play a part in the continuity between the discs and the outer plasma membrane (see Figure 33) (Han, Anderson et al. 2012). These studies shed further light on the role of PROM1 in the photoreceptor development and function.

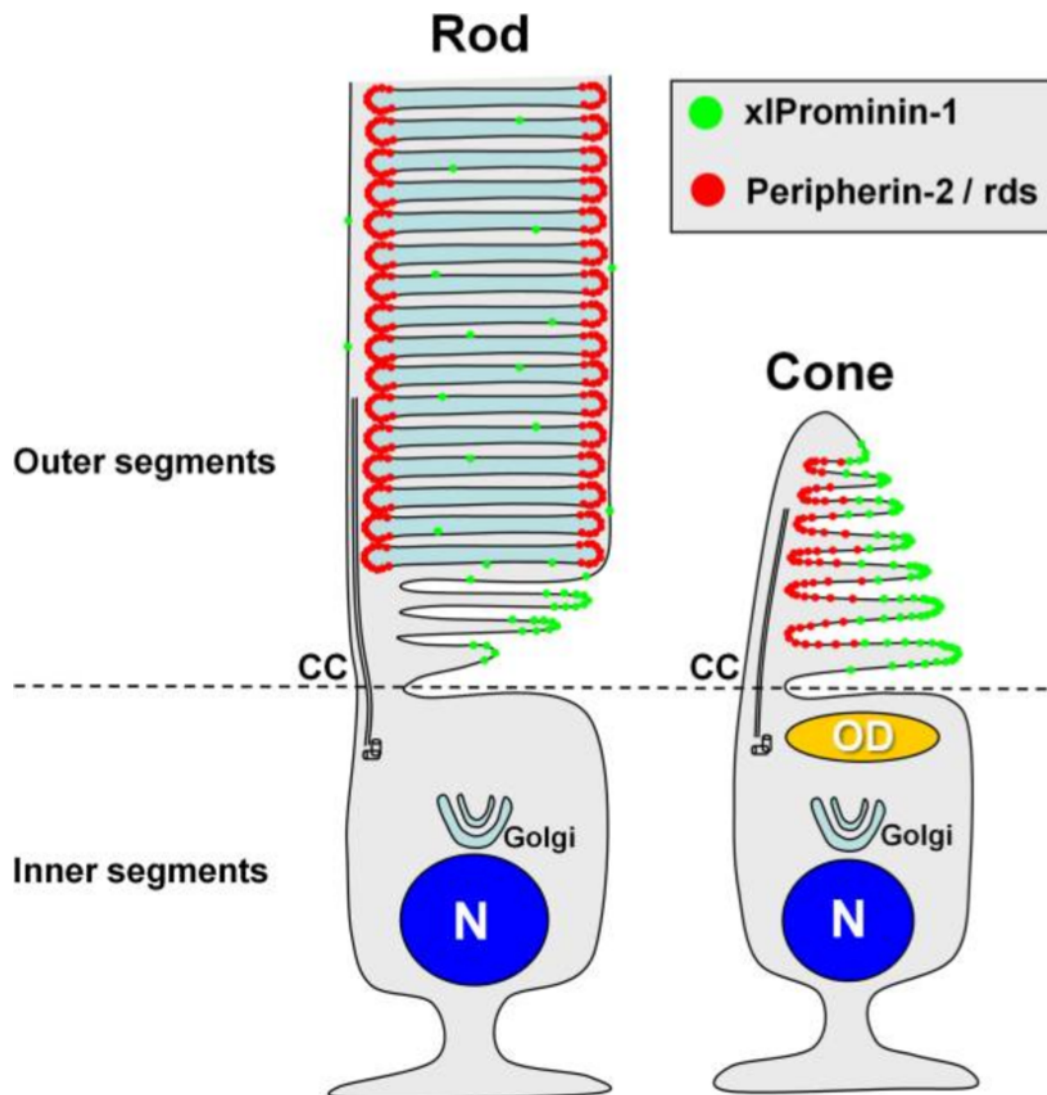


Figure 33. Prominin-1 and peripherin-2/RDS (Han, Anderson et al. 2012).

*This representation depicts the location of xIProminin-1 (Xenopus Laevis Prominin-1 homolog) and Peripherin-2/RDS throughout the rod and cone outer segments. CC= connecting cilium, OD = oil droplet, N = nucleus.*

The pedigree described in this chapter provides insight into the difficulties surrounding the interpretation of variants of unknown significance (VUS) in the case of retinal degenerations. Large numbers of possible disease-causing variants are identified and must be assessed by diverse methods to decide



whether or not they are producing the phenotype in a given case. Due to the current pace of expansion of these dataset, many VUS will in the future be re-categorised as either pathogenic or benign (Milunsky 2017).

Some variants are more readily solved, as in our second pedigree with a well described mutation and an evidence base supporting the phenotype. Deep phenotyping has been a critical step in the process of variant categorisation in ophthalmic disorders, identifying candidate genes and also allowing for the dismissal of certain variants which do not correlate with the phenotype.

In summary, we believe the variant c.1946C>T, p.S649L could possibly be disease-causing in this case of autosomal recessive cone-rod dystrophy. Further similar cases in the literature and the creation of an animal model of this substitution may elicit further evidence of pathogenicity in the future.

## **Chapter 6. Biallelic RPE65-mediated retinal dystrophy masquerading as choroideraemia in an Irish cohort**

### **6.1 Summary**

We present here a cryptic case of autosomal recessive RPE65-mediated retinitis pigmentosa found in a choroideremia pedigree. As the retinitis pigmentosa patient was male and the clinical symptoms mimicked those of other affected members of the pedigree, he was initially diagnosed with choroideremia. It was not until next-generation sequencing was carried out that it was discovered that there were two separate diseases with similar clinical presentations in the same pedigree. This case provides further evidence of the potential of next-generation sequencing to generate unexpected and clinically-relevant findings for patients with inherited retinal disease.

### **6.2 Introduction**

One of the fundamental assumptions when dealing with inherited diseases of any kind is that multiple individuals with similar disease presentations in the same pedigree will share the same underlying genetic cause. Although a reasonable assumption, on rare occasions this will turn out not to be the case.

We present here a report of such a case, in a choroideremia pedigree with multiple affected males. Choroideremia is an X-linked retinal disorder causing progressive vision loss due to degeneration of the choroid and retina. The clinical picture in affected males is of creeping, marked degeneration of the peripheral fundus, often with bare sclera visible, surrounding an island of preserved tissue around the fovea. The healthy island provides normal visual acuity, with the majority retaining normal vision until the age of 60, but becomes progressively smaller until finally this area also degenerates leaving the patient profoundly blind

(Roberts, Fishman et al. 2002). Carrier females are generally asymptomatic, but can display mild clinical features leading to visual symptoms in some cases (MacDonald 2015). Whereas other common inherited retinal diseases, such as retinitis pigmentosa, can be caused by variants in over 70 different genes, choroideraemia is monogenic, caused by mutations in the *CHM* gene. This gene encodes the protein *REP-1*, Rab escort protein-1. This 653 amino acid protein is involved in cell vesicular transport and is found throughout the body (Coussa and Traboulsi 2012). Although it is clear there is progressive degeneration of the choroid, RPE and photoreceptors, it is still uncertain as to which tissue, if any, is the site of the primary defect in the pathogenesis of the disease. Deep phenotyping with advanced imaging modalities such as adaptive optics has led us to believe that the first tissue to degenerate is likely to be the RPE (Dimopoulos, Radziwon et al. 2017). Unfurling the specifics of the pathogenesis of the disease is critical to conceptualising treatment modalities. Due to its monogenic nature, choroideraemia was the second IRD to undergo clinical trials of gene replacement therapy with the use of an adeno-associated virus as a vector. The first trial of this method of treatment commenced in 2011 (MacLaren, Groppe et al. 2014). Predating this trial was a clinical trial for the treatment of RPE65-mediated Leber's Congenital Amaurosis, the long-term 3-year results of which have now been published (Bainbridge, Mehat et al. 2015). A gene-mediated treatment for RPE65-mediated retinal degeneration, called Luxturna (Spark Therapeutics, Philadelphia, USA), is now commercially available and has both U.S. food and Drug Administration (FDA) and CE (Conformité Européene) approval as of 2018.

Retinitis pigmentosa is the most common retinal dystrophy. Unlike choroideraemia, over 80 different genes cause non-syndromic retinitis pigmentosa, and there is a much wider spectrum of phenotypes (Daiger 2019). The mode of inheritance can be autosomal dominant, recessive or X-linked. The most common features are symptoms of night blindness and visual field scotomata, with clinical retinal signs of bone spicule pigment deposition, arteriolar attenuation and pale, waxy optic discs.

We present here an unusual pedigree with multiple family members affected by choroideraemia. When a further, older male member of the family was found to have a similarly advanced retinal degeneration, a presumptive diagnosis of choroideraemia was made. Upon genetic testing, he was found to have a homozygous RPE65 mutation, causing an advanced retinitis pigmentosa phenotype.

### **6.3 Methods**

The patients were assessed at the Research Foundation, Royal Victoria Eye and Ear Hospital, and underwent clinical assessment with DNA isolation and sequencing. These processes, along with data analysis of variants provided by sequencing, are detailed in chapter 2.

### **6.4 Results**

#### **6.4.1 Clinical Findings**

The index case (IV:1) gave a history of lifelong nyctalopia with difficulties descending and ascending steps from the age of seven suggestive of visual field loss. A decrease in visual acuity commenced in the early twenties. At age 25, visual acuity was 6/12 in the right eye, with a correction of  $-12.25/+2.25 \times 45^\circ$ . Visual acuity in the left eye was 6/48, with a correction of  $-12.75/+2.00 \times 125^\circ$ . Goldmann visual fields were severely constricted, with a small central field of  $<20^\circ$  in both eyes, with some remaining islands at  $50^\circ$  from centre to the IV4e target (figure 34). Rod- and cone-isolated electroretinogram (ERG) responses were extinguished. The fundal appearances were in keeping with choroideraemia with diffuse retinal pigment epithelium (RPE) and choriocapillaris atrophy and clumping with macular sparing (figure 35). These changes were associated with optic disc pallor and attenuation of the retinal vessels. A pedigree was constructed from the information provided at this consultation (figure 36).

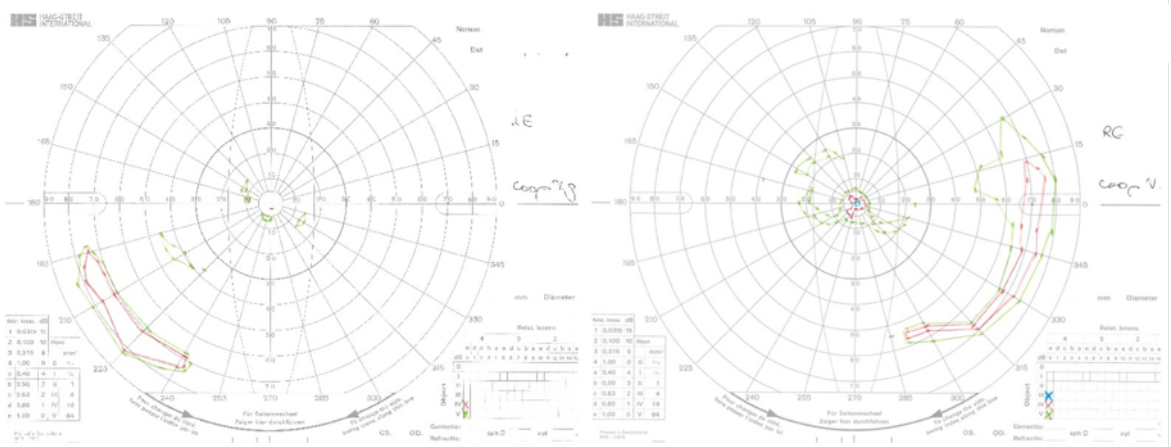


Figure 34. Proband (Patient IV:1) Goldmann visual fields

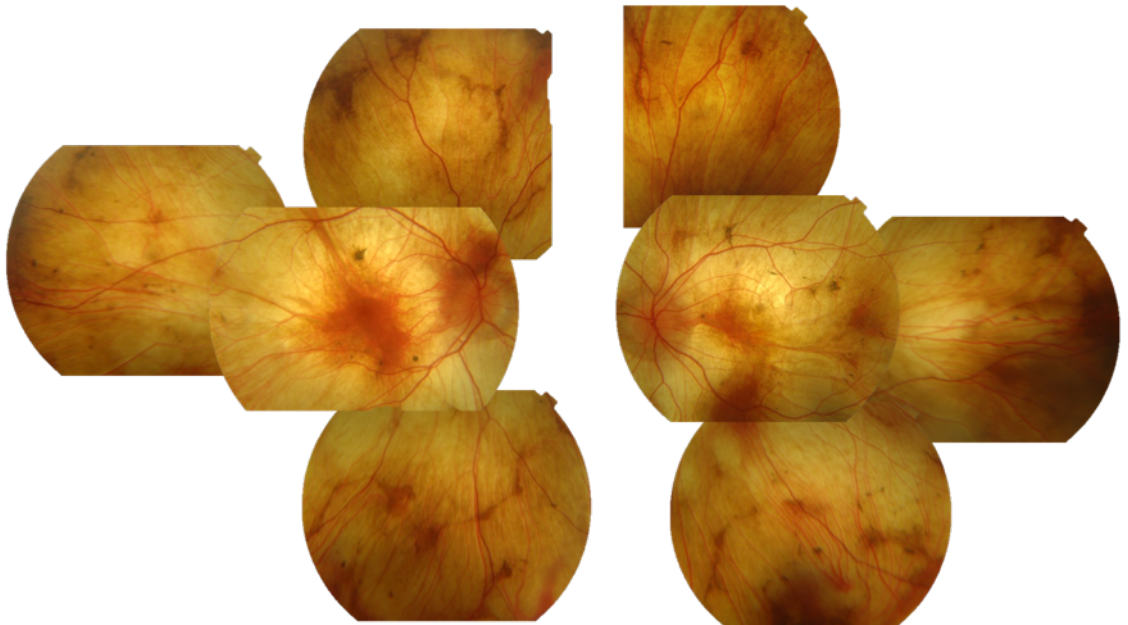
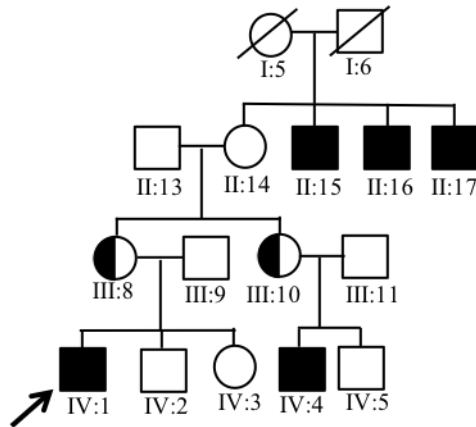


Figure 35. Proband (patient IV:1) colour fundus photographs



**Figure 36. A pedigree diagram depicting the affected patients and their relatives.**

***The index case is illustrated with an arrow.***

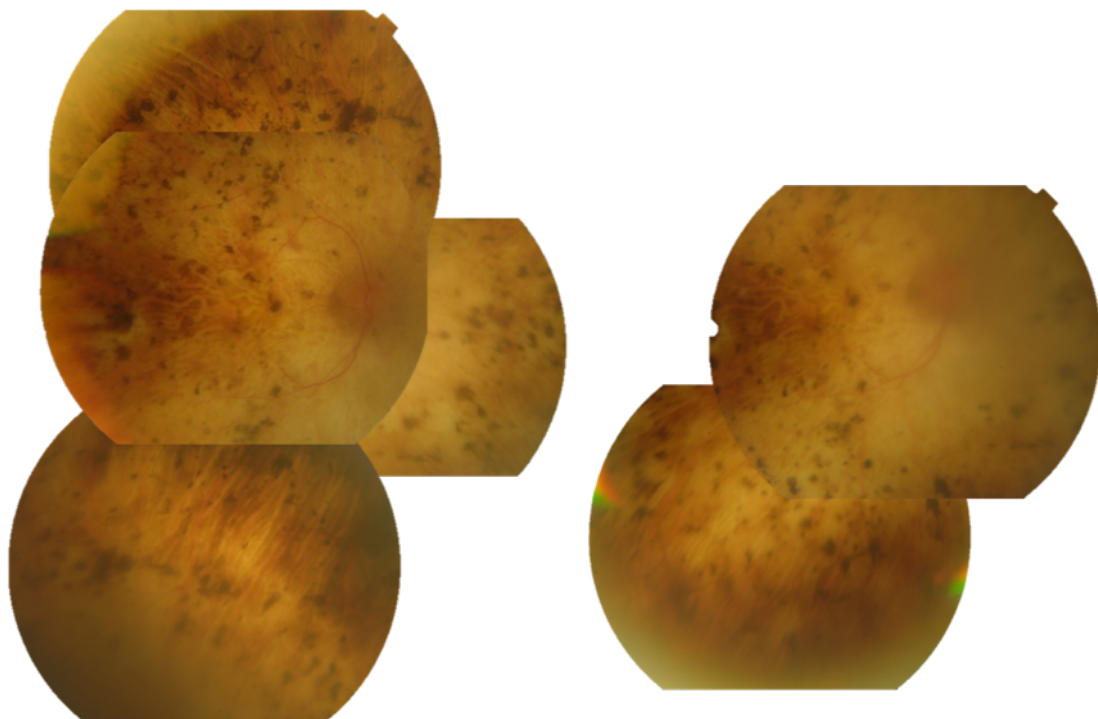
His mother (III:8), an X-linked choroideraemia carrier, had no visual symptoms. Her visual acuity in the right eye was 6/6, with a correction of  $-0.25/+1.00 \times 130^\circ$  and 6/9.5 in the left eye, with a correction of  $+0.25/+0.75 \times 65^\circ$ . Her Goldmann visual field was full in both eyes and examination of the anterior and posterior segments was unremarkable.

A first cousin of this patient (IV:4) was found to be similarly affected. He gave a history of nyctalopia since age 10, with no decrease in visual acuity. Visual acuity was 6/6 in the right eye, with a correction of  $+0.50/+0.25 \times 95^\circ$ , and 6/6 in the left eye, with a correction of  $\text{plano}/+1.00 \times 85^\circ$ . Goldmann visual fields showed mild constriction and both the rod and cone responses of his ERG were attenuated. Colour vision was diffusely abnormal. Though anterior segment examination was normal, there were cells in the vitreous and the fundal appearance was of patchy RPE and choroidal atrophy with scalloped edges and occasional pigmentary deposits. This was in keeping with X-linked choroideraemia.

His mother (III:10), another choroideraemia carrier, had no visual complaints. Her visual acuity in the right eye was 6/4.8, with no spectacle correction. Visual acuity

in the left eye was 6/4.8, with a correction of +1.75/+0.50 x 160°. Her Goldmann visual fields and colour vision were both normal. Her ERG showed normal rod and cone responses. Fundal examination revealed a diffuse, patchy disturbance of the RPE with a similar pattern of patchy hypofluorescence on autofluorescent imaging.

A great-uncle of the index case (II:15), initially thought to have X-linked choroideraemia, was interviewed at the age of 65. He described nyctalopia since the age of five and significant visual acuity disturbance from the age of six. He attended a dedicated school for the blind from the age of twelve. He had no light perception in either eye (NPL). On examination, there was a fine rotatory nystagmus with limitation of extraocular eye movements bilaterally. His right eye was pseudophakic and the left had a significant posterior subcapsular cataract. Fundal examination showed severe disc pallor and vessel attenuation with extensive RPE thinning and diffuse, blotchy pigmentary deposits (figure 37).

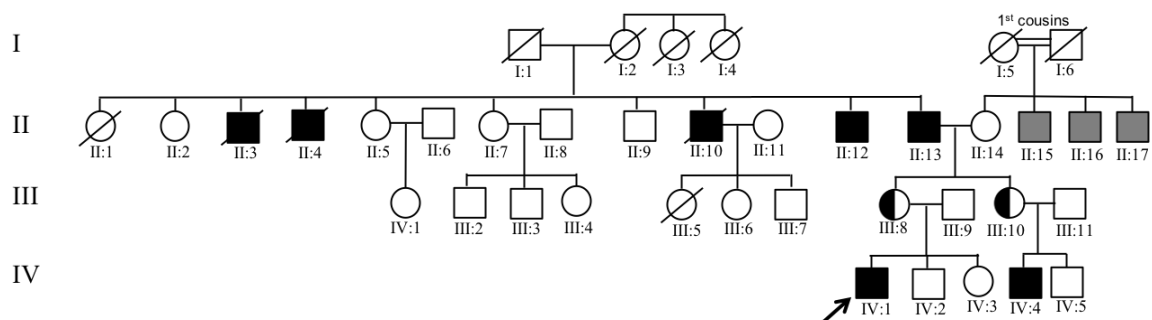


**Figure 37. Patient II:15 colour fundus photographs**

Another great-uncle and member of the RPE65 cohort (II:16), attended for interview. He described lifelong nyctalopia and a severe decrease in visual acuity from the age of 18. He also attended a school dedicated to the visually impaired. Visual acuity was perception of light (PL) bilaterally. His Goldmann visual field in the right eye was grossly constricted to  $<5^\circ$  to the V4e target and unrecordable in the left. On examination, he was found to have limitations of eye movement in all directions bilaterally with horizontal jerk nystagmus. Pupillary light response was absent. He had bilateral early cataracts and his fundal examination showed extensive RPE and choroidal atrophy with pigmentary clumping peripherally.

At this stage, next generation sequencing was performed on members of the family seen to date in what appeared to be a classical X-linked pedigree of choroideraemia. The younger members of the family had appearances in keeping with choroideraemia and their great uncles had advanced retinal degeneration which would also fit with this theory. Surprisingly, although the younger members were found to have mutations of the CHM gene, consistent with this theory, the older members were found to harbour biallelic RPE65 variants, indicating a retinitis pigmentosa genotype.

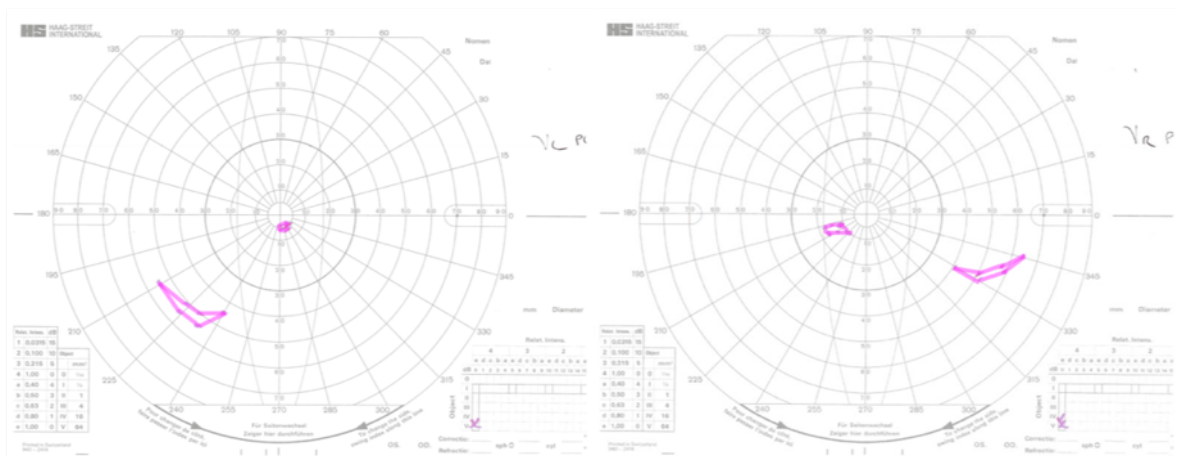
Upon further questioning, the patient mentioned that his maternal grandfather was also visually impaired. Further to this, the parents of the index case's great uncles were found to be first degree cousins (see figure 38).



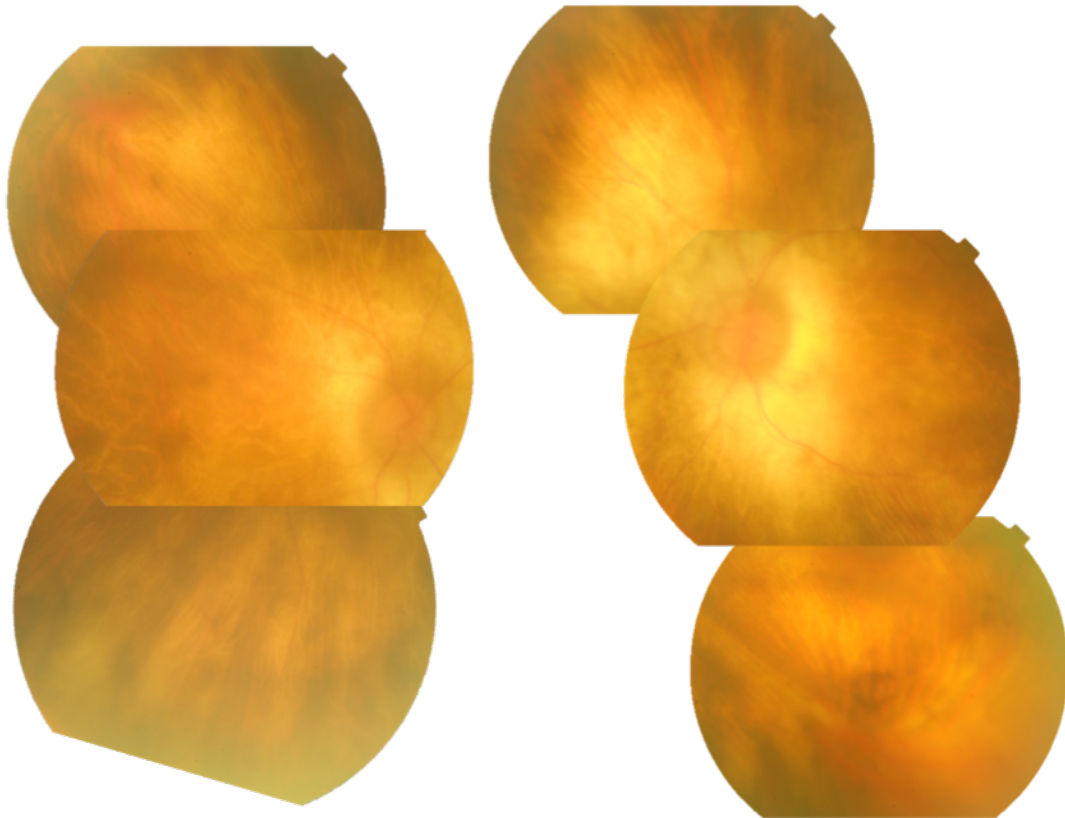
**Figure 38. Expanded pedigree incorporating maternal grandfather and siblings**



The affected patient's maternal grandfather (II:13), aged 71, had a history of at least 20 years of nyctalopia, with decreased visual acuity for the past 15 years. Four of his brothers were similarly affected. Visual acuity on examination was perception of light (PL) in both eyes. Goldmann visual fields were constricted to paracentral 10° islands (figure 39). Fundal examination revealed similar fundal changes to (IV:1), with extensive RPE and choriocapillaris degeneration, though affecting the macula in this case (figure 40).



**Figure 39. Patient II:13 Goldmann visual fields**



**Figure 40. Patient II:13 colour fundus photographs**

The maternal grandmother of the index case (II:14), aged 71, was examined. Her visual acuity in the right eye was 6/9.5, with a correction of +0.50/+0.50 x 30°. Visual acuity in the left eye was 6/19, with a correction of +3.00/+2.00 x 75°. She was developing early cataracts bilaterally. The fundal appearance of the discs, vessels, and retina were normal except for a small area of punched out RPE defects inferiorly in the left eye.

#### **6.4.2 Genetic Studies**

Two affected members from this pedigree underwent targeted genetic sequencing. Sequencing of the first patient identified, as expected, a strong mutation in the CHM gene: The 239<sup>th</sup> codon had been mutated into a stop codon,

c.715C>T, p.Arg239\*. As the REP1 protein encoded by CHM is over 600 amino acids long, this mutation would eliminate nearly two-thirds of the protein, almost certainly rendering it nonfunctional.

Sequencing of the second patient by the same method, however, did not reveal the same mutation. DNA sequence reads from this patient aligning to CHM codon 239 were manually examined, and confirmatory Sanger sequencing was performed, revealing that the mutation was definitively not present. The patient showed clear signs of advanced retinopathy, indicating that, for at least one member of the pedigree, the CHM p.R239\* mutation was not the disease-causing mutation.

This posed a diagnostic conundrum, as a premature stop mutation such as p.R239\* in CHM is virtually certain to cause choroideraemia, and no other plausible candidate mutations in CHM had been found in either patient. Nonetheless, one patient was clearly affected while definitively not carrying the p.R239\* mutation. As a result of this unexpected outcome, bioinformatics analysis was re-run on the patient lacking the p.R239\* mutation and the search was expanded beyond the X chromosome and CHM. A homozygous mutation was found in RPE65, p.R91W, which has previously reported as being causative of retinitis pigmentosa (El Matri, Ambresin et al. 2006). Outside of this pedigree, this mutation was not observed, even heterozygously, among the over 300 patients sequenced as part of the *Target 5000* study, nor was it observed in any individuals sequenced as part of the *1000 Genomes* study.

The presence of a very rare yet homozygous mutation raised a suspicion of consanguinity, which was later confirmed by a history of a first cousin marriage between (I:5) and (I:6).

## 6.5 Discussion

Choroideraemia and retinitis pigmentosa share many clinical features. Symptoms of nyctalopia, progressive narrowing of visual field and eventual loss of visual acuity are common to both disorders and peripheral retinal atrophy closing in on the central fovea and attenuated retinal vessels are characteristic findings in patients with both diseases. Although the characteristic severe atrophy of both the retina and the choroid in choroideraemia appears different initially to that of the RP classical pigment clumping, advanced retinal atrophy in RP can have an overlap in appearance with that of advanced choroideraemia. Dimopoulos et al., however, argue that choroideraemia can be clinically differentiated from advanced RP based on fundus autofluorescent imaging (FAF). The peripheral fundus in choroideraemia has characteristic islands of healthy RPE and areas of hypo-autofluorescence tend to be large and circular in shape with scalloped edges (Dimopoulos, Radziwon et al. 2017). Despite possible clinical clues to diagnosis, it is becoming apparent in the era of genetic testing that sometimes diagnoses based on phenotype can be in error. Whole exome sequencing of a large number of Chinese families with retinitis pigmentosa revealed some amongst them had CHM mutations indicative of choroideraemia (Li, Guan et al. 2014, Guo, Li et al. 2015). Similarly, a cohort of patients in Madrid initially diagnosed with other retinal dystrophies, were found to carry CHM mutations (Sanchez-Alcudia, Garcia-Hoyos et al. 2016). However, genetic testing itself is not infallible and variants of unknown significance can frustrate the diagnosis.

In some instances, where a diagnosis of choroideraemia cannot be established through genetic testing alone, an immunoblot test to ascertain the amount of the REP-1 protein in patient fibroblast cells can be performed (MacDonald, Mah et al. 1998). However, this is usually limited to a research context. Most patients with choroideraemia carry null mutations causing a complete absence of the protein product of CHM. Even those patients with missense mutations, whereby a point mutation causes a single different amino acid to be placed within the product protein, have been shown to have no difference in phenotype from those patients with null mutations and a similar lack of functional protein (Freund, Sergeev et al.

2016). This is the reason for the lack of genotype-phenotype correlations observed in this disease compared to, for example, retinitis pigmentosa. The corollary of this is that all choroideraemia patients, in theory, should be equally eligible for gene-based therapy with a replacement CHM gene.

Although genotype-phenotype correlations do not exist for choroideraemia, they are important in the case of RPE65-mediated IRD. Patients with mutations of this gene, which is essential in the visual cycle, where vitamin A derivatives are recycled and restored to the form of 11-cis-retinal to be used once more in the process of phototransduction, have different levels of 11-cis-retinal depending on the type of mutation they carry. For this reason, patients are diagnosed with diseases with a range of severity – ranging from Leber Congenital Amaurosis to a milder form of autosomal recessive retinitis pigmentosa. The R91W mutation of the RPE65 gene in our pedigree has been shown in a knock-in mouse to produce low levels of functional RPE65 and 11-cis-retinal, which would have allowed our patient with this mutation to have had good visual acuity in early life, which added to the presumption of his diagnosis being the same as other family members, that of choroideraemia (Samardzija, von Lintig et al. 2008).

It would be helpful if electrodiagnostic testing could provide further delineation between the diagnoses of choroideraemia and retinitis pigmentosa but these diseases, as measured by their electroretinographic recordings, behave similarly. The ERG in retinitis pigmentosa shows first a reduction in amplitude predominantly of scotopic responses followed by photopic responses in a “rod-cone” pattern. Both rod and cone responses slowly fall in amplitude until they are unrecordable (Lam 2005, Verbakel, van Huet et al. 2018). Similarly, in choroideraemia, the ERG shows a reduction in response in a rod-cone distribution (Mura, Sereda et al. 2007). In the advanced stages of both diseases, it is not possible to record any scotopic or photopic response. In addition, notably, milder ERG abnormalities can be found in both the carriers of X-linked retinitis pigmentosa and choroideraemia, so female relatives cannot be a clear source of evidence either (Vajaranant, Fishman et al. 2008). One source reported ERG

abnormalities in 15% of choroideraemia carriers (Sieving, Niffenegger et al. 1986), whereas ERG abnormalities are seen in up to 96% of X-linked retinitis pigmentosa carriers (Comander, Weigel-DiFranco et al. 2015).

Providing a molecular diagnosis is increasingly becoming the standard of care in the management of patients with IRDs. This pedigree is an important example of a situation where it was not possible to accurately diagnose all patients on the basis of clinical information alone. An accurate diagnosis is important for patients in the context of modern medicine and modern life for many reasons. Firstly, it gives them the ability to find more accurate information about their own condition and access to more individualised prognostic information. Secondly, in the era of an increasing number of genetic mutations being catered for by clinical trials, it allows them to know if they would be eligible for current or future planned clinical trials of gene-based therapies. Treatment trials have been carried out for the two disease entities diagnosed in this pedigree, namely choroideraemia and RPE65-mediated IRD, as mentioned above. Finally, this information is imperative for genetic counselling and family planning.

Central to the management of the genetic information which is imparted to patients is genetic counselling which is provided either by ophthalmologists themselves, genetic counsellors with experience of inherited eye disease or paediatric/adult geneticists (Branham and Yashar 2013). Access to these services is deemed highly important by patients, with one study gaining insight into this level of importance by how much patients would be willing to pay for the services as an insight into what this service is worth to them (Eden, Payne et al. 2013). Genetic counsellors can explain inheritance patterns to patients and help them estimate what possibilities exist for passing genetic diseases to successive generations of their offspring. More recently, this information has been used for preimplantation genetic diagnosis of embryos and embryo selection in conjunction with in vitro fertilisation (IVF), which has been reported in a small number of cases relating to eye disease recently in a study from Israel (Yahalom, Macarov et al. 2018). Further advances in genetic diagnostic technology and

treatments for these diseases will further drive the need for accurate diagnosis in the future.

# Chapter 7: Conclusion

## 7.1. Conclusions

The research carried out in this thesis and the work surrounding it within the structure of the Target 5000 project have helped to characterise over 1000 patients and their family members with inherited retinal disease in Ireland. During my time in the research department at the Royal Victoria Eye and Ear Hospital, I characterised over 150 patients with retinal degenerations and this has contributed to a bank of over 750 patients from the Republic of Ireland group alone.

My work has helped identify novel mutations such as that of the GNAT1 mutation c.904C>T, p.Gln302\*, a point-nonsense mutation causing a truncated protein, which is novel to the international literature. We identified the putatively disease-causing mutation c.1946C>T, p.S649L mutation of the PROM1 gene, which has highlighted the difficulties encountered in categorising variants which arise from either panel-based exome sequencing as in our case, along with whole exome sequencing or whole genome sequencing, all of which are directly relevant to current clinical practice.

We have identified a novel phenotype in the GNAT1 patient with a late-onset rod-cone dystrophy or “retinitis pigmentosa” phenotype. Previously, this gene had been associated only with congenital stationary night blindness. These novel genetic and phenotype associations will contribute to the international bank of data, which serves to help molecular scientists and clinicians solve cases of rare variants and unusual phenotypes.

We have also been able to confirm diagnoses in complicated pedigrees, which would otherwise have gone unrecognised, as in our pedigree of patients with both choroideraemia and RPE65-mediated retinal degeneration. This information is vital to provide accurate genetic counselling to families with IRDs.



## 7.2 Future Directions

The work of this thesis and of other similar groups worldwide is slowly revolutionising how patients with IRDs are diagnosed, counselled, and treated. Molecular diagnosis has now become the international clinical standard for these patients. Genetic testing and deep phenotyping over time in natural history studies have provided much more specific and reliable information about individual patients' diagnoses and clinicians are in a position to be able to provide a more evidence-based guide to prognosis in many cases. The information also allows for genetic counselling that is more factual and robust.

A recent study showing that a high percentage of patients with IRDs have actionable ophthalmic disease such as cataract, cystoid macular oedema and epiretinal membrane emphasises the importance of seeing this cohort regularly (Liew, Strong et al. 2018). Deep phenotyping and natural history studies of these diseases will allow us to characterise the ideal time for certain interventions like gene-based therapies and which patients would benefit the most from them.

Molecular diagnosis is essential for eligibility for gene-based therapies, such as the commercially available Luxturna, and enrolment in clinical trials for gene-based therapies. The number of trials of gene-based therapies is ever-increasing and alternative therapies such as stem cell treatments, retinal prostheses, optogenetics, antisense oligonucleotides, and others also continue to make visual gains in both animal and human models. Substantial functional improvements in vision have been provided by these therapies in many instances in this exciting and dynamic field and progress is constant.

There is a significant challenge to be found in categorising the pathogenicity of variants identified by gene sequencing, especially whole genome sequencing which has the ability to produce large amounts of data. Approximately, 50-60% of patients who undergo targeted panel-based sequencing of known IRD genes receive a molecular diagnosis, and several avenues of resolution are being

explored in those cases where diagnosis is not possible. The first avenue is novel genes and, in 2018, at least 5 genes novel to the literature were described for the first time (Daiger 2019). Furthermore, it is highly likely that many of the known genes harbour further mutations that have not yet been identified. One piece of evidence for this lies in the high number of patients with a phenotype suggestive of Stargardt disease, for example, who have one identified mutant variant in ABCA4 and the other, which we know is likely to lie within the other allele, remains unsolved. Intronic variants which are present in non-coding DNA and affect splice-site regions, along with regulatory site variants which code for promoter sequences or microRNAs and finally copy number variants which are difficult to identify using current NGS techniques are all areas of high priority for investigating these unsolved cases (Farrar, Carrigan et al. 2017). The fact that there is a constant expansion of the international dataset of described pathogenic variants, along with an increasingly in-depth knowledge of more unusual variants and their locations, will hopefully make this process incrementally more straightforward in the future.

Just over thirty years ago, no single gene had been identified that caused an inherited retinal degeneration, no effective treatment existed for these diseases, and the human genome had not yet been sequenced. We now know of over 250 genes that cause retinal degenerations, with thousands of known mutations and an abundance of knowledge of the physiological mechanisms that underlie the pathology of these diseases. Whole genome sequencing is widely and inexpensively available and trials of treatments with many different approaches to restore vision are underway across the world. To achieve a more accurate interpretation of WGS data, to further understand how these deleterious variants cause disease and to provide the best possible levels of vision to those who suffer from inherited retinal disease are aims which must be addressed by further research. One study estimates the increase in diagnostic yield of inherited retinal degenerations with WGS, compared to NGS, to be 29% (Ellingford, Barton et al. 2016).

There are specific challenges to the provision of services to these patients in the Irish health service. Traditionally, many patients with IRDs were discharged from clinics, as there was deemed to be no treatment of benefit to these patients. With the advent of treatment modalities, there will be increased pressure on the services to incorporate these patients into the clinical environment with appropriate specialist input. The modern approach to management of patients is also more holistic and, ideally, there would be genetic counselling services, refraction services, low visual aid services, and social support services available to patients in an accessible and streamlined manner through the health service. The cost of treatment will be raised as an important issue as more treatments become available. The cost of Luxturna, which has been approved by the European Medicines Agency for the treatment of RPE65-mediated retinal degenerations, is in excess of \$400,000 dollars per eye. No patient in Europe has yet been treated and the UK awaits NICE guidance on this issue. In the meantime, we must endeavour to provide equitable access to treatments.

Finally, there is a need to train further clinicians and scientists in this area to continue this work and move towards a future where a clear diagnosis and treatment are available for every patient.

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