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Modulation of mitochondrial function as a therapeutic
strategy for Stargardt disease and an exploration of the
genetic architecture of Stargardt disease in Ireland.

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

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Under the supervision of Prof. Jane Farrar

Declaration

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A handwritten signature in black ink, appearing to read 'Iris Post', is written over a horizontal line.

Iris Johanna Maria Post

17th of August, 2023

Summary

Inherited retinal degenerations (IRDs) are an extremely heterogeneous group of disorders, together representing the most common cause for vision loss in young people. An estimated 5.5 million people globally are living with an IRD. The most common IRD caused by a single gene is Stargardt disease type 1 (STGD1), which is caused by biallelic mutations in ABCA4 and vision loss can occur as early as the first decade of life. No therapies are available to cure or alleviate symptoms for this IRD. One of the main limiting factors in designing gene therapies is transport of the transgene into *in vivo* cells. AAV vectors, which are able to transduce post-mitotic cells such as retinal cells well and are low immunogenic, can only carry a gene with a maximum size of 4.5kb. The cDNA of ABCA4 encompasses 6.8kb, so a different approach is essential for designing a therapy for STGD1.

This PhD thesis investigates the possibility to modulate mitochondrial function as a therapeutic strategy for Stargardt disease. ABCA4 dysfunction leads to the accumulation of toxic vitamin A aggregates (A2E) in photoreceptor cells and retinal pigment epithelium. A2E is a major component of the autofluorescent pigment complex lipofuscin, which causes downregulation of mitochondrial function, increased levels of oxidative stress, and ultimately, cell death. Utilising a codon-optimised, humanised yeast homolog for NADH Dehydrogenase, NDI1, we investigated if the effect of ABCA4 dysfunction on mitochondrial welfare could be ameliorated.

Chapter 3 of this thesis describes the exploration of AAV.CAG.ophNDI1 as a therapeutic strategy in a variety of cell models of disease. Models were based on RPE-like cell line ARPE19 cells and primary porcine RPE cells that were insulted with either NaIO₃ or A2E. Treatment with AAV.CAG.ophNDI1 improved mitochondrial function and reduced levels of Reactive Oxygen Species (ROS) in all models. We then extended the investigation to mouse models of disease, which are presented in chapter 4. The model, *Abca4*^{-/-} mice, was first characterised extensively. Then, they received subretinal injections with various doses of AAV.CAG.ophNDI1 in one eye, while the second eye was only injected with PBS to serve as a control. Here, we found that cellular ROS levels could be significantly reduced with the therapy. Furthermore, we showed, for the first time, that lipofuscin levels can be reduced through the modulation of mitochondrial function.

Additionally, we recognise that it is extremely important to genetically diagnose everyone living with IRDs or other forms of sight loss in order to know if any such individual is eligible for approved gene therapies or clinical trials. The Target 5000 project aims to provide clinical care and genetic diagnoses for all Irish IRD patients. A total of 212 people have been successfully diagnosed with biallelic, ABCA4-related Stargardt disease through Target 5000. Utilising Target

5000 data, chapter 5 of this thesis provides an extensive overview of the genetic architecture of Stargardt disease on the island of Ireland. A case study is presented on a variant that appears to be enriched in the Irish population, p.Cys54Tyr. Furthermore, a short overview is given on the prevalence of PROM1, PRPH2, and ELOVL4 in the Target 5000 cohort, as dysfunction in these genes can give rise to Stargardt-like phenotypes.

This PhD thesis outlines a gene therapy strategy that is independent of the gene involved in disease. Employing a transkingdom complex I gene therapy (NDI1) to modulate mitochondrial function can provide therapeutic benefit in *in vitro* and *in vivo* models of disease. Strategies targeting common pathways of disease may provide a therapeutic option for degenerative conditions whose genetic diagnosis remains elusive.

This thesis is dedicated to 'Papa John'
I can only hope you're proud of the person I've become
Miss and love you always

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List of abbreviations

11-cis-RAL	11-cis-retinaldehyde
11-cis-ROL	11-cis-retinol
A2E	N-retinylidene-N-retinylethanolamine
A2PE	N-retinylidene-N-retinyl-phosphatidylethanolamine
AAV	Adeno-Associated Virus
ABCA4	ATP-Binding Cassette, subfamily A, member 4
AD	Autosomal Dominant
ADP	Adenosine Diphosphate
AMD	age-related macular degeneration
AR	autosomal recessive
at-RAL	all-trans-retinaldehyde
at-ROL	all-trans-retinol
ATP	Adenosine Triphosphate
BCVA	Best Corrected Visual Acuity
CRD	Cone-Rod Dystrophy
DNA	Deoxyribonucleic acid
ECAR	Extracellular Acidification Rate
ELOVL4	Elongation of very long chain fatty acids protein 4
EtBr	Ethidium Bromide
iMD	inherited Macular Dystrophy
IMM	Inner Mitochondrial Membrane
LB	Luria-Bertani medium
LCA	Leber Congenital Amaurosis type
LE	Left eye
LHON	Leber's Hereditary Optical Neuropathy
LRAT	Lecithin retinol acyltransferase
MD	Macular Degeneration
MOI	Multiplicity Of Infection
NaIO ₃	Sodium Iodate
N-ret-PE	N-retinylidene- phosphatidylethanolamine
NDI1	NADH Dehydrogenase Internal (yeast)
NFW	Nuclease Free Water
NGS	Next Generation Sequencing
OCR	Oxygen Consumption Rate
OMM	Outer Mitochondrial Membrane
OS	Outer Segment (of photoreceptor cells)
OXPHOS	Oxidative Phosphorylation
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
POS	Photoreceptor Outer Segment
PROM1	Prominin-1
PRPH2	Peripherin 2 (also known as RDS)
RDH8	All-trans-retinol dehydrogenase
RE	Right eye

RNA	Ribonucleic acid
ROM1	Retinal Outer Segment Membrane Protein 1
ROS	Reactive Oxygen Species
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse Transcriptase polymerase chain reaction
smMIPs	small molecule Molecular Inversion Probes
SRC	Spare Respiratory Capacity
STGD1	Stargardt Disease type 1
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

1. General introduction

1.1. Eye Physiology

The human eye is the result of millions, if not billions, of years of evolution. While the first life forms were only able to sense the direction light was coming from, our eyes allow us to see depth, details, and colours in the world around us. Through all those years, the eye became increasingly complicated, which is evident in the anatomy and physiology of the eye. Figure 1.1 depicts how light enters the eye and what cell types are involved in the phototransduction.

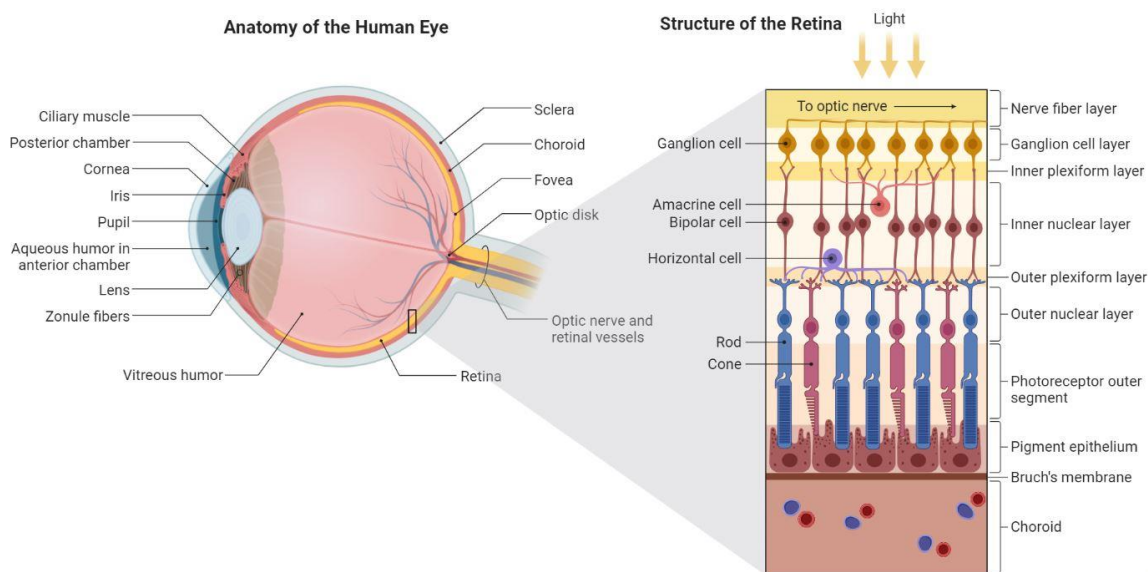


Figure 1.1. Schematic overview of the eye. Light passes the cornea and enters the eye through the pupil onto the lens. The lens directs the light onto the retina, where the light is converted into neuronal signals, that are conducted to the brain via the optic nerve. A schematic overview of organisation of the retina shows all cell types. The light enters the retina in this picture from the top and is caught by rod and cone photoreceptors. Figure created with Biorender.com

Light particles called photons enter the human eye through the cornea, travel through lens and intravitreal space and then reach the retina. The retina is located at the back of the human eye and consists of several layers of cells, each with their own important function. Phototransduction is a cascade that typically does not work effectively unless all key components are functional, and it starts with post-mitotic light sensitive photoreceptor cells that detect the presence of light. Photoreceptor cells detect light using opsin proteins and can be divided into rod and cone cells.

Rhodopsin, the opsin found in rod cells, is very sensitive to lower light levels and facilitates the dark-light vision associated with rod photoreceptors. Cone cells, in humans, contain one of three different types of cone opsin. These three types of opsins are responsive to light with different

wavelengths, corresponding to blue, green, and red light. Opsins convert these specific light signals into electrochemical signals that can be transmitted via bipolar cells and ganglion cells to the optic nerve and the brain.

The retinal pigment epithelium, or RPE, consists of a single layer of pigmented cells on the outside of the retina. The RPE has many functions, all of which are vital for natural functioning of the visual cycle. Melanin in the RPE absorbs light (Boulton 1998), protects the cells against photo-oxidation (Strauss 2005) and has been shown to quench some types of Reactive Oxygen Species (ROS) (Hu *et al.* 2008). The RPE cells also transport nutrients, ions, and components of the visual cycle between the choroidal blood vessels and photoreceptor cells (Strauss 2005). Furthermore, a variety of growth factors to support growth and cell survival in photoreceptors and other retinal cells, such as VEGF and PEDF, are produced by the RPE (Dawson *et al.* 1999). The RPE also helps with photoreceptor outer segment renewal. Photoreceptor outer segments consist of a stack of 'discs' that need to be renewed systematically to keep them functional. At the base of the outer segment, new discs are made, while at the top of the outer segments the old discs are shed. The RPE cells absorb the old discs, recycle the important molecules and deliver those back to the photoreceptors. Of course, the shedding and re-growing of the discs needs to be tightly regulated in order to keep the outer segments functional (Strauss 2005). In human eyes, the entire outer segment is renewed approximately once every ten days (Gao *et al.* 1992).

The macula is a pigmented zone located centrally in the retina, which is densely populated with photoreceptor cells. Its name is derived from the Latin *macula lutea*, which translates to yellow spot. The macula's yellow colour comes from macular pigments such as Lutein, zeaxanthin, and meso-zeaxanthin, whose function is to absorb blue and ultraviolet light and to prevent photooxidative damage to RPE cells. The macula is the focal point of the lens and the cornea, and therefore enables high acuity vision. The fovea centralis is located at the centre of the macula and contains the highest concentration of cone photoreceptors in the eye. The visual sharpness it supplies allows humans to perform activities such as reading (Iwasaki *et al.* 1986).

After photoreceptor cells have translated photons into an electrical signal, it is transmitted to the brain via other retinal cell layers. Horizontal cells assess the levels of illumination on the retina and use that to adjust the output of photoreceptor cells and to implement an inhibitory feedback loop to the photoreceptors. Bipolar cells can receive synapses from either horizontal cells or photoreceptor cells and can transmit them directly to retinal ganglion cells (RGC) or indirectly via amacrine cells. Amacrine cells are suggested to be involved in detection of moving objects and in the regulation of sensitivity via inhibitory feedback loops to bipolar cells (Masland 2012). Transmission of visual signals via these different cell types allows for the generation of

functional subunits that improves contrast, light adaption and sensitivity. Last, bipolar and amacrine cells transmit information to the retinal ganglion cells. The RGCs have long axons that form the optic nerve and transmit the visual information they received directly to the brain. Approximately half of the nerves running through the optic nerve to the brain, transport visual information originating from the fovea, highlighting the density of cells present in this area (Masland. 2012).

The eye has been at the forefront of translational gene therapies and that is not without reason. As mentioned before, most retinal cells are fully differentiated and post-mitotic. In principle, this feature of the retina is beneficial for gene therapies, as the absence of cell division means that the supplemented gene will not be diluted with each cell division. Therefore, the supplemented gene does not require some form of replication *in vivo* or re-administration of the therapy, as would be required in dividing cells. However, the post-mitotic status of retinal cells also has a downside because some vectors used for gene delivery, such as retrovirus, can only transduce cells during cell division, which means they are not suitable as vectors for IRD therapies (Ziccardi *et al.* 2019).

Another advantage of gene therapies for retinal disease, is that the retina is a relatively small target tissue, and therefore, smaller quantities of therapeutic compounds are needed, reducing manufacturing costs. The eye is also an accessible organ, with surgeries and imaging techniques being minimally invasive. Bilateral vision provides the option to treat only one eye at a time with a specific treatment and use the other eye as a negative control, representing another advantage. Inherited retinal degenerations (IRDs) often show similar disease progression in both eyes, so in principle efficiency and potential side effects of therapies can be compared if only one eye is treated (Ziccardi *et al.* 2019).

An additional advantage of using (gene) therapies in the retina is that the retina is immunoprivileged because of the blood-retina barrier (BRB). The RPE maintains the outer BRB and regulates transport of water, proteins and ions between the choroid and retina. As a result, the immune responses in the eye are far less extensive (akin to the brain) compared to the rest of the body. Given the immune privilege of the retina, the use of viral vectors to deliver gene therapies has typically been well tolerated, causing minimal immune responses, making the eye tolerate this type of therapy relatively well (Ziccardi *et al.* 2019). However, the quality of the viral vector, the dose of the therapy, the nature of the ocular disease being treated, and the route of administration can all influence immune responses to the therapy. Additionally, it should be noted that the BRB can become more fragile over time and thus reduce the immune privilege found in the eye. (Zhu *et al.* 2023)

1.2. Visual Cycle

Remarkably, the visual cycle converts a light signal into an electrical signal that can be interpreted by the brain to form an image. Vitamin A plays a key part in this cycle. Rhodopsin and cone-opsin proteins capture photons in the outer segments of their respective photoreceptor cells and then convert 11-cis-retinaldehyde (11-cis-RAL) into all-trans-retinaldehyde (at-RAL). This activates the opsin, after which the at-RAL dissociates from the pigment and merges with phosphatidylethanolamine (PE) to form N-retinylidene-phosphatidylethanolamine (N-ret-PE). ATP-Binding Cassette subfamily A4 (ABCA4) moves N-ret-PE to the cytoplasm, where it becomes a substrate for retinol dehydrogenase (RDH8). RDH8 reduces the at-RAL to all-trans-retinol (at-ROL) before it is released into the disc bilayer. It then gets transported to the RPE where the RPE65 enzyme isomerises all-trans-retinol into 11-cis-retinol (11-cis-ROL) (Choi *et al.* 2021). Mutations in the RPE65 gene are associated with Retinitis Pigmentosa (RP) and Leber Congenital Amaurosis type 2 (LCA2) (Morimura *et al.* 1998; Thompson *et al.* 2000). The final step of the visual cycle is the oxidation of 11-cis-ROL back into 11-cis-RAL by the 11-cis-RDH enzyme (Kiser *et al.* 2012). Due to the complicated nature of these events and the large number of enzymes and other components involved in the visual cycle, it comes to no surprise that there are a large variety of visual impairments that stem from mutations in the genes encoding components of the visual cycle.

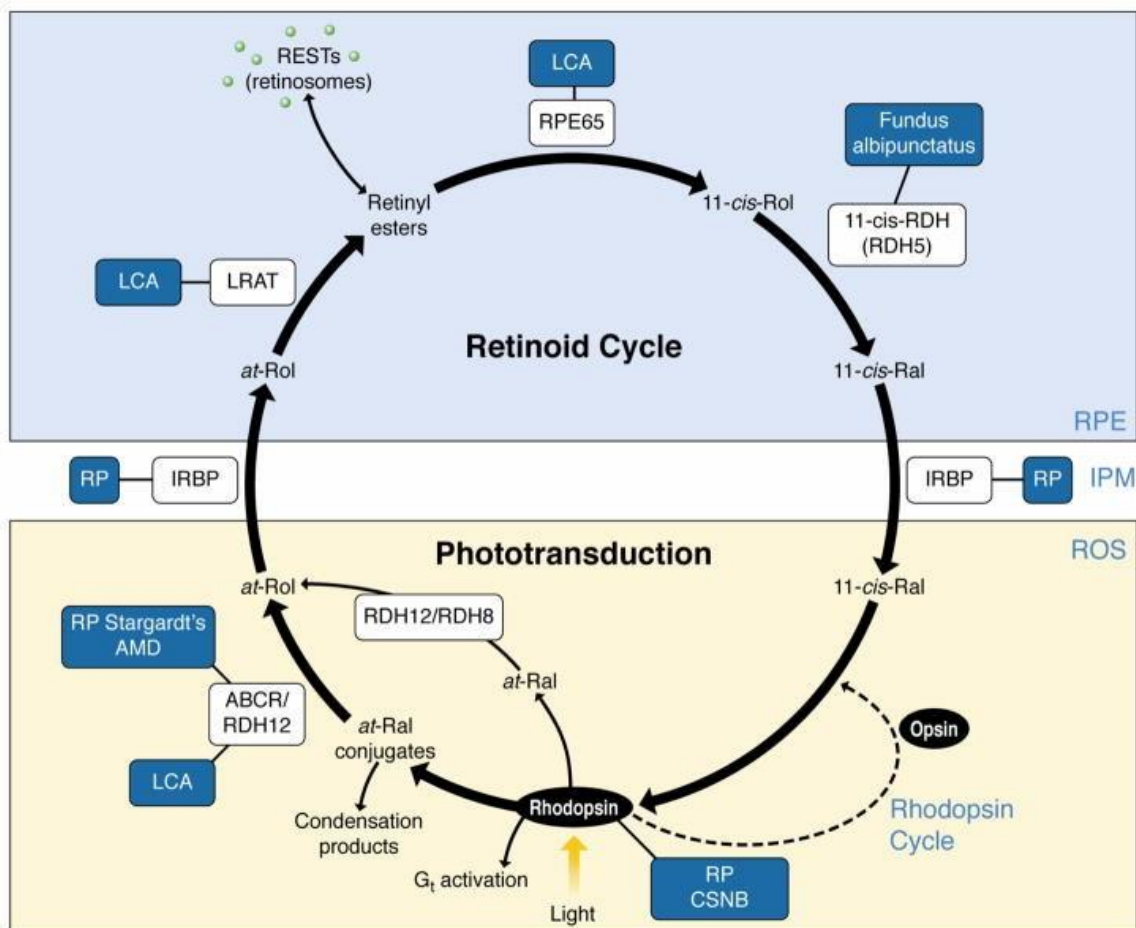


Figure 1.2. The phototransduction cycle recycles vitamin A in in the photoreceptor outer segments and the RPE. Many types of inherited visual impairment are caused by mutations in genes involved in this cycle. Source: Kiser *et al.* 2012.

1.3. Stargardt disease and the function of ABCA4

Stargardt Disease is an inherited retinal degeneration (IRD) that presents its phenotype relatively early in life. This condition was first described in the early 1900s (Stargardt. 1909) and it is characterised by an irreversible and progressive degeneration of the macula, slowly causing a deteriorating central vision loss. Though a relatively rare disease, Stargardt is the most common inherited form of macular degeneration caused by mutations in a single gene, with an estimated incidence of 1 in 10,000 (Blacharski. 1988). As of today, there is no treatment available for Stargardt Disease.

In 1997 mutations in the *ABCA4* gene were found to be causative for Stargardt Disease type 1 (STGD1) (Allikmets *et al.* 1997), which follows a recessive inheritance pattern. Stargardt Disease types 3 and 4, which are characterised by similar phenotypes, are caused by dominant defects in other genes, *ELOVL4* and *PROM1*, respectively (Zhang *et al.* 2001; Michaelides *et al.* 2010), but these are less common. Though Stargardt disease type 1 follows a classic mendelian inheritance pattern, there's a wide variability in disease progression and age of onset due to differences in

penetrance and severity of alleles. Combinations of various alleles show a wide variability in severity of disease (Cornelis *et al.* 2017; 2022)

ABCA4 is mainly expressed in the outer segments of photoreceptor cells (PR), however, recent findings suggest that it is also present and active in the RPE (Lenis *et al.* 2018). ABCA4 is a transporter protein that consists of two large extracellular domains, two sets of six transmembrane domains and two nucleotide binding domains (NBD) that protrude into the cytoplasm. The NBD binds to ATP molecules, which gives the protein the energy to carry out its function. It is suggested that ABCA4 functions as a flippase, which means that it can transport its substrate across a membrane by 'flipping' its own conformation (Bungert *et al.* 2001).

ABCA4 is a transmembrane protein located on the bilayer PR disc membrane and transports N-retinyl-phosphatidylethanolamine (N-ret-PE) from the PR disc lumen to the PR cytoplasm. Here, the visual cycle will continue, and N-ret-PE will eventually be reduced into vitamin A and transported to the RPE, where it will be converted into 11-cis-retinal so that it can be recycled in the visual phototransduction cascade.

When *ABCA4* is mutated and not functioning as effectively as it should, the transfer of N-ret-PE from the lumen to the cytoplasm is reduced. Eventually this leads to a build-up of N-ret-PE in the OS of the rod photoreceptor cells, which allows the formation of a condensation product of N-ret-PE and retinaldehyde, called N-retinylidene-N-retinyl-phosphatidylethanolamine (A2PE). In phagolysosomes of the RPE, A2PE undergoes phosphate hydrolysis, yielding N-retinylidene-N-retinylethanolamine (A2E), which accumulates and typically can cause further problems over time (Sparrow *et al.* 2003).

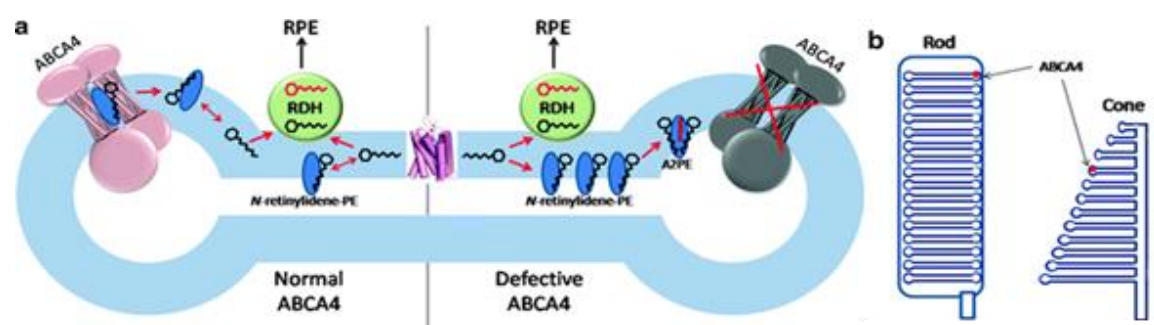


Figure 1.3. ABCA4 is located on the disc membrane of photoreceptor cells. Source: Tsybovsky et al 2010.

Accumulation of A2E negatively affects the RPE cells in many ways; it impairs cholesterol discharge from endosomes and lysosomes (Zhou *et al.* 2006), it disrupts the 11-cis retinal supply to the retina (Moiseyev *et al.* 2010), and it activates the retinoic acid receptor, which leads to upregulation of pro-angiogenic factors, which then leads to the development of new blood vessels in locations where they are not supposed to develop, contributing to disorders like wet

AMD (Iriyama *et al.* 2008). A2E build-up also destabilises cellular membranes, and it impairs degradation of phospholipids from phagocytosed OS (Sparrow *et al.* 2012). Furthermore, A2E is part of lipofuscin, an autofluorescent, pigmented complex composed of oxidised and degraded lipids and proteins. Indeed, up to 25% of free cytoplasmic space in retinal cells can be filled with lipofuscin granules in elderly people (Mazzitello *et al.* 2009). However, it is important to note that these experiments have purely been done on mouse eyes and on a large variety of cell models (such as cell lines, primary RPE cells and patient-derived iPSCs), but ophthalmoscopic imaging has also shown RPE cells to be affected (Strauss *et al.* 2016; Westeneng-van Haaften *et al.* 2012).

There are also indications that in cells with high A2E concentrations autophagy is reduced, leading to accumulation of toxic products and cell death (Saadat *et al.* 2014). Autophagy is a cellular process that removes dysfunctional components, such as lipofuscin, from the cell. The autophagy process involves the formation of so-called phagophores, which engulf damaged proteins, organelles or cellular components. The phagophore closes and forms a double-membrane autophagosome, which ultimately fuses with a lysosome into a autolysosome. In the autolysosome, the damaged proteins and other cellular components are broken down into amino acids, sugars and fatty acids (Parzych and Klionsky. 2014).

Autophagy is a highly energy-consuming process, requiring a lot of ATP for proper function. However, A2E accumulation also disrupts mitochondrial function. The oxidative stress caused by A2E build-up leads to damage of mitochondrial DNA, which then leads to production of reactive oxygen species (ROS) and reduced mitochondrial function (Saadat *et al.* 2014). Accordingly, A2E inhibits ATP synthesis by mitochondria, reducing the energy that is available to RPE cells for functions such as autophagy, among many others. Considering the retina, with high energy demanding specialised neurons such as photoreceptors and retinal ganglion cells, is one of the tissues in the human body with the highest oxygen-consumption rate and energy needs, the relationship which has recently been highlighted between mitochondrial dysfunction and vision loss is not remarkable (Saadat *et al.* 2014; Sivapathasuntharam *et al.* 2019).

All in all, the accumulation of A2E in RPE cells ultimately leads to lower cell survival rates. Every RPE cell provides vital nutrients and visual cycle components to 30 to 40 photoreceptor cells (Gao *et al.* 1992), so it is understandable that the death of a few RPE cells can have a large adverse impact on the photoreceptor cell population. Eventually, photoreceptor cells will also die as a result of RPE cell death, causing loss of vision. Given that retinal cells are post-mitotic, once cells die, it is impossible to regenerate these cells. As *ABCA4* is a very large gene, the cDNA alone comprising 6.8kb, it is challenging to generate a gene replacement therapy and deliver this gene

or its encoded protein to photoreceptor cells. Dual vector *ABCA4* gene therapies are in development, but currently still face several obstacles (Dyka *et al.* 2019; McClements *et al.* 2020; Trapani 2018). However, since the A2E accumulation in Stargardt disease damages mitochondria and reduces their function, providing an overarching therapeutic strategy by augmenting mitochondrial function that is relevant to all patients with Stargardt disease, irrespective of the genetic subtype of disease, is being considered. Indeed, in this study our aim is to improve metabolic function of the RPE cells by directly modulating mitochondrial function.

1.4. Mitochondrial Function and the electron transport chain

A sentence almost every science student has heard in their lives is “the mitochondria is the powerhouse of the cell”. To understand how mitochondria provide the cells with energy we need to look at the morphology of mitochondria. The outer mitochondrial membrane (OMM) encloses the entire organelle. It contains many pores and channels for transport of nucleotides, ions and metabolites between the cell and the mitochondrion. Damage to the OMM can cause leakage of mitochondrial proteins into the cellular cytosol, which can at times even elicit cell death. The perimitochondrial space, or intermembrane space, is the space between the OMM and the inner mitochondrial membrane (IMM). The concentration of small molecules in this space is equal to the concentration in the cellular cytosol. The IMM houses several proteins with multiple functions, including oxidative phosphorylation (OXPHOS), ATP synthesis, the transport of proteins and mitochondrial fusion and fission. Invaginations which significantly expand the surface area of the IMM are called cristae. A higher ATP demand of a tissue typically correlates to more cristae in the mitochondria in that tissue and also more mitochondria per cell. The matrix, the space within the mitochondrion, encapsulated by the IMM, contains the mitochondrial genomes. There are between 2-10 mitochondrial genomes per mitochondrion (Robin *et al.* 1988). Energy conversion through the Krebs cycle and production of ATP occur in the matrix.

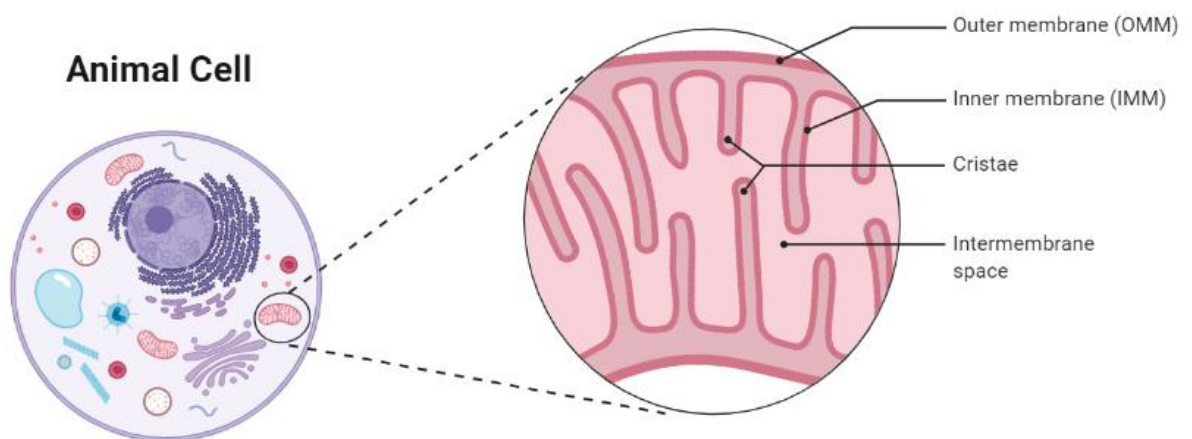


Figure 1.4. Mitochondrial Structure in animal cells. Figure created with Biorender.com.

The Electron Transport Chain (ETC) drives the synthesis of ATP through the creation of an electrochemical proton gradient. This gradient is maintained via the transfer of H^+ -ions across the IMM. For a functional ETC, five mitochondrial complexes work in concert. Complexes 1, 3 and 4 pump H^+ -ions from the mitochondrial matrix to the intermembrane space to create the electrochemical proton gradient. The ATP synthase complex uses this gradient by allowing H^+ -ions to return into the matrix and to simultaneously generate ATP from an ADP-molecule and phosphate. Complex I is a NADH Dehydrogenase and it is involved in the reduction of NADH (using NADH produced by the Krebs cycle) and a H^+ -ion to form NAD^+ to oxidise Coenzyme Q into $CoQH_2$. At the same time, it pumps four H^+ -ions into the intermembrane space. Complex II similarly reduces $FADH_2$ into FAD for the oxidation of Coenzyme Q, but this complex does not transport H^+ -ions across the membrane. The reduction of $FADH_2$ goes hand in hand with the conversion of succinate into fumarate. Two $CoQH_2$ molecules are reduced by Complex III, whereafter 4 H^+ -ions are transported to the intermembrane space, 2 electrons are used in cytochrome C redox-reactions and 2 electrons are used (together with 2 H^+ -ions from the matrix) by the same complex to oxidise one CoQ back into $CoQH_2$. Complex IV ultimately uses the free electrons from cytochrome c oxidation and H^+ -ions to turn oxygen into water. The (unofficial) fifth complex is ATP synthase. The electrochemical gradient created by transport of H^+ -ions over the IMM generates a potential energy that is used by ATP synthase for the production of ATP.

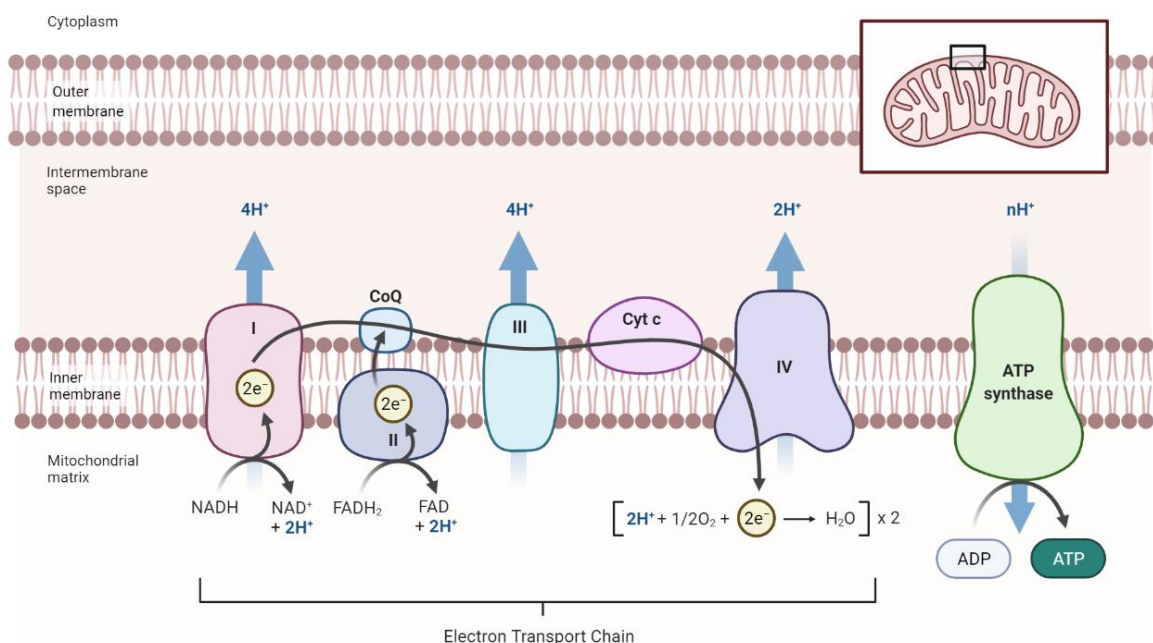


Figure 1.5. Schematic view of mitochondrial Electron Transport Chain. Five mitochondrial complexes form an electrochemical proton gradient by transporting hydrogen across the inner mitochondrial membrane. This gradient is required for ATP-production. Figure was generated with biorender.com.

Photoreceptor inner segments contain the highest concentration of mitochondria, and they have the highest energy demand of any tissue. The high ATP consumption is necessary for ionic pump function, which generates the membrane potential that is needed for synapses that send visual signals to the brain. Loss of mitochondrial function is related to loss of photoreceptor and RPE cells (Okawa *et al.* 2008; Sivapathasuntharam *et al.* 2019). However, due to the high rate of OXPHOS in the retina, the retinal cells are very susceptible to oxidative stress. The accumulation of ROS can cause damage to mitochondrial DNA (mtDNA) and the mitochondrial structure (Eells *et al.* 2019). Damaged mtDNA might produce dysfunctional or damaging proteins, that may lead to more mitochondrial dysfunction. In addition, increased oxidative stress levels are related to more inflammation in RPE cells in dry age-related macular degeneration (Brown *et al.* 2018).

In humans ATP production requires many enzymes and protein complexes, each of which are built from several proteins. Complex 1 for example contains 45 different subunits, which can be divided into three categories. There are 38 subunits encoded by nuclear DNA, and 7 subunits encoded by mtDNA; additionally, 6 proteins aid in assembly or maintaining stability (Sharma *et al.* 2009; Zhu *et al.* 2016). Therefore, given this complexity, a gene therapy based on human ETC complexes would be very difficult to achieve. However, there may be other options available when we consider ‘borrowing’ genes from other life forms. Yeast (*Saccharomyces cerevisiae*) is a single celled fungus that has been extensively utilised for research in the life sciences. Of note, instead of using 45 subunits to form a complex for NADH reduction and CoQ oxidation, it has a single gene encoding for one protein that can reduce NADH, namely NADH Dehydrogenase Internal or NDI1 (Marres *et al.* 1991). Previous studies by the Farrar lab have optimised the NDI1 gene for studies on gene therapies for a variety of IRDs (Chadderton *et al.* 2013; Millington-Ward *et al.* 2022). In the current project, this optimised NDI1 variant will be investigated as a candidate for therapy for Stargardt Disease, a macular degeneration that follows a mendelian inheritance pattern, however, the approach is potentially relevant to other ocular conditions as well.

1.5. Gene therapy

When designing treatments for diseases, one can generalise that two broad options may be available: symptom relief or addressing the underlying cause of the disease. Many therapeutic developments have largely focused on symptom relief for most of human history, however with the information from the human genome project and an emerging understanding of the genetic basis of many disorders, new potent treatment options have appeared on the horizon.

In 1971 a conference titled Prospects for Designed Genetic Change was held by the US National Institute for Health. During this conference, it was estimated that within 5 to 10 years “science

fiction” could become non-fiction, and that “certain inborn errors” could be treated with the administration of the affected gene (Mukherjee, 2016). While it took much longer than 10 years, we have finally reached the point where gene therapies are being used as a treatment in patients.

In 2017, the FDA approved the use of the novel gene therapy Luxturna™ that supplements the RPE65 gene in patients with autosomal recessive IRDs due to RPE65 mutations (FDA, 2017; Russell *et al.* 2017). As explained before, the RPE65 enzyme is involved in the visual cycle and isomerises atROL into 11-cis-ROL. Mutations in this gene disrupt the visual cycle and can therefore cause a progressive IRD phenotype. The Luxturna™ gene therapy, that has the scientific name Voretigene neparvovec-rzyl, comprises an adeno-associated virus (AAV) that has been modified to contain the cDNA of the human RPE65 gene expressed from a chicken beta actin (CBA) promoter (Maguire *et al.* 2008; Maguire *et al.* 2019). The AAV is the vessel/vector to transport the gene into the cells, where cellular mechanisms will transcribe the cDNA into mRNA enabling cells to subsequently produce RPE65 protein. This is a typical example of treating genetic disorders with gene augmentation: a defective gene is supplemented with a functional copy of that gene. Another example of an FDA and EMA approved gene augmentation therapy is onasemnogene abeparvovec (sold under brand name Zolgensma™), which supplements the survival motor neuron 1 (SMN1) gene in children with spinal muscular atrophy (SMA), a progressive neuromuscular disorder that leads to severe physical disability or death (Mendell *et al.* 2017; Groen *et al.* 2018; Hoy. 2019). As of the 15th of May 2023, 7 classic gene therapies have been approved by the EMA and/or FDA, which have been summarised in Table 1.1.

However, not all genetic disorders are caused by recessive lack-of-function mutations. In some cases, dominant negative mutations occur, meaning a mutated gene product impacts the wild type gene product in a negative way. This can happen when the protein forms polymers or is part of a larger protein complex and the mutation still allows the protein to form this complex but renders the complex inactive. Another example is when a dominant mutation causes an encoded protein to become too active or changes the function of the protein (gain-of-function). These types of genetic disorders would require different approaches such as gene silencing or ‘suppression and replacement’. Here, the target gene must be suppressed through provision of an inhibitor, in combination with provision of a wild type gene that has been modified subtly so that it is refractory to suppression.

Table 1.1. Overview of in vivo gene therapy products approved by FDA and/or EMA

Name	Method	Vector type	Disease	Approved?	Source
Luxturna	Supplementation of RPE65	AAV	LCA	EMA + FDA	Maguire <i>et al.</i> 2008; 2019.
Adstiladrin	Supplementation of Interferon α -2b	adenoviral	BCG-unresponsive non-muscle invasive bladder cancer	FDA	Boorjian <i>et al.</i> 2021
Hemgenix	Supplementation with Factor IX	AAV	Factor IX-deficiency haemophilia b	FDA + EMA	Pipe <i>et al.</i> 2023
Imlygic	Supplementation with oncolytic gene	Herpes virus	melanoma	FDA + EMA	Ferrucci <i>et al.</i> 2021
Roctavian	Supplementation of factor VIII	AAV5	Factor VIII-deficiency haemophilia a	FDA + EMA	Blair. 2022 (review)
Upstaza	Supplementation of AADC gene	AAV	aromatic L-amino acid decarboxylase (AADC) deficiency	EMA yes, FDA is accessing	Keam. 2022
Zolgensma	Supplementation of SMN1 gene	AAV	Spinal muscular dystrophy	FDA + EMA	Nishio <i>et al.</i> 2023 (review)
Elevidys	Supplementation with recombinant DMD gene	AAV	Duchenne Muscular Dystrophy	FDA	Zaidman <i>et al.</i> 2023
Vyjuvek	Supplementation with COL7A1 gene	Herpes virus	Epidermolysis Bullosa	FDA, EMA is assessing	Dhillon. 2023 (review)

Recently therapeutic strategies involving mutation-specific techniques have been developed. Indeed, many of these employ antisense technologies which were initially developed decades ago. Due to significant improvements in antisense oligonucleotide (AON) chemistries that have resulted in improved longevity and toxicity profiles for AONs, AON-based therapies have become realistic therapeutic options for many disorders. AONs are small RNA or DNA molecules that can be used to suppress expression of a target gene or can correct specific mutations, such as mutations involved in aberrant pre-mRNA splicing (reviewed in Ferencak *et al.* 2021). For example, AONs can prevent the spliceosome from binding to wrongly induced spliced sites. Aberrant splicing can cause the encoded protein to be truncated, out of frame and/or misfolded due to the deletion of exons or inclusion of introns, leading to dysfunctional proteins.

Other types of gene therapy technologies focus on correcting DNA (or RNA) *in vivo* or *ex vivo*. One such technique is the recent Nobel prize awarded CRISPR/Cas9 technique, which uses bacterial enzymes to precisely introduce double-stranded cuts in DNA in a sequence-specific location and then relies on naturally occurring DNA repair systems to fix the mistake by non-homologous end joining (NHEJ) or correction of the DNA by homology directed repair (HDR) when a donor template is provided (Jinek *et al.* 2012). Recent advances also enable the engineering of single nucleotide variants (SNVs) without the introduction of double stranded

DNA breaks (DSBs) in living cells, with a technique called base editing (Nishida *et al.* 2016). Even more recently prime editing was described; a technique that uses Cas9 endonucleases to introduce SNVs, deletions, and insertions in DNA without DSBs or the need for template DNA (Anzalone *et al.* 2019). Though these newest techniques are predicted to give rise to fewer by-products and are highly efficient, these techniques may still come with off-target DNA editing as a major side effect, making them not safe for use in humans yet. Nevertheless, these safety matters do not concern everyone, as became clear when Chinese scientist He Jiankui used CRISPR/Cas9 to genetically edit embryos to be resistant to HIV before transferring them into their mother's uterus. This led to the births of twins in October 2018 and a third baby in 2019, all of whom He claims to be perfectly healthy (summarised in Marx 2021).

Another technique commonly used is to extract cells, such as T-cells, either from patients or donors (autogenic or allogenic), edit their genome *ex vivo* so they can battle disorders such as cancer better, and to transplant those modified cells into patients (Gowing *et al.* 2017). In fact, the majority of Gene and Cell Therapy products approved by FDA and/or EMA fall into this category. An example here is Breyanzi, a therapy to treat patients with a variety of white blood cell cancers. Patient CD4+ and CD8+ T cells are purified from patient blood and genetically modified to produce CAR (chimeric antigen receptor) which has oncolytic properties. These cells are then transplanted back into the patient, where the CAR protein aids in destruction of cancer cells (Siddiqi *et al.* 2023).

Though precision medicine techniques like base and prime editing seem promising to treat genetic disorders by repairing specific mutations, the human genome project has highlighted that many inherited disorders are highly genetically heterogeneous. Because so many different types of pathogenic genetic mutations exist for many conditions, it is difficult to design gene therapies that would be relevant to and beneficial for many patients. For example, while LCA type 2 can be treated with Luxturna™, Leber Congenital Amaurosis (LCA) in general is a wildly genetically heterogeneous disorder. At the moment of writing, OMIM, the Online Mendelian Inheritance in Man database, recognises 19 types of LCA that are each caused by mutations in a different gene, but show somewhat similar clinical phenotypes (OMIM, 2021 <https://www.omim.org/entry/204000?search=lca&highlight=lca>). Over 260 genes have been found to be involved in IRDs so far (Farrar *et al.* 2017), showing how widely varied this category of disease is. Therefore, for some rare and/or genetically heterogenic disorders, it may be more effective to design a gene therapy that targets disease pathways that are common to multiple forms of the condition instead of targeting individual IRD genes causative of disease. In addition, this strategy may also be valuable as an approach for disorders involving large genes that can be difficult to deliver. Indeed, the latter is also the case for aspects of my project. The *ABCA4* gene

is too big to fit into a single AAV viral vector. Some teams are developing dual vector approaches to deliver the *ABCA4* gene, which thus far have not reached human clinical trial and will be discussed elsewhere. Others are looking at developing overarching strategies that modulate cellular pathways disturbed in Stargardt disease (reviewed by Cremers *et al.* 2020). Indeed, in the current study, I have adopted the latter approach, and propose to improve cell viability by enhancing pathways involved in cellular metabolism that are adversely affected by A2E build-up in Stargardt disease.

1.6. Aims of this PhD project

As briefly mentioned before, the aim of this PhD is to develop a possible gene therapy that may slow down progression of the retinal degeneration related to Stargardt Disease type 1. As the *ABCA4* gene, which is responsible for STGD1, is very large and does not fit into a single AAV vector (the current vector of choice for many ocular gene therapies), this project is focused on targeting common pathways of disease involved in IRDs. Indeed, the emerging observations of significant mitochondrial dysfunction including complex I deficiency in Stargardt disease and other ocular conditions provides a potential therapeutic target for IRDs independent of a specific IRD gene. Furthermore, in principle a single gene yeast homolog for the mitochondrial complex 1 represents a means to augment OXPHOS using a single gene. Therefore, in this study the *NDI1* gene has been cloned into an AAV vector to generate high titre AAV-ophNDI1 and is being evaluated as a means to improve cellular bioenergetics. Various AAV serotypes and several promoters are being explored to investigate the most efficient gene delivery vector providing effective expression of the therapeutic transgene. AAV vectors will be utilised to explore the potential therapeutic benefit provided by this therapy in several *in vitro* and *ex vivo* cell models mimicking aspects of the disease, and also *in vivo* in a mouse model of Stargardt disease. Additionally, this thesis explores the genetic landscape of Stargardt disease in the Irish population, focusing on the most prevalent pathogenic mutations in Irish Stargardt patients and on genes causing pseudo-Stargardt phenotypes.

2. Materials and Methods

2.1. Constructs

The following construct was generated and used for this study:

- pAAV-CBA-ophNDI1

The following constructs that were previously generated by our lab were used for this study:

- pAAV-CBA-eGFP
- pAAV-CMV-eGFP
- pCBA-RP14
- pCHOP-ophNDI1

The sequences and maps of these constructs can be found in appendices I and II

2.2. Bacteria

2.2.1. Strains of *Escherichia coli*

For transformations and plasmid preps the chemically competent XL1-Blue strain of *E. coli* cells was used.

2.2.2. Growth of bacteria

Luria-Bertani (LB) medium (1% NaCl (BioSciences), 1% Tryptone (Sigma), 0.5% yeast extract (Sigma)) was used to grow XL1-Blue *E. coli* cells in suspension. To grow colonies, 1.5% of agar (Sigma) was added to the LB medium to make LB-agar plates. Both LB medium and LB agar were sterilised in an autoclave at 121°C for 20 minutes. Antibiotics were added to LB medium and LB agar to ensure careful selection of only bacteria containing the desired construct. Table 1 shows the concentrations of each antibiotic used in this study.

Table 2.1. Antibiotic concentrations used in bacterial work

Antibiotic	Stock Concentration (in NFW)	Working concentration (in LB or LB/agar)
Ampicillin (Sigma)	20 mg/mL	50 µg/mL
Kanamycin (Sigma)	20 mg/mL	50 µg/mL
Tetracycline (Sigma)	10 mg/mL	25 µg/mL

2.2.3. Multiplication of competent cells

To generate more competent XL1-Blue *E. coli* cells, stocks cells were streaked out on an agar plate with tetracycline and grown overnight at 37°C. A colony was picked from this plate and cultured overnight in 2 mL LB medium with tetracycline and incubated overnight at 37°C under constant agitation. This suspension was transferred to 500 mL of LB with tetracycline and grown until it reached late exponential phase (OD_{600 nm} = 0.6-0.7), after which it was centrifuged at 6000rpm at 4°C for 15 minutes. The pellet was washed with 500 mL of 100 mM MgCl₂ and

centrifuged again at 6000rpm at 4°C for 15 minutes. Cells were then resuspended and washed with 250 mL of 50 mM CaCl₂ (Thermo Scientific) and incubated on ice for 30 minutes, before being centrifuged again at 6000rpm for 15 minutes. The resulting pellet was resuspended in 25 mL of 50 mM CaCl₂ + 5 mL of sterile glycerol. This suspension was divided into 1 mL aliquots and each aliquot was snap-frozen in liquid nitrogen before being stored at -80°C.

2.2.4. Transformation

Approximately 10 to 20 ng of plasmid DNA was added to 200 µL of competent XL1-Blue E. coli cells. In negative controls, nuclease free water (NFW) was used instead of plasmid DNA. This mix was incubated on ice for 30 minutes to ensure good distribution of the plasmids and then heat shocked for 45 seconds at 42°C. After a 2-minute rest on ice, 1 mL of LB medium is added to the cells. After incubation for 1 hour at 37°C under constant agitation (at 100rpm), 200 µL of this mixture is plated on a LB agar plate with selecting antibiotics. All these actions were performed under sterile conditions (Bunsen burner). The cells are then grown overnight at 37°C.

2.3. Plasmid preps

2.3.1. Miniprep

The GeneJET Plasmid Miniprep Kit (Thermo Scientific; Cat. No. 10242490) was used to generate small plasmid preps, resulting in up to 20 µg of a desired, purified plasmid. The plasmid was transformed according to section 2.2.4. Single colonies were picked from each plate and cultured overnight in LB media with a selecting antibiotic at 37°C under constant agitation at 220rpm. The culture was centrifuged for 5 minutes at 12000rpm to harvest the cells. The plasmids were then purified from the pelleted cells according to the manual of this kit. The plasmid DNA was eluted in 50 µL of nuclease free water.

2.3.2. Megaprep

Plasmid preps for larger DNA yields up to 2 mg were carried out with the NucleoBond PC 2000 EF, Mega kit (Macherey-Nagel; cat. no. 740549). A desired plasmid was transformed according to section 2.2.4. A colony was picked and grown in 10 mL of LB with a selecting antibiotic for 8 hours at 37°C at 220rpm. This suspension was then transferred to a volume of 500 mL of LB with the same selecting antibiotic in a 2 L Erlenmeyer flask and grown for 16-20 hours at 37°C at 220rpm. This volume was then centrifuged for 15 minutes at 4°C at 6000g. Next, DNA was isolated from the cell pellet according to the instruction manual provided with the kit. The purified plasmids were eluted in a total of 3 mL of nuclease free water.

2.4. DNA manipulation

2.4.1. Restriction digests

Restriction digests of plasmids or other DNA fragments were performed with restriction enzymes from New England Biolabs were used. A digestion mix was made consisting of 5 µg of DNA, 2 µL

of restriction enzyme, 2 μ L of the corresponding 10x NEB buffer and the mix was topped up with NFW to a total volume of 20 μ L. In case larger quantities of DNA were digested, the reaction volume was adapted accordingly. The reaction mix was incubated at 37°C for 90 minutes. A small volume of each reaction was run out on a 1% agarose gel with Ethidium Bromide (EtBr) to verify if the digestion was effective.

2.4.2. Blunting

A 5' overhang after restriction digestion could be blunted with the Klenow enzyme (New England Biolabs). A reaction mixture was made with 2 μ g of DNA, 1 μ L of Klenow enzyme, and 2 μ L of 1mM dNTP mix (Sigma) in a total volume of 50 μ L NFW. The reaction was then incubated for 30 minutes at 37°C before being heat inactivated at 75°C for 10 minutes.

2.4.3. Ligation

Ligations of two different DNA products were carried out at different insert:vector ratios depending on the size of both fragments. The bigger the insert, the lower the ratio and vice versa. A ligation mix was made up in a total volume of 15 μ L of NFW, with 1 μ L T4 Ligase (Roche), 1.5 μ L 10X T4 Ligase buffer, 50 ng of vector DNA and varying quantities of insert DNA. The reaction is carried out overnight at 18°C and then used for transformation. A negative control with only the vector and no insert was included. Multiple colonies from transformation were used for miniprep plasmid extraction as per section 2.3.1. Restriction digests were used to analyse if the ligation was successful.

2.4.4. Primer design

Primers were designed according to the following rules: GC content between 40-60%, preferably ending and starting with G or C, length between 18-24 nucleotides, melting temperature between 55 and 67 °C with a maximal difference of 2°C between matching forward and reverse primers, but preferably lower. All primers were manufactured by Sigma Aldrich. Primer stock solutions (100 μ M) were made up with NFW and kept at -20°C; working solutions were made up from stock solutions through a 1 in 10 dilution to achieve a concentration of 10 μ M. A list containing all sequencing primers and their sequences can be found in Appendix III

2.4.5. DNA purification from liquid

DNA products from digestions or PCRs or other similar techniques usually had to be purified before they could be used in consecutive steps. Purification was carried out with the GeneJET PCR Purification Kit (ThermoScientific; Cat. No. 10578360) according to the kit manual.

2.4.6. DNA purification from gel

In order to isolate specific products from a restriction digest, the DNA was separated on an agarose gel with EtBr. Products of interest were excised carefully with a clean scalpel on a UV-

based transilluminator. The DNA was then purified from the agarose through use of the GeneJET Gel Purification Kit (Thermo Scientific; Cat. No. K0691). The manufacturer's instructions were used to carry out the isolation.

2.4.7. DNA isolation from tissue

For the isolation of mice DNA ear clips or retinal tissue was used. The tissue was snap frozen in liquid nitrogen right upon collection and then stored at -20°C until further use. The DNeasy Blood and Tissue kit (Qiagen) was used to extract and purify DNA from the samples according to the instruction manual that came with the kit. DNA was eluted in NFW and stored at -20°C .

2.4.8. DNA isolation from patient blood

Blood from IRD patients and their family members is stored at -20°C in tubes labelled with patient-specific barcodes. At any point while handling human blood a dedicated lab coat was worn. Two pairs of gloves were worn so that in case of any type of spillage, one could be taken off easily and replaced. DNA was isolated in a laminar flow cabinet that was thoroughly cleaned with ethanol, disinfected with Virkon in ethanol, and cleaned again with ethanol before and after use to prevent cross-contamination of samples and to keep researchers safe from blood-borne diseases. Additionally, all individuals handling blood are vaccinated against hepatitis A and B.

DNA was isolated from $200\mu\text{L}$ of blood with either the DNeasy Blood and Tissue kit (Qiagen) or the Nucleospin Blood DNA extraction kit (Machery-Nagel) or the GeneJET Whole Blood Genomic DNA Purification kit (ThermoFisher) according to the protocol provided in each respective kit. DNA was eluted in NFW and stored at -20°C until further use. Aliquots were always taken by a senior member of the team before subsequent experiments were done to prevent contamination of the original sample.

2.4.9. RNA isolation from tissue

RNA was purified from retinal tissue taken from euthanised mice. As the tissue was taken it was placed in a 1.5 mL eppendorf tube and snap frozen in liquid nitrogen for storage at -20°C . If RNA was to be extracted straight away, the tissue would be placed in 1 mL of buffer RLT supplemented with $10\mu\text{L}$ of beta-mercaptoethanol (BME). Buffer RLT is part of the RNeasy mini kit (Qiagen) that was used for RNA isolation from tissue. The manufacturer's protocol was used to extract the RNA and RNA was eluted in NFW.

2.4.10. RNA isolation from cells

Cells were grown in 96-well plates as described in section 2.6. Cells were removed from the 96-well plate with trypsin (section 2.6.2) and then collected through centrifugation. The pellet was resuspended in ($100\mu\text{L}$) buffer RLT (Qiagen) + 10% BME and RNA was further isolated with the RNeasy mini kit (Qiagen) according to manufacturer's protocol. DNA was removed through a 3-hour digestion with DNase (Qiagen). RNA was eluted in NFW.

2.4.11. Polymerase Chain Reaction

Specific regions of DNA would be amplified with polymerase chain reactions. The enzyme used was either Q5 High-Fidelity (2X), NEB or Firepol® (5X), Solis Biodyne and working solutions were made according to manufacturer's protocol. Either 35 or 40 thermal cycles were employed to amplify DNA; 35 for Q5, and 40 for Firepol, respectively.

Table 2.2 PCR reaction mixes

	Q5	Firepol
Mastermix	12.5 µL	4 µL
Primermix (F+R, 10µM)	2.5 µL	1 µL
H₂O	8 µL	13 µL
DNA	2µL (corresponding to approx. 50-100 ng)	2µL (corresponding to approx. 50-100 ng)
Cycles	35	40

2.5. Sanger sequencing

2.5.1. Having constructs sequenced

Sanger sequencing of constructs and DNA fragments was performed by the TubeSeq Service of Eurofins Genomics (Ebersberg, Germany). The samples and primers were supplied in 1.5 mL Eppendorf tubes. To sequence entire plasmids, 1 µg of DNA in 20 µL was submitted for every 4 reactions. To sequence specific PCR amplified DNA products, 100 µg of DNA in 20 µL was supplied. Primers were designed as described in section 2.4.4 and for every 4 reactions, 20 µL of 10µM primer was supplied.

2.5.2. Interpreting sequences

The sequence files returned to us from Eurofins were then compared to construct sequences with Benchling software (2021). Sequences of mouse DNA were compared to mouse genome GRCm38 (mm10, Mus musculus). Sequences of human DNA were compared to the human reference genome GRCh38 (hg38, homo sapiens).

2.6. Cell culture

2.6.1. Cell lines and their media

Multiple retinal cell lines were used in this study. Immortalised cell lines hTERT-RPE1 (ATCC® CRL-4000™) and ARPE-19 (ATCC® CRL-2302) were both maintained in DMEM:F12 media (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Merck) and 1% sodium pyruvate (Biosciences) in T75 flasks (Sarstedt). Primary porcine RPE cells were expanded in DMEM (Gibco) supplemented with 20% FBS (Merck) and 0.1% gentamycin (BioSciences) in 5 cm Petri dishes (Sarstedt). All cells were grown in an incubator at 37°C with 5% CO₂. Sterilised water was kept at the bottom of the incubator to keep humidity levels high.

2.6.2. Passaging cells

Cells would be passaged at 90-100% confluency. Cells were washed with PBS (Gibco) and detached with 0.25% Trypsin-EDTA (BioSciences). The trypsin was inactivated by adding 4 volumes of media with FBS and mixed and diluted into clean T75 flasks (Sarstedt) or petri dishes (Sarstedt).

2.6.3. Freezing cells for storage

Cells were prepared for long term storage as follows: Cells would be washed and detached as in section 2.6.2. The trypsin would be inactivated by 4 volumes of media containing FBS. The cell suspension was centrifuged in a 15 mL centrifuge tube for 5 minutes at 300g. The supernatant was discarded, and the pellet resuspended in a mixture of 90% FBS and 10% DMSO (Sigma-Aldrich), the volume of which depended on specific cell lines. This mixture was then divided into 1 mL aliquots in 2 mL cryovials (Nalgene). The tubes were frozen in a Mr Frosty freezing container (Nalgene), which enables a 1°C per minute decline in temperature to minimise crystallisation of FBS and DMSO to reduce cell death. Once the cell suspension had frozen solidly, it would be transferred to water-resistant cardboard boxes to be stored in a -80°C freezer long term.

2.6.4. Thawing cells for use

Cryovials with frozen cells were defrosted in a 37°C water bath. Once thawed, the cells were slowly decanted into 5 mL of pre-warmed media in a 15 mL centrifuge tube. This tube was then centrifuged for 5 minutes at 300 rpm. The supernatant was discarded, and the pellet resuspended in 10 mL of prewarmed media, before being plated on a clean T75 flask. The cells were then grown at 37°C.

2.6.5. Counting cells

In order to seed cells at specified densities, they must be quantified first. After cells were washed, trypsinised and neutralised as described in section 2.6.2, two aliquots of 20 µL of the cell suspension were taken and put in two separate Eppendorf tubes. To each tube, 20 µL Trypan Blue (BioSciences) was added and mixed in, after which 20 µL was pipetted onto a haemocytometer cell counting slide. The living cells in the four outer corners of the cell counter were counted manually and the average was taken from these four. This number was doubled to account for Trypan blue dilution and multiplied by 1×10^4 to find the number of cells per mL of cell suspension.

2.6.6. Primary post-mortem porcine cells

RPE cells were isolated from pig eyes since pig eyes are very similar to human eyes. The eyes were directly taken from post-mortem pigs at an abattoir, and placed into CO₂-independent medium (Biosciences, cat. no. 18045054) and kept on ice during transport. In a 20cm petri dish the eyes were then cleaned, i.e. all muscle and fatty tissues were carefully removed from the eye

with curved scissors (SLS), after which the eyes were washed in Pursept disinfectant (Thermofisher, cat. no. 11814110) twice for two minutes, then in CO₂-independent medium for two minutes and last in serum-free DMEM (Gibco).

After the washing steps, the eyes were dissected. With a 16G needle (BD®), a small hole was poked in the eyeball at the ora serrata. With a pair of curved, sharp scissors (SLS) placed in that hole the cornea and lens were cut from the eye. At this point the inside of the eye is visible, and only fully pigmented eyes were used for subsequent steps. The vitreous body and retina were removed by carefully peeling them away from the inner eye to avoid scratching the RPE and the eye cup was filled with PBS to prevent any cells from drying out. The remaining pieces of the retina around the optic nerve were then removed with forceps and sharp scissors, after which the eye cup was washed three times with PBS. Approximately 2mL of 0.25% Trypsin-EDTA (Biosciences, cat. no. 25200056) was added to each eye cup and the eyes were incubated for 95 minutes at 37°C to detach the RPE cells from the inner eye cup. After this digestion, the RPE cells were loosened with a Pasteur pipette by pipetting the trypsin up and down 10-20 times, after which cell suspension was added to a 15mL centrifuge tube (Sarstedt), along with 4 mL of DMEM + 20% FBS and centrifuged for 5 minutes at 1000rpm (56g). The supernatant was removed and the cells were resuspended in 5mL DMEM + 20% FBS + 0.1% gentamycin and transferred to a 5cm Petri. Cells were incubated at 37°C overnight and on day 2 and day 4, the cells were washed roughly with PBS before the media was changed. On the fifth day, the cells were ready to be used in further experiments.

2.6.7. Transduction

Transductions of cells with viral vectors took place under low serum conditions. Media was removed from the cells and replaced with only half the volume of media containing only 2% of FBS. For XFe96 Seahorse tissue culture plates this volume was 50 µL and for 8-well chamber slides (Miltenyi Biotec) and 48-well tissue culture plates (Sarstedt) this volume was 150 µL. Subsequently, virus was added to the media and the cells were left to incubate at 37°C for 4 hours. After this incubation step, the same volume of media containing a double FBS concentration was added. For ARPE19 and hTERT cells, this means 20% FBS in DMEM-F12 (Gibco) and for primary pig RPE cells it's 40% FBS and 0.2% gentamycin in DMEM (Gibco).

2.6.8. Sodium Iodate treatments

A fresh stock solution of 100 mM NaIO₃ was prepared by dissolving 99 mg of powdered NaIO₃ in 5 mL of media (DMEM or DMEM-F12, respectively). The stock solution was filtered with a sterile syringe and a 0.22 µm filter (MilleX) before being diluted in media to form a working solution of 5mM. To treat cells with NaIO₃ the original media was replaced with the working solution.

2.6.9. A2E treatments

All work with A2E was done in darkness with only a small amount of red-filtered light as A2E is sensitive to white and blue light. Stock solutions of 10mM A2E were stored at -80°C. Working solutions of 30-50µM A2E were made up in serum free media, respective of the cell type it was used on, and therefore control cells also received serum free media. After A2E was applied to the cells, they were placed in blue light (430nm) of ~1 mW/cm² (80-90 Lux) at 37°C. Depending on downstream assay, the timing for blue light exposure varied from 30 minutes to 3 hours.

2.6.10. Bradford assays for normalisation

All media was removed from the plate and replaced with 50 µL of lysis buffer (0.1% Triton X-100 (Sigma) in PBS). The plate was placed on a shaker at room temperature for 10 minutes to lyse the cells. A dose curve was prepared with Albumin Standard (Thermo Scientific, cat. no. 23209) in lysis buffer, with protein contents ranging from 25 µg/mL to 2000 µg/mL. Ten microliters from each dose were added to 40 µL of lysis buffer in a new 96-well plate (Sarstedt). Background measurements would be taken through the incorporations of a series of blank wells, containing only the Bradford reagent and lysis buffer. 150 µL of Bradford reagent (Thermo Scientific) was added to each well. Then, 100 µL of Bradford reagent would be mixed with the lysed cells on the old plate, before the entire volume would be transferred into corresponding wells on the new plate. The ladders and blanks also received another 100 µL of reagent. Small air bubbles on the surface of the liquid were removed with a spray of ethanol before the absorption in each well was measured with a Fluostar Microplate Reader (Optima) at 595nm. A graph of the dose curve and corresponding equation were generated using Excel software. This equation was then used to calculate protein levels in each sample so the Mito stress test could be normalised.

2.7. Virus generation

2.7.1. Triple Transfection

Triple transfection methodology was used by Dr. Naomi Chadderton to generate high titre AAVs in house. pHelper, pAAVserotype, and pCAG-ophNDI1 were propagated using Megaprep (as described in section 2.3.2.). These plasmids were then transfected at a ratio of 2:1:1 into HEK293 cells (CRL-1573; ATCC) growing on 50 150mm plates of using polyethylenimine (PEI) in DMEM. The media was refreshed after 4 hours and the plates were maintained at 37 °C for 48 hours. The newly generated AAVs were purified from the cells with thiophosgene (CSCI₂) gradients. Multiple centrifugation steps were followed by the selection of the samples whose light fraction was most likely to contain higher AAV quantities. These fractions were then dialysed in PBS to wash away all residual chemical traces, and then harvested, before being stored at -80°C. A sterility test was carried out on all fractions by depositing a small volume of each sample into a sterile 20mL white cap tube containing 5 mL of LB media (generated as in section 2.2.2) and leaving it for incubation at 37°C, under constant agitation for several days.

2.7.2. Titration

Once the AAV was purified the genomic titre, or the viral genomes per mL, was determined. This was done with a real-time reverse transcriptase PCR with SYBR green reagents (Qiagen) as further described in section 2.8.2. A dose curve was made with the pCAG-ophNDI1 that was also used to generate the viruses. The AAV was digested with DNase (Qiagen) and Proteinase K (Qiagen) to eliminate all DNA and protein contaminations according to manufacturer's protocols. The following reaction mixture was made for quantification: 10µL of SYBR Green reagent, 0.5 µL of Forward Primer (10 µM), 0.5 µL of Reverse Primer (10 µM), 4.5 µL of diluted AAV sample, 0.15 µL of RT enzyme mix (Qiagen cat. no. 204243) and 4.35 µL of NFW. The PCR itself started with a 15-minute boiling step at 95°C and was then followed by 40 cycles of 10 seconds at 95°C and 60 seconds at 60°C. StepOne Software (v2.3) was used to calculate the titres of each AAV fraction based on the dose curve of the corresponding plasmid.

2.8. Verification of Expression

2.8.1. Fluorescence microscopy of cells

The transduction efficiency of different AAV serotypes and the expression levels of different promoters in all cell lines used in this study were investigated with AAVs expressing EGFP. Cells were seeded in 8-well chamber slides (Miltenyi Biotec) with densities adapted to each cell lines size as can be found in table 2.3.

Table 2.3. Cell densities for seeding 8 well imaging slide per cell type

Cell type	Seeding density (cells/well)
hTERT-RPE1	$2.5 * 10^3$
ARPE-19	$5 * 10^4$
Primary porcine RPE	$1.375 * 10^4$

After overnight incubation at 37°C the cells were transduced as described in section 2.6.7 with various AAV-EGFP serotypes and vectors. After 48 hours the wells were washed carefully with PBS and then fixed for 20 minutes with 4% paraformaldehyde in PBS. The wells were washed 3 more times with PBS before a 1 in 10000 dilution of HOECHST in PBS was applied to stain the cell nuclei. After 15 minutes the wells were again washed 3 times with PBS. The walls of the chamber slides were taken off the imaging slide and a few drops of OCT mounting media (VWR) was applied. A coverslip was carefully placed on top of the slide and the slides were then placed at 4 °C overnight to dry.

The slides were imaged with an Olympus IX83 microscope. The HOECHST stain was measured at an emission wavelength of 455 nm and the EGFP was registered at an emission wavelength of 508 nm.

2.8.2. RT-PCR

With real-time reverse transcriptase PCR the presence of specific strands of RNA in a sample could be quantified. Samples were diluted 1 in 50 in NFW before being used and each sample was quantified in trifold. A master mix consisting of 10 μ L SYBR Green (Qiagen), 0.15 μ L RT enzyme mix (Qiagen), 0.5 μ L 10 μ M Forward Primer, 0.5 μ L 10 μ M Reverse Primer and 4.35 μ L NFW per reaction was prepared. 15.5 μ L of this mix was put into the wells of a MicroAmp® Fast 96-Well Reaction Plate (applied biosystems®, cat. no. 4346907). A dose curve was made with plasmid DNA, also in trifold. 4.5 μ L of either diluted sample or dose curve was added to each well. First, the samples were denatured for 15 minutes at 95°C and cooled down to 60°C for 1 minute. After that, 40 cycles consisting of 15 seconds at 95°C and 1 minute at 60°C followed.

2.8.3. Mitochondrial Stress Test

To examine the effects of the AAV-CAG-ophNDI1 on the energy metabolism of cells, mitochondrial stress tests were performed on the ARPE-19 cell line and primary pig RPE cells. The mitochondrial stress test is carried out by the Seahorse XF96 Analyzer (Agilent) and it measures oxygen consumption rate (OCR) of live cells after the addition of several chemicals that influence mitochondria.

In a XF96 cell culture plate, either $1.5 \cdot 10^4$ ARPE-19 cells or $1.375 \cdot 10^4$ primary pig RPE cells were seeded in 100 μ L of media. The four corner wells (A1, A12, H1 and H12) were only filled with media. After overnight incubation at 37°C the cells would be almost fully confluent. They were then transduced with AAVs in varying multiplicities of infection (MOI) as described in section 2.6.7. After 48 hours at 37°C the cells were treated with NaIO₃ as per section 2.6.8. The sensor cartridge was hydrated with 200 μ L XF Calibrant (Agilent) per well and incubated overnight at 37°C in a low CO₂ environment. The following morning the cells were washed with clear XF DMEM medium (Agilent) supplemented with 10 mM D-glucose (Sigma), 1 mM Sodium Pyruvate (Gibco) and 2 mM L-Glutamine and then left to incubate in 180 μ L of the aforementioned media at 37°C under low CO₂ conditions. The supplemented XF DMEM medium was also used to dilute chemicals that induce mitochondrial changes. Table 2.4 gives an overview of the concentrations of each chemical that was used in this assay.

Table 2.4. Chemicals and their concentrations used in mitochondrial stress test.

Compound	Port	Volume of chemical stock (µl)	Seahorse medium Vol (µl)	Stock conc. (in EtOH)	Added chem conc.	Final well conc.	Vol added to port (µl)
Oligomycin	A	30	2970	1 mM	10 mM	1.0 mM	20
FCCP	B	6.6	3993.4	10 mM	13.75 mM	1.25 mM	20
Rotenone	C	24	3976	1 mM	6 mM	0.5 mM	20
Antimycin A	D	2.6	7197.4	10 mg/ml	3.61 mg/ml	0.28 mg/ml	20

The sensor cartridge has four ports, and a different chemical is added to each port. Before the cartridge was placed into the machine, it was moved up and down a few times to get rid of any air bubbles that may have formed overnight. The mitochondrial stress test was then set up to take 5 baseline measurements and to then take 5 measurements after injection with each chemical.

When the assay was finished, the media was carefully taken of the cells and the plate was either frozen at -20 °C or used directly for a Bradford assay (section 2.6.9) to normalise the data against the protein content in each well. After normalisation, the data set was analysed using Agilent Wave software (v2.6.1.53) and any wells in which chemical injections failed were removed.

2.9. In vivo studies

All research that includes animals has been performed in accordance with Irish and EU legislations. The Protection of Animals used for Scientific Purposes Regulations (S.I. no. 543 of 2012) were abided carefully. Mice were kept in the animal facility within the Smurfit Institute of Genetics. The mice had access to water and food ad libitum and were housed under 12-hour light-dark cycles.

In this study the Abca4^{-/-} mouse model from the Jackson Laboratory (Jax #023725) has been used. Six female and three male mice were purchased from this lab and used for breeding to establish a colony in our facility. These mice have a 129s background, so for our control group wild type 129s mice were used.

2.9.1. Subretinal Injections

Subretinal injections were performed by Dr Paul Kenna to administer AAV.CAG.NDI1 to RPE and photoreceptor cells in 1- and 2-month-old mice. Via intraperitoneal injections with ketamine (1µg/10g body weight) and xylazine (0.1 µg/10g body weight) mice were anaesthetised. Mouse pupils were dilated with 1% tropicamide and 2.5% phenylephrine. Local analgesia was applied before the sclera was punctured and 1 µL of PBS containing viral genomes was administered to

the subretinal space with a 10 µL Hamilton syringe and a 34-gauge blunt-ended microneedle. With an intraperitoneal injection of atipamezole hydrochloride (100µg/10g body weight) the anaesthesia was reversed. The body temperature of the mice was maintained with a homeothermic heating device during the procedure and recovery. Mice body weight and general health and welfare were monitored at 4 hours, 24 hours, 48 hours, and 1 week post-procedure.

2.9.2. Electroretinograms (ERG)

ERGs measure the electric activity in retinal cells, which represents functionality of these cells. Overnight, the mice were dark adapted and thereafter prepared for the ERG under weak red light conditions. ERGs were generally carried out by Dr Sophia Millington-Ward. Via intraperitoneal injections with ketamine (1µg/10g body weight) and xylazine (0.1 µg/10g body weight) mice were anaesthetised. Mouse pupils were dilated with 1% tropicamide and 2.5% phenylephrine. A Ganzfeld bowl was used to introduce standardised flashes of light to the mice. Contact lens electrodes were used to record the ERG responses. Reference and ground electrodes were placed subcutaneously, approximately 1 mm from the temporal canthus and anterior to the tail, respectively. Rod-isolated responses were recorded using a dim white flash with a maximal intensity of 25 dB of light, where maximal flash intensity was 3 candelas/m²/s) presented in the dark-adapted state. Then, the maximal response that combines the rod and cone responses with the maximal intensity flash was recorded. The mice were then light adapted for 10 minutes to a background illumination of 30 candelas/m², after which cone-specific responses were recorded to the maximal intensity flash. This intensity was initially presented as a single flash and subsequently as 10-Hz flickers. For each response a-waves were measured from the baseline to the trough and b-waves from the baseline to the top.

2.9.3. Histology

Mice were euthanised via CO₂-inhalation and subsequent cervical dislocation. The eyes were enucleated using McPherson forceps, and then fixed in 4% paraformaldehyde (VWR) in PBS for 4 hours or overnight at 4°C. The lens and vitreous were removed from the eyes, before being washed in PBS, and cryoprotected consecutively in 10, 20 and 30% sucrose (Sigma-Aldrich) in PBS solutions. The cryoprotected eyes were snap-frozen in a 1:1 blend of 30% sucrose and OCT medium (VWR) using liquid nitrogen. The Leica CM1900-1-1 cryostat was used to section the frozen eyecups at a thickness of 12µm per section. Sections were mounted on polysine slides (Epredia™) and stored at -20°C until further use.

2.9.4. Microscopy

Fluorescent microscopy images were taken with an Olympus IX83 inverted microscope.

2.9.5. Cone counts

Retinal sections (prepared as described in section 2.9.3.) were stained with Cy3 and DAPI as described by Palfi *et al.* (2022). Images of 4 sections per eye were taken with an Olympus IX83 (Mason Technology, Dublin, Ireland) inverted motorized microscope. For each image, two sections were analysed on each side of the optic nerve, respectively. Photopea online photo editor software was used to mark each individual cone on a separate layer, which was transported as a jpeg and analysed in ImageJ. Then, the length of the measured area was calculated in pixels with ImageJ to normalise the cone number. Averages were taken for each section and the mean of all sections per eye was used for statistical analysis (paired and unpaired t-tests).

2.9.6. Quantification of lipofuscin-based autofluorescence

Lipofuscin aggregation was quantified by measuring the intensity of its native autofluorescence in sections of *Abca4*^{-/-} mouse whose eyes were injected with PBS or 2/8AAV.CAG.NDI1. Retinal sections were prepared as described in section 2.9.3. and stained with DAPI. Per eye, 4 sections were imaged with an Olympus IX83 (Mason Technology, Dublin, Ireland) inverted motorized microscope. The DAPI stain was visualised at an emission wavelength of 455 nm and the autofluorescence was registered at an emission wavelength of 508 nm.

For autofluorescence quantification only the 508nm wavelength was used. The display histogram was fixed to 1000-15000 on all images before being saved as a jpg document. Each of these images was then cut into four sections, which were then uploaded to imageJ. On each section the raw intensity was measured in a 20 by 200 pixel selection that was placed directly over the RPE layer.

2.9.7. Quantification of Reactive Oxygen Species in mouse retina

Wild type and *Abca4*^{-/-} mice were euthanised via CO₂-inhalation and the retinas were dissociated by making an incision in the front of the eye and using McPherson forceps to pull them out. The retinas were dissolved in 900µl HBSS (Gibco, 14175) + 100µl Trypsin (Merck, T1005) for 20 minutes at 37°C. After 5 minutes (out of these 20), 10µl DNase (Sigma D5025) was added and the retinas were pipetted gently up and down with a 1 ml pipette. At the end of the incubation period 100µl of trypsin inhibitor (SDS-019) was added and cells were centrifuged at 600g (2500rpm) for 5 minutes. Cell pellets were resuspended in 250 µl HBSS with 2.5 µl DNase and 2 µl CellRox™ Green Reagent (Invitrogen by Thermo Fisher Scientific, Eugene, Oregon). Retinal cells were incubated in the dark at 37°C for 2.5 hours. Propidium Iodate was administered to each sample shortly before analysis to aid in the distinguishment between live and dead cells. Median levels of CellRox™ in cell samples, representing relative ROS levels, were recorded. The gating strategy was based on Chandler *et al.* 2021 and is presented below.

2.9.8. Quantification of Reactive Oxygen Species in cell models

The CellRox™ assay was also utilised on cellular models, in which case cells were seeded into 48-well plates at a density of 5×10^4 (ARPE19 and pRPE) in their respective media. The cells were transduced the next day as described in section 2.6.7. After 48 hours the cells were insulted with A2E and subjected to blue light (1 mW/cm^2 (80-90 Lux) at 430nm) for 30 minutes. Twenty hours later the cells washed with PBS and then trypsinised with 200µL of trypsin (Biosciences) until cells were detached from the well surface, and the reaction was stopped by adding 800 µL of media. Media is then removed via centrifugation (5 minutes at 600g) and the pellet was resuspended in HBSS for a wash step and centrifuged again. Cell pellets were resuspended in 250 µl HBSS with 2.5 µl DNase and 2 µl CellRox™ Deep Red Reagent (Invitrogen by Thermo Fisher Scientific, Eugene, Oregon) and incubated for 1.5 hours at 37°C.

2.10. Informed Consent

As the studies described in chapter 5 of this PhD thesis are based on genomic and clinical data of patients, informed consent from patients was requested prior to their participation in these studies. Ethical approval was awarded to the hospitals (Royal Victoria Eye and Ear Hospital (Dublin, Ireland); Mater Misericordiae University Hospital (Dublin, Ireland); Mater Private Hospital (Dublin, Ireland); The Belfast Health and Social Care Trust Hospitals (Belfast, Northern Ireland)) and to the research labs (Trinity College Dublin (Dublin, Ireland); Radboud University Medical Center (Nijmegen, The Netherlands) that played a role in this study.

2.11. Statistics

Data sets were pooled per construct in cells and in mice and also per genotype in mice. Statistical analyses were performed with GraphPad Prism software (version 9.0.2. for Windows). When comparing two eyes of one mouse, for example when the left eye served as a control and received a PBS injection, a paired t-test was used. Findings were considered to be significant only if p-values were below 0.05.

3. An exploration of the therapeutic potential of AAV.NDI1 on cell models of disease

3.4. Introduction

Many gene therapies are based on the supplementation of a gene that is faulty in a patient and therefore causes disease. This means that these therapies are very specific and can only be used in patients with mutations in that specific gene. Gene supplementation strategy would therefore perhaps not be optimal for very rare diseases, conditions with great genetic heterogeneity, or multifactorial diseases, as generating the therapy and performing clinical trials would be extremely costly and time-consuming. A potential solution for this is the generation of non-specific gene therapies (therapies independent of the causative gene) that target more general aspects of retinal conditions such as, for example, apoptosis, oxidative stress and/or mitochondrial dysfunction.

One of the main obstacles in gene therapy design is delivery *in vivo*. Common strategies involve the use of viral or non-viral vectors. Viral vectors include viruses such as retroviruses, adenoviruses, adeno-associated viruses (AAV) or lentiviruses (Merentie *et al.* 2016; reviewed by Papaioannou *et al.* 2023). Viral vectors can transduce tissues with high efficiency, but depending on the vector used, can cause undesirable immunological responses. Additionally, as RPE and photoreceptors are post-mitotic cells and therefore do not divide, it is vital that the viral vector of choice is able to infect cells regardless of its status in the cell cycle. Lentiviruses, for example, which are derived from the (human) immunodeficiency virus, have a larger cargo capacity than AAV, namely 8kb, and a low immunogenic response. They can even transduce non-dividing cells, but they, like others in the retrovirus genus they are part of, routinely integrate into the host genome, which can give unpredictable and potentially oncogenic results (reviewed by Poletti and Mavilio, 2021). Adenovirus is another popular gene therapy vector that can carry large transgenes of up to 37kb and don't integrate into the host genome. However, because various adenoviruses also cause a broad spectrum of illnesses, such as common colds, that patients potentially have been exposed to in the past, the immunological response to adenoviral vectors can be problematic (reviewed by Lee *et al.* 2017). Indeed, the immune reaction to adenovirus was employed as a vaccine strategy against SARS-CoV-2; a transgene encoding for the covid spike protein was cloned into adenoviral vectors in order to reinforce the immune reaction (Bos *et al.* 2020; Folegatti *et al.* 2020; Ikegame *et al.* 2021). In this study AAVs were chosen for delivery of the NDI1 gene to cells and tissues.

3.4.1. Adeno-Associated Viruses

Adeno-associated viruses are small, non-enveloped parvoviruses that, though they are often found in company of pathogenic viruses, do not cause disease themselves (Atchison *et al.* 1965; Carter. 2004; Basner-Tschakarjan *et al.* 2014). Aside from not being pathogenic, AAVs yield a low immune response in humans (Chimule *et al.* 1999; Basner-Tschakarjan *et al.* 2014; Ziccardi *et al.* 2019) and are able to transduce non-dividing cells (Flotte *et al.* 1994; Podsakoff *et al.* 1994), which make them ideal candidate vectors for gene therapy. Indeed, AAVs have already been used in many clinical trials and FDA- and EMA-approved gene therapies (Russell *et al.* 2017; Maguire *et al.* 2019; Mendell *et al.* 2017).

AAVs consist of single stranded DNA (ssDNA) that is packaged in a capsid. The capsid proteins contain variable surface receptors, which allow the virus to infect different cell types. At least twelve different natural serotypes have so far been discovered (reviewed in Kotterman *et al.* 2014), each with their own tropism. Combinations of two serotypes, containing the replication genes of one serotype and the capsid of another, are widely used for the optimisation of gene therapies. In the past, recombinant vectors have been engineered with techniques such as PCR, domain-swapping, and molecular cloning. This is called pseudotyping and the combination of

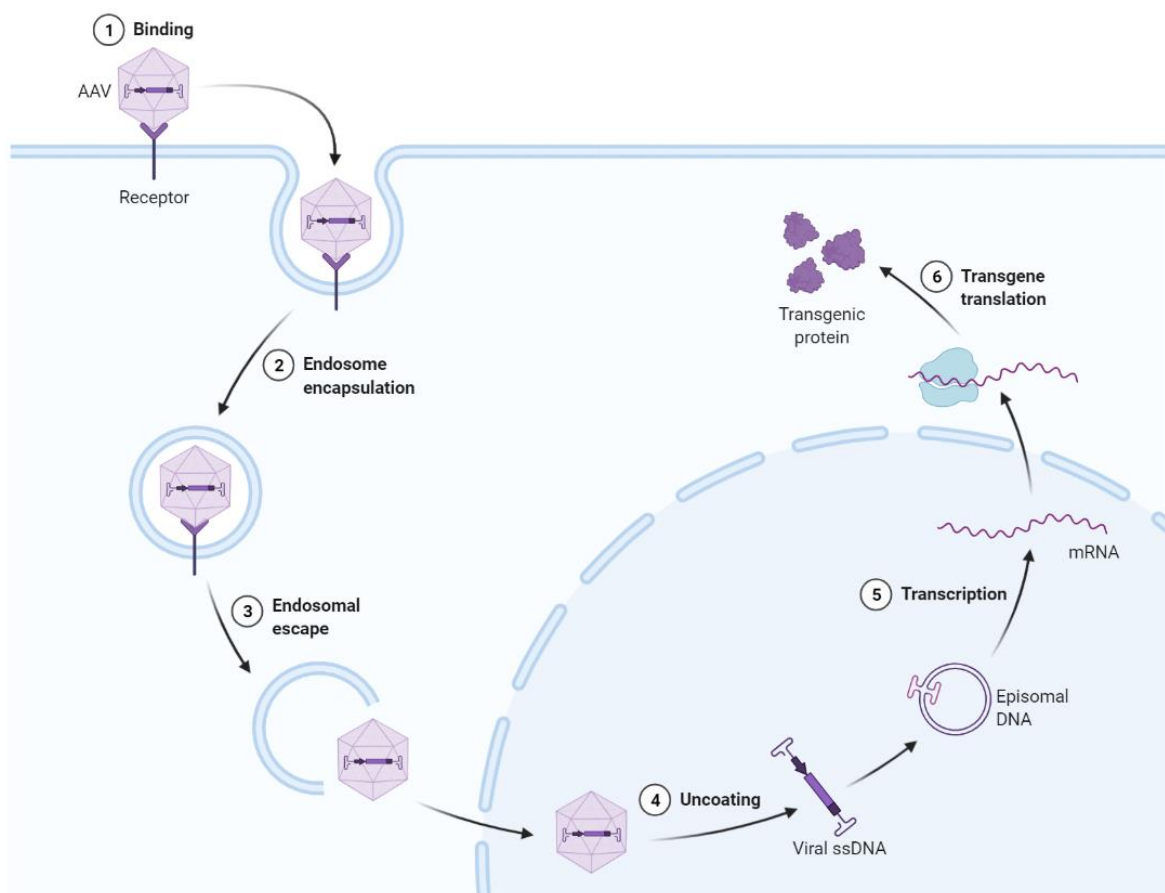


Figure 3.1. Infection of a cell with a recombinant AAV vector. The AAV particle enters the cell via encapsulation and is then transported to the nucleus. Here, the viral DNA will be released, transcribed and translated. Figure was created with Biorender.com

different serotypes can improve transduction for specific tissues or cell types (Wu *et al.* 2006). For example, AAV serotypes 2, 5 and 8 show tropism toward the CNS and since the retina developed from neural cells, these serotypes can also effectively transduce photoreceptor cells and RPE (Wu *et al.* 2006). A hybrid serotype is usually annotated as AAV X/Y in which X represents the serotype of the ITRs and replication genes and Y the serotype of the capsid. Directed evolution is a high-throughput approach that imitates natural evolution. Variants of proteins and genes are created through mutagenesis of nucleic acid, via the inclusion of random point mutations, insertions and deletions, and shuffling of DNA. These variants are then screened under selective pressure, which allows the creation of a library containing functional novel variants. This technique can be used to generate novel AAV capsid genes with specific desired characteristics, which is extremely useful for designing novel gene therapies (Bartel *et al.* 2012).

Infection of animal cells by AAVs happens as follows: First, the virus binds to receptors on the cellular membrane which vary depending on the AAV serotype. Via endosomal encapsulation the AAV then enters the cell. After the AAV escapes from the endosome, it will enter the nucleus, where the capsid will be removed (uncoating). After uncoating the single stranded AAV undergoes second strand DNA synthesis leading to the formation of a replicative double-stranded form of the viral genome, that can be transcribed by the cellular RNA polymerases. These mRNAs will then be translated by the cells to form the viral particles needed to create new virions.

AAVs are usually found in presence of other viruses since AAV gene expression is suppressed in the absence of helper viruses. Furthermore, it is rare for AAVs to be able to lyse cells on their own. AAVs can infect cells alone, but they become latent as they cannot replicate or release any viral particles without the presence of other viruses.

AAVs can be genetically manipulated and used for gene therapy by removing the viral genes and substituting them for a gene of interest (termed recombinant AAV (rAAV) vectors). Depending on the serotype and promoter, the virus can then deliver the gene of interest to specific target cells for long-term gene expression. Both the Luxturna and Zolgensma gene therapies rely on recombinant AAVs to deliver the RPE65 and SMN1 therapeutic genes into the target cells (Mendell *et al.* 2017; Maguire *et al.* 2019). Although recombinant AAVs do not replicate or integrate within the host genome, they have been shown to provide long term expression of the therapeutic genes in clinical trials (Maguire *et al.* 2019.) and even as long as a decade in dogs after a single administration of the AAV therapy (Cideciyan *et al.* 2013). As retinal cells are post-mitotic, one injection with an AAV therapy could theoretically allow indefinitely long-term transgene expression.

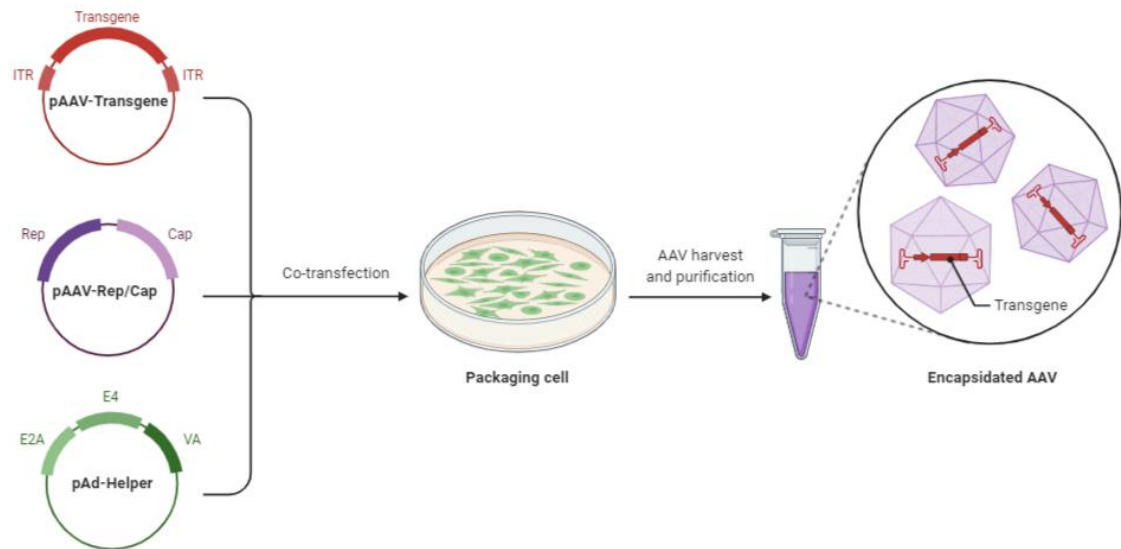


Figure 3.2. Triple Transfection approach for the generation of recombinant AAV vectors. Figure created with biorender.com

As mentioned before, AAVs need a helper virus for co-infection. Examples of these helper viruses are herpes or adenovirus, but it is difficult to filter them out of AAV preparations completely and these viruses can have adverse side effects *in vivo*. For manufacturing AAVs, another option is the triple transfection approach, in which the gene of interest, flanked by Inverted Terminal Repeats, (ITR), is cloned into one plasmid, another plasmid carries the replication and capsid genes, and a third plasmid carries additional functions required for the replication process (Matsushita *et al.* 1998; Xiao *et al.* 1998; Nyguen *et al.* 2021). These genes, E2A, E4, and VA, were identified in adenovirus to play an essential role for AAV replication (Jay *et al.* 1981; Huang *et al.* 1989; Janik *et al.* 1989). All three plasmids are transfected into HEK293 cells, which will generate the single stranded AAV genome and produce the viral capsid proteins to encapsulate that genome. After several purification steps, the AAV particles are ready for downstream experiments. This method is also used in the Farrar lab for the generation of recombinant high-titre AAVs.

Unfortunately, the size of the gene is a limiting factor, since the capacity of AAV vectors is approximately 4.5 to 5 kb (Wu *et al.* 2010b) and many genes of interest are bigger than that. The cDNA alone of *ABCA4*, for example, is 6.8 kb, and therefore it cannot fit into a single AAV vector. To overcome this problem, various dual vector systems for the *ABCA4* gene have been designed, such as overlapping vectors, trans-splicing, or a hybrid approach. In the overlap approach, the *ABCA4* gene is split into two vectors with a 1.35kb overlap between them. In the trans-splicing method, the two vectors do not overlap, but artificial splice acceptor and donor sites are introduced to ligate the products of two vectors into one protein. The hybrid model also has no

overlap of *ABCA4* but utilises a recombinogenic element of the alkaline phosphatase gene to merge two vectors into one *ABCA4* protein (Tripani *et al.* 2014; Tripani *et al.* 2018; Dyka *et al.* 2019; McClements *et al.* 2020). Photoreceptor cells are post-mitotic, therefore the homologous recombination system that's required for recombination of the dual vectors is downregulated. As a result, full length *ABCA4* levels remain barely detectable *in vivo*. Plus, it should be mentioned that there are still some long-term safety concerns for both dual vector systems, as they also appear to produce non-specific part-length proteins (Dyka *et al.* 2019; McClements *et al.* 2020).

3.4.2. Yeast mitochondrial complex 1 gene, *NDI1*

Given the potential limitations of dual vector AAV delivery of *ABCA4* gene therapies and an increasing interest in common pathways of disease in ocular therapies, the focus of this study has been to modulate a disease pathway present in many ocular conditions including Stargardt disease. Indeed, the emerging observations of significant mitochondrial dysfunction, including complex I deficiency, in Stargardt disease and other ocular conditions provides a potential therapeutic target for inherited ocular disorders independent of specific IRD genes (Brown *et al.* 2018; Eells *et al.* 2019; Sivapathasuntharam *et al.* 2019).

In the Farrar lab, we have undertaken research on the potential of a yeast mitochondrial gene, *NDI1*, to improve mitochondrial function including ATP production in different retinal cell types to reduce symptoms of inherited retinal diseases such as Leber Hereditary Optic Neuropathy (Chadderton *et al.* 2013; 2023) and AMD (Millington-Ward *et al.* 2022; 2023). *NDI1*-based gene therapies have also been shown to improve visual function in multiple sclerosis mouse models (Talla *et al.* 2020) and to protect against neurodegeneration in a rat model for Parkinson's disease (Marella *et al.* 2008), showing its wide applicability.

The yeast NADH Dehydrogenase Internal gene (*NDI1*) is a homolog to the mitochondrial electron transport chain complex I. Where the human complex 1 comprises 46 subunits, 7 of which are encoded in the mitochondria (Zhu *et al.* 2016), the yeast homolog is encoded by a single nuclear gene, *NDI1* which encodes a protein of 518 amino acids which is transported to mitochondria using a mitochondrial localisation signal (MLS) (Marres *et al.* 1991). These characteristics make it ideal as a potential gene therapy targeting mitochondrial function since the *NDI1* gene fits into commonly used gene therapy vectors such as AAV, can be expressed in the nucleus and is transported to mitochondria. Like the mitochondrial complex 1, *NDI1* functions as a NADH-ubiquinone oxidoreductase, meaning it converts NADH into NAD⁺ and electrons.

In principle a single gene yeast homolog for the mitochondrial complex 1 (*NDI1*) represents a means to augment mitochondrial function. In this study a codon-optimised version of *NDI1*, which expresses 3 times higher than wild-type *NDI1* (Millington-Ward *et al.* 2022; Chadderton *et*

al. 2023), was cloned into an AAV vector to generate high-titre AAV.CAG.ophNDI1 and evaluated as a means to improve cellular bioenergetics. In this chapter, gene delivery via various AAV serotypes is optimised and the use of AAV.CAG.ophNDI1 vectors as a potential therapy for Stargardt Disease is explored. EGFP has been used extensively in research studying expression provided by specific AAV serotypes or specific promoters, in proof-of-concept studies, among many other applications (Arun *et al.* 2005; Chudakov *et al.* 2005; Maddalena *et al.* 2018; Millington-Ward *et al.* 2020). Therefore, as part of the research described in this chapter, AAV vectors driving EGFP expression were used to investigate the transduction efficiency of various AAV serotypes and gene promoters to optimise gene delivery in various cell models. AAV.CAG.ophNDI1 vectors were then tested *in vitro* on different RPE cell types and models of disease to assess potential beneficial effects of this novel gene therapy targeting mitochondrial dysfunction.

3.5. Results

In this chapter the use of NDI1 as an AAV-based gene therapy for Stargardt disease is tested on cell models of disease. First, the transduction efficiency of various AAV serotypes on a variety of RPE-related cell types is tested with EGFP-based transduction assays. That information was then used to determine what serotypes the therapeutic AAV.o-phNDI1 would be produced in. Then, cell models of disease were optimised before the effect of the therapeutic vector was extensively investigated.

3.5.1. AAV serotype assays

To explore the efficiency of AAV transduction in different cell lines, EGFP vectors were used. Recombinant AAV vectors containing EGFP and the ubiquitous CBA or CMV promoters previously available in our lab were utilised to compare several promoters and AAV serotypes. Various RPE-like cell lines and porcine primary RPE cells were seeded onto microscope slides and transduced with the AAV-EGFP vectors as described in section 2.8.1 with varying multiplicities of infection (MOI). Cells were fixed and stained with HOECHST 48 hours post-transduction as described in section 2.8.2.

Initially, hTERT-RPE1 cells were transduced with AAV2/5.CBA.EGFP vector, with MOI's 5×10^5 , 1×10^6 and 5×10^6 , however this did not produce enough expression of EGFP to yield visible fluorescence in the cells. The dose was then increased to MOI's of 5×10^7 , 1×10^8 and 1.5×10^8 . Again, this did not drive enough gene expression to show fluorescence. Images were taken at an emission wavelength of 455 and 508nm and displayed in Figure 3.3.

Therefore, other AAV serotypes were examined, such as AAV2/2 and AAV2/8. Since AAV vectors for these serotypes with EGFP driven by a CMV promoter were readily available in the lab and we were primarily interested in defining the AAV serotype that was able to transduce hTERT-RPE1 cells, the available vectors using the CMV promoter were used for these transduction assays instead of generating AAV2/2 and AAV2/8.CBA.EGFP vectors. hTERT-RPE1 cells were transduced with MOIs of 1×10^6 and 2×10^6 of AAV2/2-CMV-EGFP, and a MOI of 2×10^7 , 4×10^7 and 8×10^7 of AAV2/8-CMV-EGFP, respectively. In contrast to the prior experiments, both serotypes showed clear EGFP fluorescence when analysed via fluorescence microscopy (Figure 3.4). Of note, these transduction assays clearly demonstrated that the AAV2/5 serotype does not transduce efficiently in hTERT-RPE1 cells.

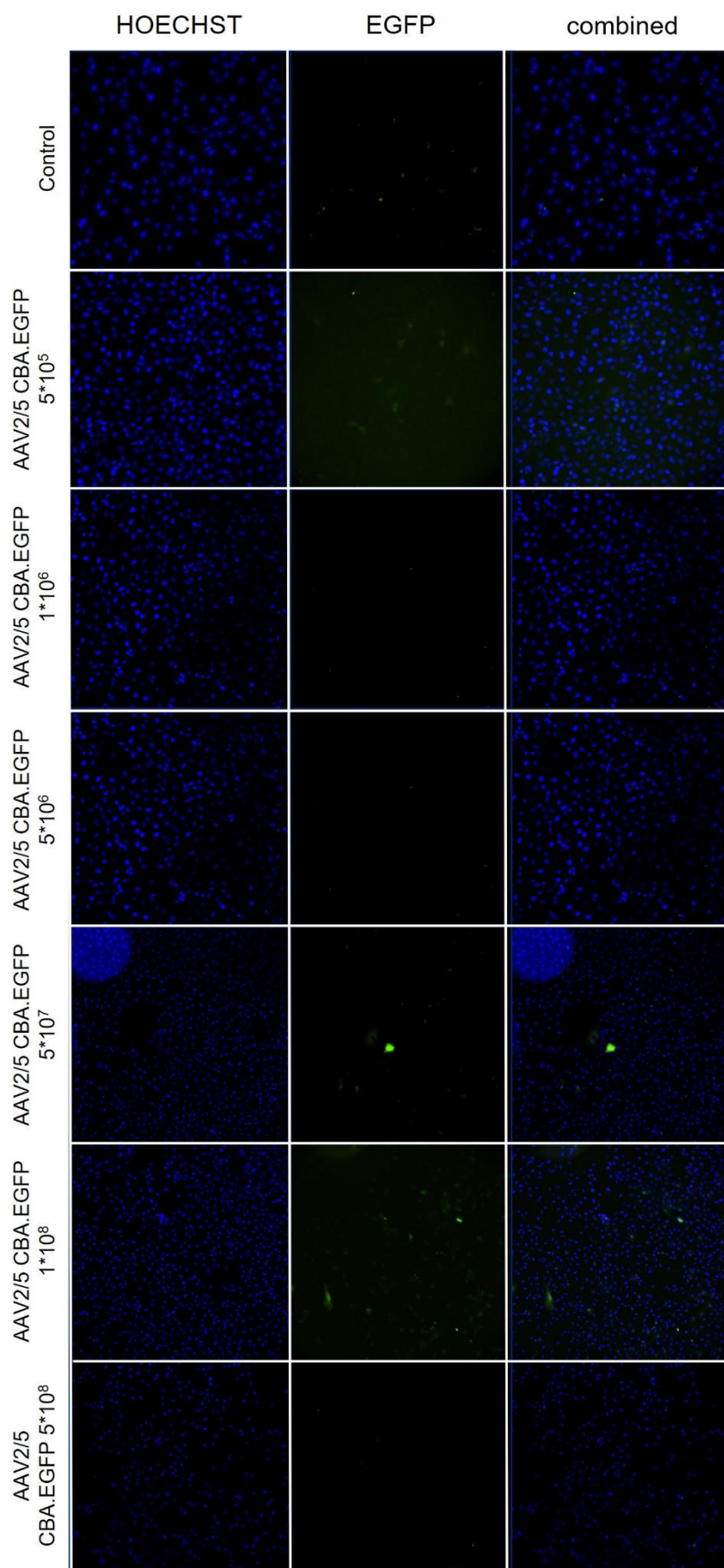


Figure 3.3. Transduction assay on hTERT-RPE1 cells with 2/5 CBA-EGFP AAV vector. A dose curve ranging from 5×10^5 to 1.5×10^8 viral particles per cell (MOI) was used to transduce hTERT-RPE1 cells. The left column in this figure shows the HOECHST-stained cell nuclei at an emission wavelength of 455nm. The middle column shows that no GFP was visible in any of the AAV doses used at an emission wavelength of 508nm. The right column

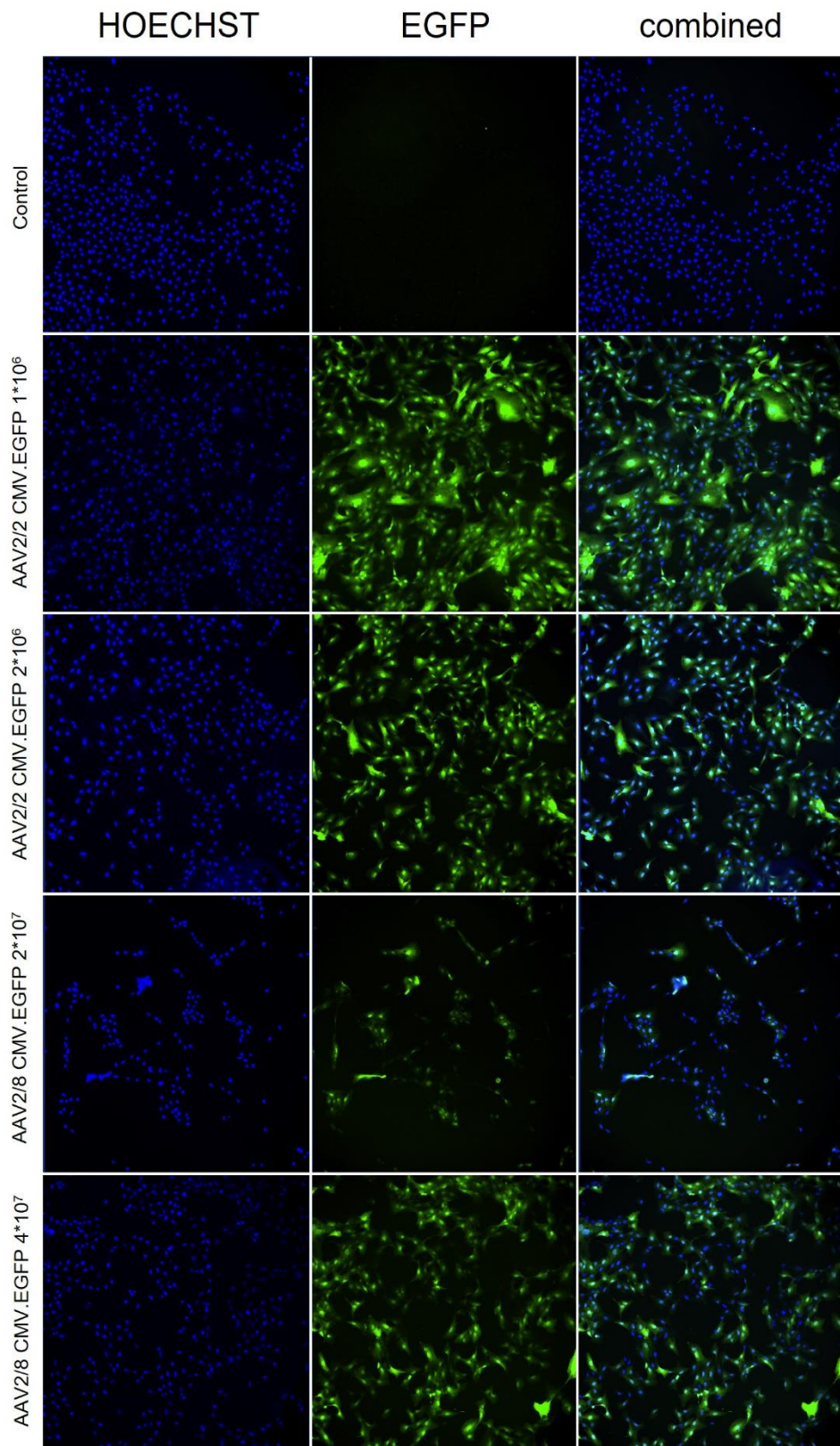


Figure 3.4. Transduction assay on hTERT-RPE1 cell line with AAV2/2 and AAV2/8 CMV-EGFP. The left column in this figure shows the HOECHST-stained cell nuclei at an emission wavelength of 455nm. The middle column shows that GFP at an emission wavelength of 508nm. The right column merges the HOECHST and GFP images.

However, upon further review of the literature it was decided that the hTERT-RPE1 cell line may not be the best replica of the RPE and the experimental plan was adapted to using the ARPE19 cell line and to employing porcine primary RPE cells. The ARPE19 cell line is more commonly used in other studies researching Stargardt disease (Saadat *et al.* 2014; Westlund *et al.* 2009; Marie *et al.* 2018), which allows for better comparison of our future results with other studies. While the induced immortality of cell lines almost certainly changes some specific characteristics of cells and tissues, ARPE19 cells exhibit gene expression profiles approaching more resemblance to stem cell-derived RPE and native RPE, while hTERT-RPE1 cells are more divergent (Samuel *et al.* 2017). Therefore, we think that combining the data that will be generated from experiments on ARPE19 cells with data that will be generated from experiments on primary cells can give a broader and more in depth overview of the possible effects AAV.oPhNDI1 can have on retinal cells.

First, the transduction assay was repeated on ARPE19 cells. These cells were seeded in 8-well chamber slides with a cell density of 4.5×10^4 as described in Materials and Methods section 2.8.1. The cells were transduced with AAV2/2.CMV.EGFP (M.O.I. of 10^3 and 10^4), AAV2/5.CMV.EGFP (M.O.I. of 10^5 and 10^6), and AAV2/8.CMV.EGFP (M.O.I. of 10^4 and 10^5). This assay was performed *in duplo* and then repeated. We found that AAV2/2 transduces the cells most efficiently, with potent expression of the EGFP protein at an MOI of 1×10^3 , whereas AAV2/8 required 1×10^4 viral particles per cell for similar fluorescence levels. The AAV2/5 serotype only showed very mild fluorescence even at a MOI as high as 1×10^6 , which means it is a thousand times less efficient at transducing these cells than AAV2/2. A representative image is shown in Figure 3.5.

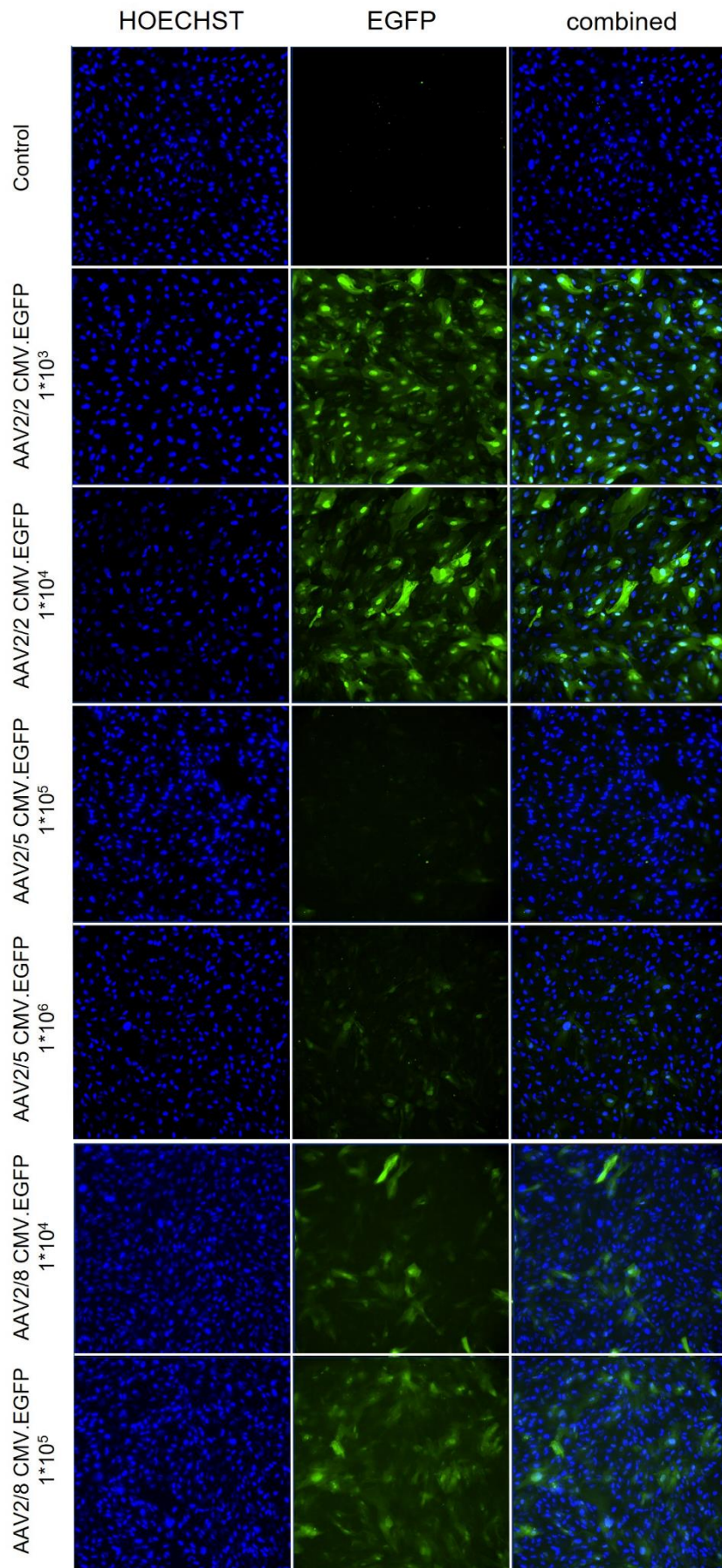


Figure 3.5. ARPE19 cells transduced with AAV.CMV.EGFP vectors of serotypes 2/2, 2/5 and 2/8. The left column in this figure shows the HOECHST-stained cell nuclei at an emission wavelength of 455nm. The middle column shows that GFP at an emission wavelength of 508nm. The right column merges the HOECHST and GFP images.

In experiments previously published by our lab it was established that AAVs carrying the RPE-specific VMD2 promoter were able to successfully drive transgene expression in the cells we collected from post-mortem pig eyes (Millington-Ward et al. 2023). Combining this information with the morphology of the primary cells, we deduced that these cells are indeed primary porcine RPE cells.

Additionally, the previously described experiments were repeated utilising primary RPE cells. Initially the method to grow primary RPE cells from pig eyes had to be optimised as this method was new to our lab. These cells were isolated from eyes taken from pigs killed at an abattoir outside of Dublin, so handling of the eyes before and during transport to our lab was optimised so the eyes would be in better condition by the time they arrived in the lab for further processing. Additionally, the extraction and maintenance of the primary RPE cells was improved through trial and error, which the final protocol described in materials and methods section 2.6.6. For this experiment, primary RPE cells were transduced with AAVs carrying EGFP driven by a CMV promoter using AAV serotypes 2/5 and 2/8, which is represented in Figure 3.6. From this work we established that AAV2/5.CMV.EGFP requires a dose that is approximately 30 times higher than AAV2/8.CMV.EGFP to achieve similar EGFP expression levels in these primary RPE cells.

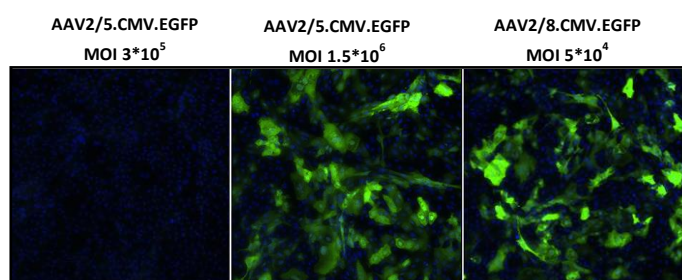


Figure 3.6. Transduction of primary porcine RPE cells with 2/5 and 2/8 serotype AAV vectors. Cells were treated with AAV and fixed and stained with HOECHST two days later to visualise EGFP fluorescence.

In summary, in this section of the project, AAV vectors driving EGFP expression were used to establish that serotypes AAV2/2 and AAV2/8 are well-suitable for transducing RPE-like cell lines. Therefore, these serotypes were chosen for subsequent studies with therapeutic vectors.

3.5.2. Cloning of pCAG-ophNdi

Once it was established that AAV2/2 and AAV2/8 can efficiently transduce the RPE-like ARPE19 cell line and post-mortem primary porcine RPE cells, therapeutic viruses carrying a codon-optimised yeast NDI1 gene were generated in these serotypes. As the triple transfection method is used in the Farrar lab for the generation of AAVs, the first step was to clone a plasmid that encodes for NDI1.

The CMV promoter is a promoter derived from cytomegalovirus. It drives robust gene expression in many tissues and is highly efficient. The CBA-promoter, also known as the CAG-promoter, is a hybrid promoter composed of the early enhancer element of the CMV promoter followed by the chicken beta-actin promoter. This promoter also drives ubiquitous gene expression, but is slightly less effective and more stable than the CMV promoter, which allows for better regulation of expression by gene therapy vectors. In addition, the CAG promoter has already been proven to be safe for use in clinical settings in gene therapies such as Zolgensma and Luxturna, respectively. Therefore, the original pCHOP-huNDI1-I82V plasmid used by the Farrar lab in previous studies was changed to be driven by the CAG promoter instead of the CMV promoter.

A plasmid was generated by excising the CMV promoter out of a pCHOP-ophNdi-I82V plasmid generated by the Farrar lab and replacing it with the CAG promoter from a pCBA-RP14 plasmid generated by the Farrar lab. The original sequences for both plasmids are available in appendix I. Maps of these plasmids that depict all components and the location of restriction sites used in this project can also be found in appendix I. The therapeutic NDI1 transgene utilised in this study is a codon-optimised version, which provides higher expression levels than the wild type gene (Millington-Ward *et al.* 2022; Chadderton *et al.* 2023).

First, a linker DNA fragment was ordered from Sigma-Aldrich to introduce additional restriction sites into pCBA-RP14 since this plasmid did not contain appropriate restriction enzyme sites on one side of the promoter to allow future manipulations of this plasmid and/or promoter. The sequence of this linker was as follows:

```
5'      ATCGACT  ACATGT  CGCACTG  GAATTC  CGTATAGC  CTTAAG  TCAGGTC
CTCGAG  GATCCAGT  AAGCTT  ACGTAGG      3'
```

The highlighted sequences represent restriction sites. Yellow is for the AflIII enzyme site, red for EcoRI, blue is for AflII, pink is for XhoI and green for HindIII. All enzymes were obtained from NEB. The insertion of this linker was performed by digesting both the plasmid and the linker, separately, with AflIII (NEB, cat. no. R0541S). Digested products were purified with the GeneJET PCR purification kit as described in section 2.4.6. The plasmid and linker were then, again separately, digested with HindIII (NEB, cat. no. R0104S). The digested linker and plasmid now had compatible 'sticky ends' and were ligated and transformed into competent XL1-Blue *E. coli* cells as described in section 2.4.3. and then purified from the cells via miniprep methodology (section 2.3.1.). Verification of successful insertion of the linker into the plasmid was done via digestion with EcoRI (NEB, cat. no. R0101S). Successful ligation would yield two bands, whereas self-ligation of the plasmid would only result in a single band (see Figure 3.7); clear evidence of successful ligation was obtained.

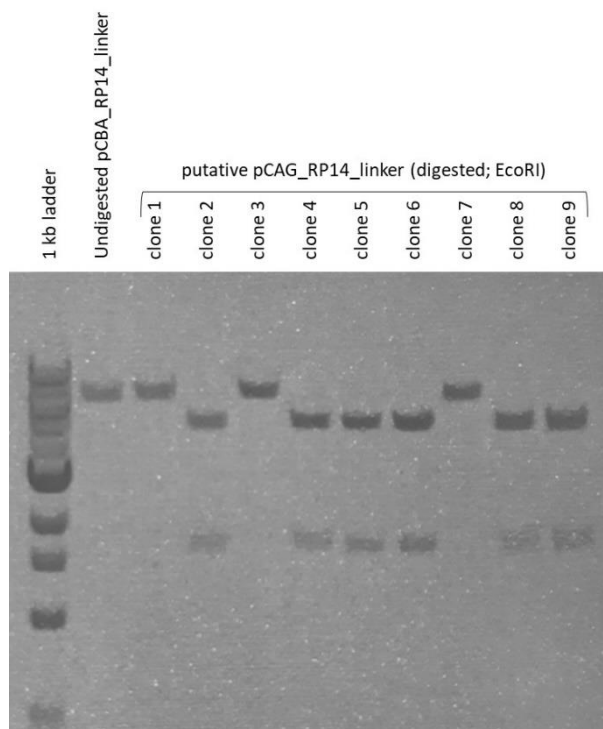


Figure 3.7. Digest of putative pCBA-RP14-linker plasmids. Plasmids were digested with EcoRI (NEB) and then run on a 1% agarose gel. If the linker was inserted correctly, bands should be 1698 and 5050 bp. If the linker was not inserted, the plasmid would only be cut once, resulting in a single band of 6748 bp.

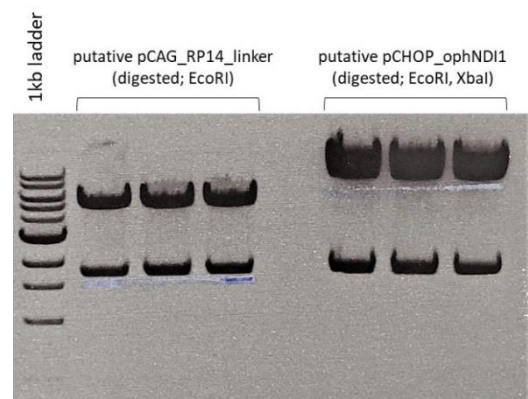


Figure 3.8. Digestion of pCBA-RP14-linker and pCHOP-ophNDI1 plasmids. The pCBA-RP14 plasmid was digested with EcoRI, which yielded bands of 1.7kb and 5kb. The pCHOP-huNDI1-I82V was digested with XbaI and EcoRI, which yielded bands of 1.8kb and 8kb.

Next, pCBA-RP14-linker was digested with EcoRI and pCHOP-huNDI1-I82V was digested with EcoRI and XbaI and digestion products were separated on an agarose gel (Figure 3.8). The 1.7kb fragment from CBA-RP14-linker plasmid was expected to carry the CAG promoter, while the 8kb fragment from the CHOP-huNDI1-I82V plasmid carries the optimised humanised NDI1 gene. Purified fragments were blunted with the Klenow reaction, ligated, and transformed in

competent *E. coli* cells. The insertion and orientation of the product was verified with restriction digest (NdeI and BamHI) (Figure 3.9.).

The production of recombinant AAV vectors from plasmids requires the desired DNA fragment to be flanked by two Inverted Terminal Repeats (ITRs). Because the EcoRI site, in addition to XbaI, was used to remove the CMV promoter from pCHOP_huNDI1_I82V, the left ITR was also removed from the sequence. Another linker carrying the left ITR sequence was therefore obtained from Genewiz:

```

5'   AGCGAATCTC TTAAGCTGTC AAGTCTAGAG CTTAACGCCT GCAGGCAGCT
      GCGCGCTCGC TCGCTCACTG AGGCCGCCCG GGCAAAGCCC GGGCGTCGGG
      CGACCTTTGG TCGCCCGGCC TCAGTGAGCG AGCGAGCGCG CAGAGAGGGA
      GTGGCCAACT CCATCACTAG GGGTTCCTTC AGCCAAGCT CAGGACATT
      TTAAGGACAA TGCCG          3'

```

The colour-highlighted sequences represent different digestion sites. Yellow is the restriction enzyme site for AflII, red for XbaI and blue for SacI. The underlined sequence corresponds to the left ITR, which was copied from another AAV-plasmid; pAAV-CBA-EGFP (previously generated by the Farrar lab).

This linker and pCBA-huNDI1-I82V were both digested with AflII (NEB). The digested products were separated on an agarose gel, excised from the gel and purified. Next, a ligation and transformation were carried out to introduce the linker into the plasmid and expand it. The insertion and orientation of the product was verified with restriction digest (SacI and SacII) (Figure 3.10). The sequence of the full plasmid was verified through Sanger Sequencing (Appendix III for primers). A map of the final construct is portrayed in Figure 3.11.

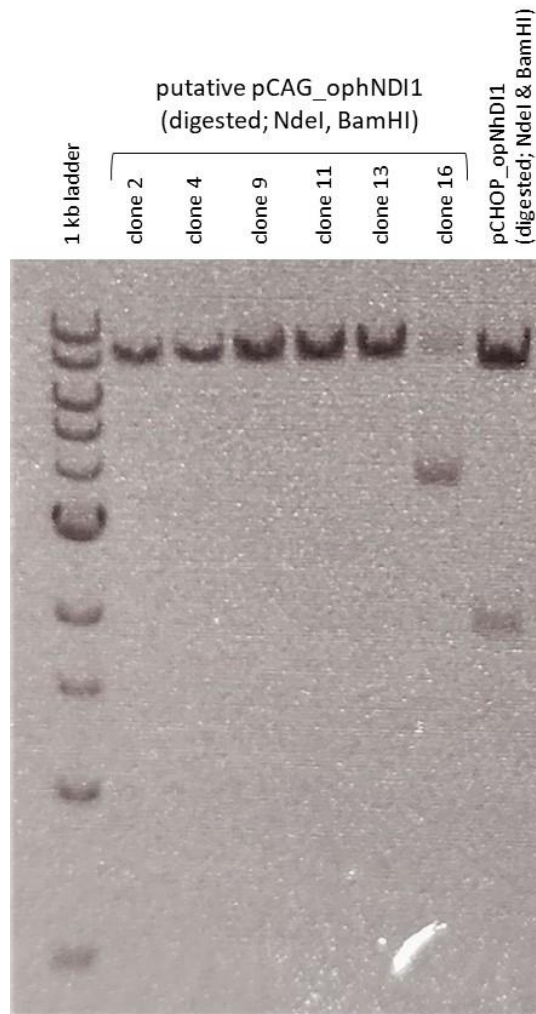


Figure 3.9. Digestion of the putative pCAG-ophNDI1 plasmid. The clones were digested with NdeI and BamHI to verify orientation of ligated product. In the correct orientation, this should yield bands of 3kb and 6.8kb. Control bands should be 300bp, 1.9kb and 7.6kb, unfortunately the 300bp band ran off the gel when separating larger bands.

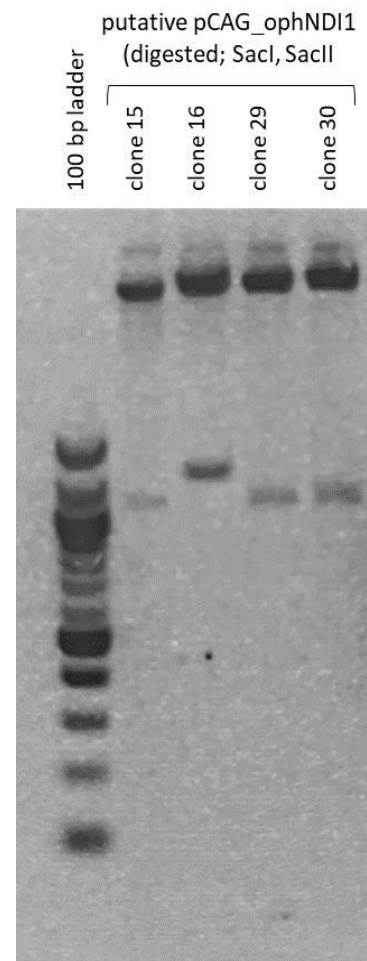


Figure 3.10. Digest of putative pCAG-ophNdi plasmids. The plasmids were digested with SacI and SacII (NEB) and separated on an agarose gel, of which four are depicted in this figure. If the ITR linker was inserted in the right orientation, the bands should be 1069 and 8948 bp, wrong orientation the bands will be 1230 and 8780bp. Clones 15, 29 and 30 have the right orientation, whereas clone 16 is the wrong orientation.

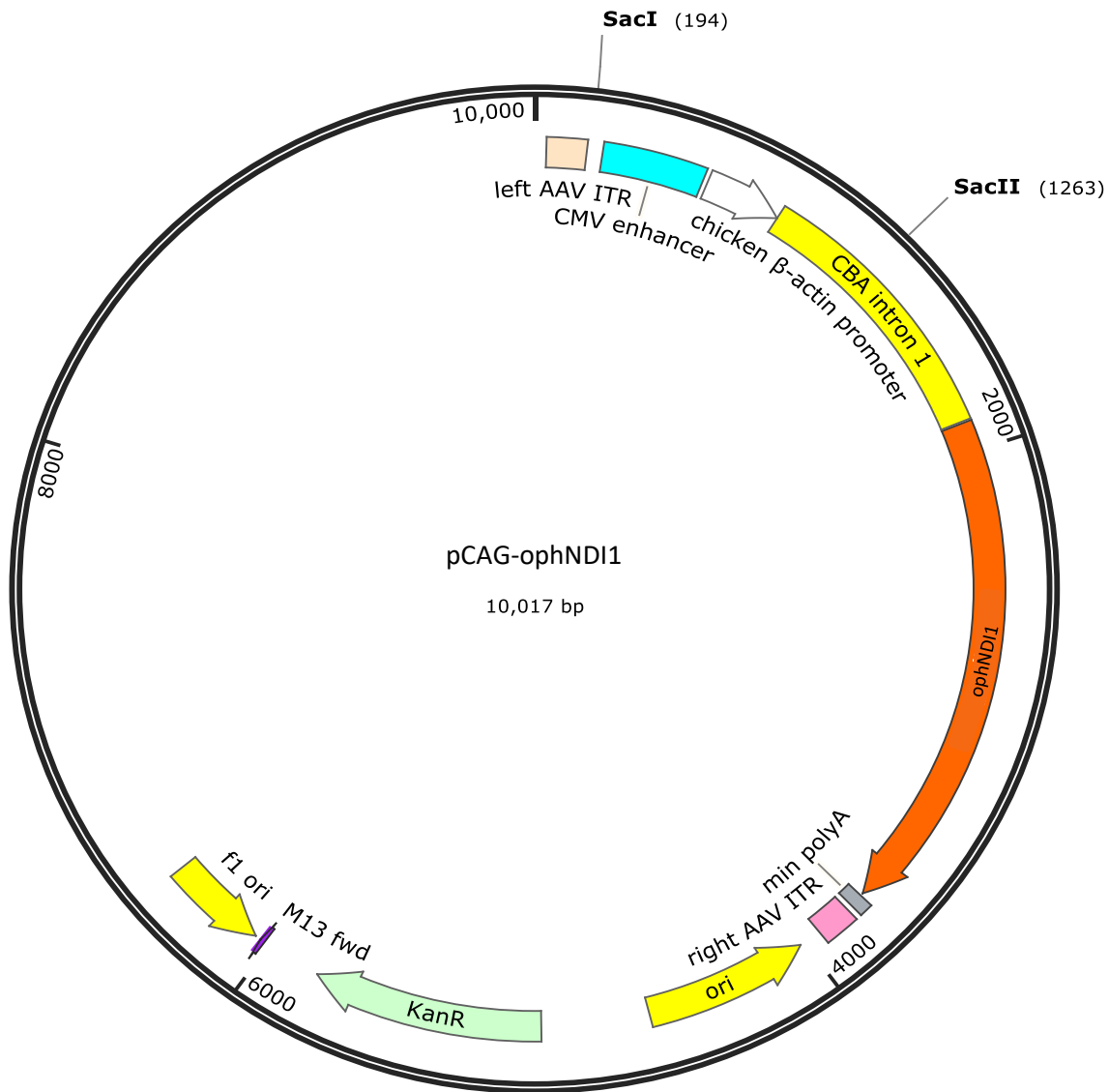


Figure 3.11. Map of pAAV-CAG-ophNDI1 construct. Restriction sites for digestion enzymes SacI and SacII are shown because these enzymes were used to verify the orientation of the ITR insert. Other restriction sites are hidden for clarity. Important features, such as the kanamycin resistance gene, the CAG promoter, and our gene of interest, optimised humanised NDI1, are shown. This map was generated using SnapGene Viewer 5.3.1 software.

3.5.3. Generation of AAV.CAG.ophNDI1

Using the pCAG-huNDI1-I82V plasmid, various serotypes of high-titre AAVs were generated via the triple transfection method as described in section 2.7.1 by Dr Naomi Chadderton, our in-house virus expert.

Then, the expression of NDI1 driven by AAV.CAG.ophNDI1 in ARPE19 and primary porcine RPE cells was verified via RT-PCR. Both cell types were seeded at a density of 2×10^4 cells per well in a 96-well plate and transduced after 24 hours with AAV2/2.CAG.ophNDI1 (MOI 1.5×10^5) and AAV2/8.CAG.ophNDI1 (MOI 2.5×10^6) in duplicate and grown for an additional 24 hours at 37°C, after which RNA was isolated from each sample according to M&M section 2.4.9. RNA levels per sample were then quantified by RT-PCR as described in section 2.8.2, with 3 replicates per duplicate sample, and then normalised to relative beta-actin quantities per well. Results are displayed in Figure 3.12, in which primary RPE cells from three different pigs are presented separately as their genetics are not identical. The dosage of each serotype was chosen based on efficacy of EGFP expression following transduction with AAV.EGFP vectors, as it was shown there that AAV2/2 transduces ARPE19 cells approximately 10 times more efficiently. Both serotypes were able to robustly drive NDI1 expression, though with the doses used here NDI1 expression was higher in cells transduced with AAV2/8.CAG.ophNDI1. However, if these data were relativised to the MOI that was used, AAV2/2 would drive expression more efficiently. Interestingly, the primary RPE cells transduce much more efficiently than ARPE19 cells in both serotypes. Indeed, we also found a similar effect in the aforementioned EGFP transduction studies. This information is used to determine dosage of AAV.CAG.ophNDI1 in subsequent *in vitro* experiments.

Once it was established that the 2/2 and 2/8 AAV-serotypes transduced the ARPE19 cell line and the primary porcine RPE cells most efficiently, this viral vector was extensively investigated for potential therapeutic benefits in cell models of disease.

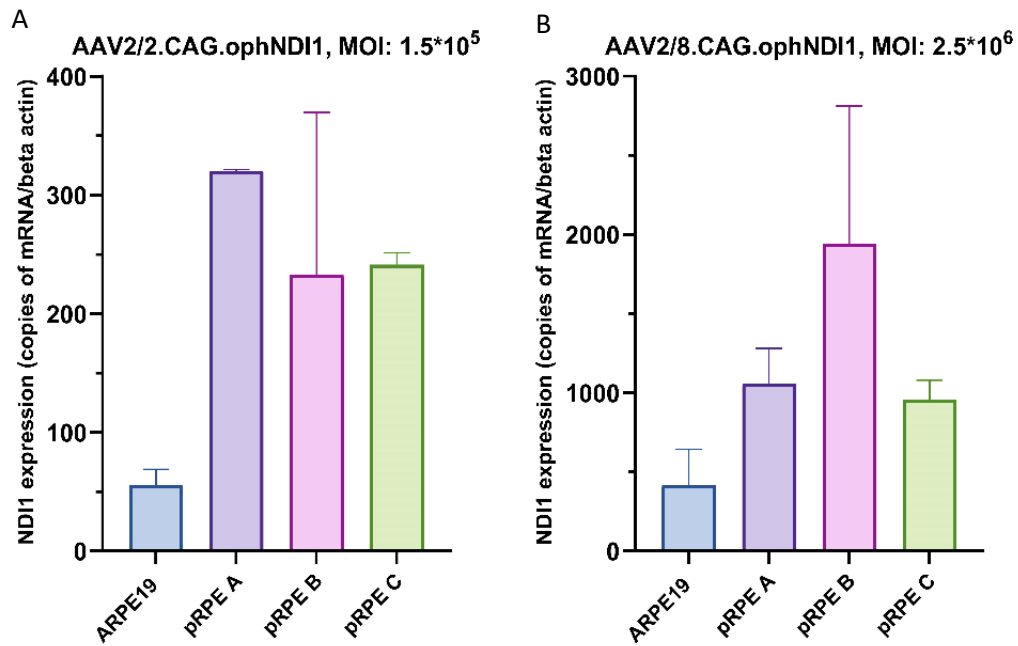


Figure 3.12. Expression of transgene mRNA in vitro. Cells were seeded at a density of 2×10^4 in 96-well plates and transduced with AAV2/2.CAG.ophNDI1 at a MOI of 1.5×10^5 or AAV2/8.CAG.ophNDI1 at a MOI of 2.5×10^6 and harvested 24 hours later. RT-qPCR was used to quantify mRNA expression of NDI1. **A.** Transduction with AAV2/2.CAG.ophNDI1 caused robust expression in all samples (ARPE19: 55.42 ± 13.4 ; pRPE pig A: 320.0 ± 1.4 ; pRPE pig B: 232.7 ± 136.6 ; pRPE pig C: 241.1 ± 10.09 copies of NDI1 mRNA relative to beta actin mRNA) **B.** Transduction with AAV2/8.CAG.ophNDI1 also yielded robust expression of NDI1 in all cell lines (ARPE19: 413.1 ± 231.0 ; pRPE pig A: 1055.0 ± 225.5 ; pPRE pig B: 1940.0 ± 872.7 ; pRPE pig C: 956.3 ± 122.9 copies of NDI1 mRNA relative to beta actin mRNA). Bar plots represent the mean, error bars represent standard deviation. No statistical test was used as the only goal of this experiment was to show expression of each serotype.

3.5.4. Evaluation of the effect of AAV.CAG.ophNDI1 on cell models of disease

A Seahorse XFe96 analyser was used to assess mitochondrial function of cells in real time. This assay was performed on various cell models of Stargardt disease. First, the RPE-like cell line ARPE19 and primary porcine RPE cells were insulted with 5mM NaIO₃ to mimic oxidative stress, mitochondrial dysfunction and RPE cell degeneration. All cell models received treatment with AAV.CAG.ophNDI1 and a mitochondrial stress test was performed to examine the effects of this candidate therapy. Additionally, primary porcine RPE cells were insulted with A2E, a dimerised vitamin A derivate that accumulates in RPE and photoreceptor cells in patients with Stargardt disease type 1 and leads to oxidative stress, mitochondrial damage, and ultimately cell death. The Agilent XFe96 Seahorse analyser allows analysis of the mitochondrial function of cell culture in 96 well plates in real-time. Initially, the basal oxygen consumption rate (OCR) of cells is measured, after which specific chemicals that influence the OCR are added sequentially. In the experiments described in this chapter, the Agilent XFe96 Seahorse analyser was utilised to interrogate mitochondrial function in RPE cells. First, oligomycin was added to the cells, which blocks the proton channel of ATP synthase, which then prevents the oxidative phosphorylation of ADP into ATP (Salomon *et al.* 2000). This reduces the OCR and the mitochondrial ATP production can be calculated from this reduction. Second, FCCP is added. FCCP is a potent uncoupler of the mitochondrial oxidative phosphorylation, in the sense that it transports protons across the inner mitochondrial membrane without the need for transport proteins, which gives the cells an unlimited number of protons and therefore allows them to maximise their respiration (Heytler *et al.* 1962; Kane *et al.* 2018). Last, rotenone, an inhibitor for mitochondrial complex 1 (Palmer *et al.* 1968), and antimycin A, which inhibits cytochrome c reductase, complex

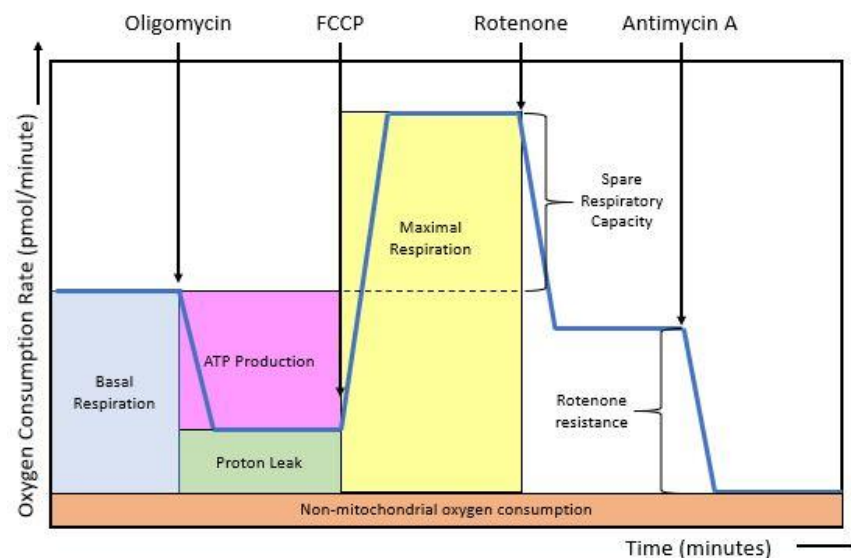


Figure 3.13. Example of Seahorse analyser data set. The oxygen consumption rate is measured by the Agilent Seahorse analyser while a variety of chemicals that affect the electron transport chain is added. Figure adapted from Agilent

III (Huang *et al.* 2005), were added to the cells. The combination of these two chemicals completely blocks mitochondrial oxygen consumption. However, rotenone and antimycin A were added sequentially, since the yeast complex 1 protein NDI1 is resistant to rotenone (de Vries *et al.* 1988), but the human complex 1 protein is not. This enabled evaluation of a functional NDI1 gene, delivered to cells via AAV, and whether any increase in OCR could be due to NDI1 expression.

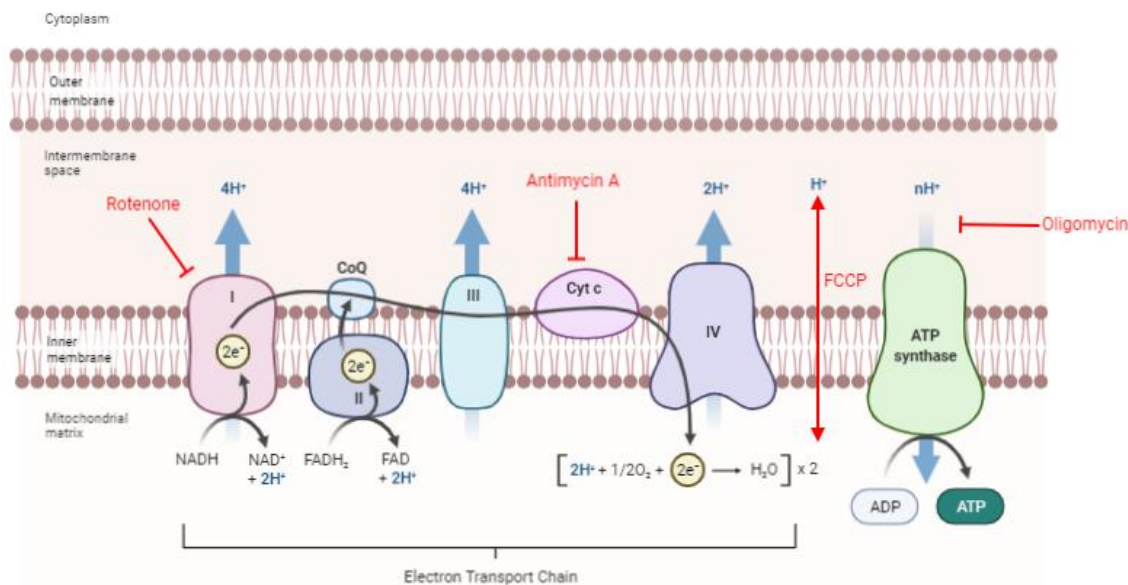


Figure 3.14. Mitochondrial electron transport chain and the effects of chemicals used in mitochondrial stress test on specific protein complexes in this chain. Figure created with Biorender.com.

ARPE19 cells were seeded onto a Seahorse XFe96 analyser plate at a cell density of 5×10^4 as described in section 2.8.3. After overnight incubation at least 5 wells were transduced with AAV2/2.CAG.ophNDI1 at a multiplicity of infection of 3.4×10^5 as described in section 2.6.7. The following day an insult with 5mM NaIO₃ was administered to the AAV-treated cells and 16 wells of untransduced control cells (section 2.6.8). Sixteen hours later mitochondrial stress test was performed on all cells utilising a Seahorse analyser. The results were normalised to the protein levels present in each well as described in section 2.6.9. This assay was repeated on 3 separate occasions.

The NaIO_3 insult significantly reduced the basal oxygen consumption rate (OCR) and the maximal OCR, spare respiratory capacity (SRC) and ATP production (Figure 3.15). Of note, transduction with AAV2/.CAG.ophNDI1 rescued the basal OCR, maximal OCR and ATP production. The SRC, however, which is calculated from the difference between maximal and basal OCR and represents how much extra energy can be produced via oxidative phosphorylation when the demand for energy suddenly increases, was decreased even further in transduced cells due to the high basal OCR in AAV.CAG.ophNDI1 treated cells. Rotenone and Antimycin A were administered to the cells during the mitochondrial stress test to block the electron transport chain. The difference in OCR post-rotenone and post-antimycin A treatment demonstrated OCR rescue via NDI1, since this protein is resistant to rotenone, but the human complex 1 is not.

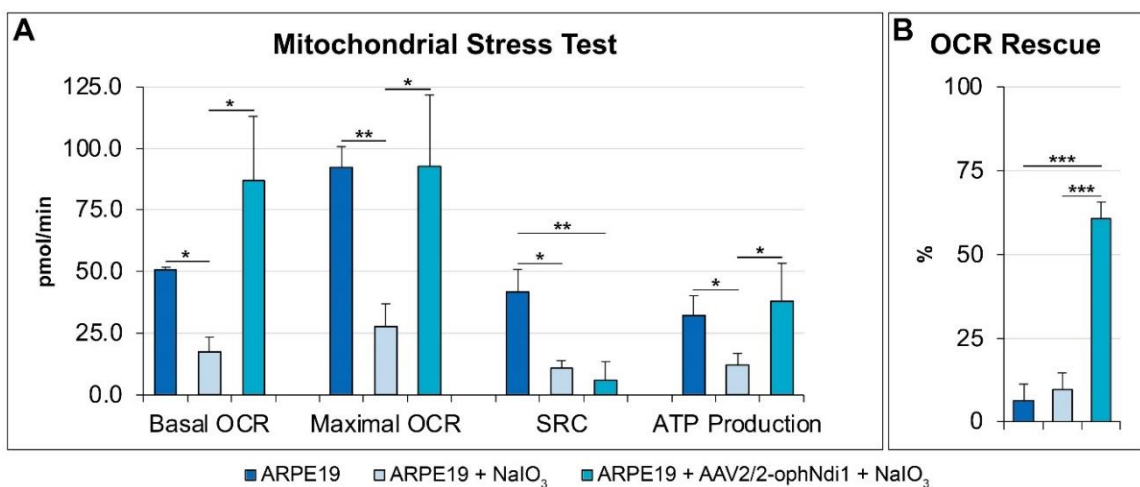


Figure 3.15. Rescue of mitochondrial function of NaIO_3 -insulted ARPE19 cells by AAV2/2.CAG.ophNDI1. A. The basal Oxygen Consumption Rate, Maximal OCR, Spare Respiratory Capacity and ATP production were significantly reduced in ARPE19 insulted with NaIO_3 . The basal OCR, Maximal OCR and ATP production were rescued in cells transduced with AAV.CAG.ophNDI1. B. Rescue of OCR by AAV.CAG.ophNDI1 post treatment with rotenone. Bars represent the mean and error bars indicate standard deviation. $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$. Source: Millington-Ward *et al.* 2022

To further explore the potentially beneficial effects of AAV.CAG.ophNDI1, primary RPE were utilised. Primary porcine RPE cells were extracted from post-mortem pig eyes as described in section 2.6.6. Then 1.375×10^4 cells were seeded onto a Seahorse XFe96 analyser plate (section 2.8.3). One day later the cells in at least 5 wells were transduced with AAV2/2-ophNDI1 at a multiplicity of infection of 3.4×10^5 (as described in section 2.6.7). The next day the transduced cells and a minimum of 15 untransduced wells were treated with 6mM of NaIO_3 (a dose informed by the prior experiments). Sixteen hours later the Seahorse analyser mitochondrial stress test was performed on all cells. The results were normalised to the protein levels present in each well as described in section 2.6.9. The assay was repeated 3 times.

In each repeat the basal and maximal OCR and the ATP production was significantly reduced in NaIO₃-insulted cells (Figure 3.15). The spare respiratory capacity (SRC), which is calculated from the difference between maximal and basal OCR and represents the energy reserves in cells, is also significantly impacted after an insult with NaIO₃. Cells transduced with AAV2/2-ophNDI1 have significantly improved basal and maximal OCRs, ATP production and SRC after NaIO₃-insult as compared to the cells that only received the insult (Figure 3.16). Treatment with rotenone revealed that the OCR-improvement was NDI1-dependent.

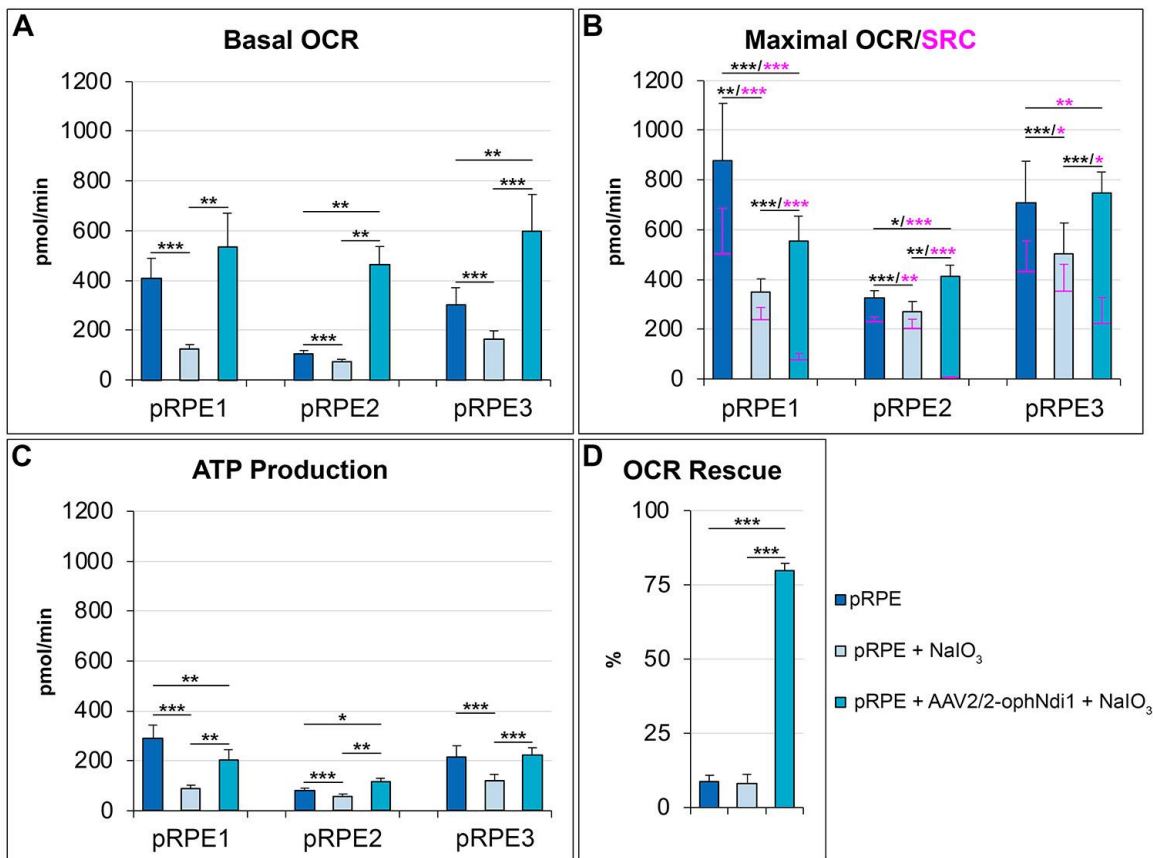


Figure 3.16. AAV.CAG.ophNDI1-mediated rescue of mitochondrial function in primary porcine RPE cells insulted with NaIO₃. A. Basal oxygen consumption rate (OCR). B. Maximal OCR and spare respiratory capacity (SRC). C. ATP production. D. OCR Rescue after treatment with rotenone. Bars represent the mean and error bars indicate standard deviation. $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$. Source: Millington-Ward *et al.* 2022.

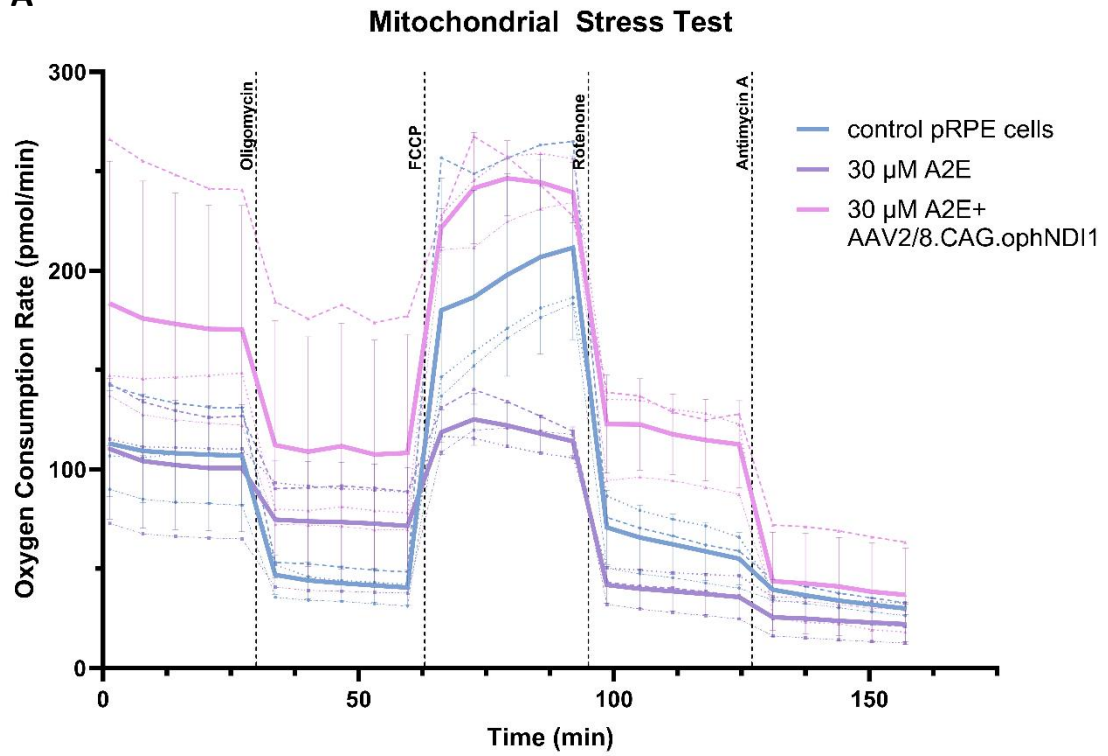
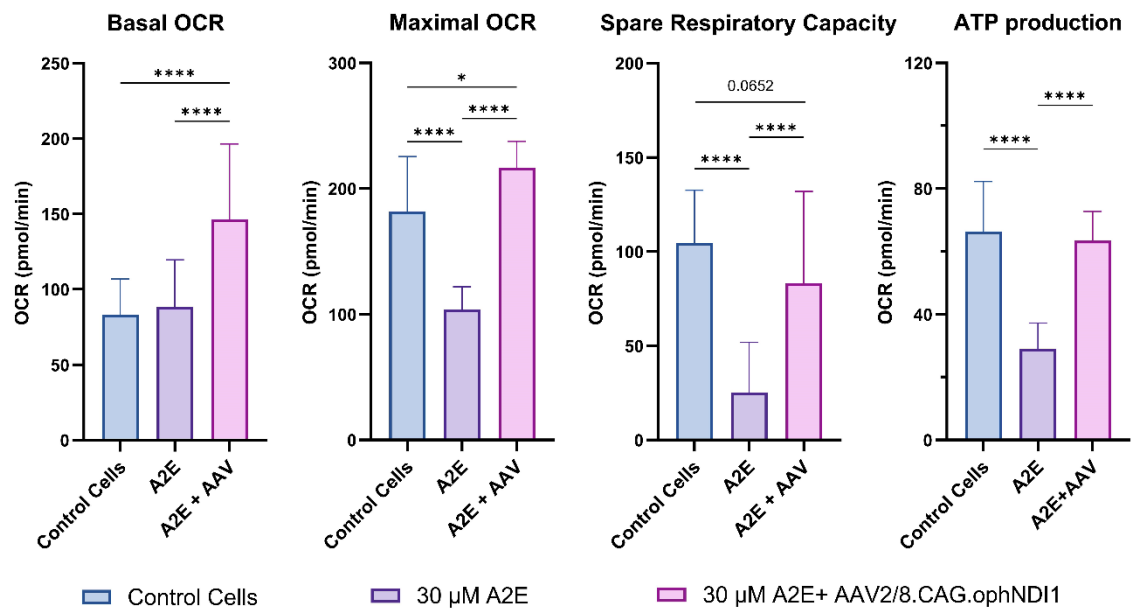
3.5.5. Utilisation of A2E as a cellular insult

In STGD1, dysfunction of ABCA4 leads to accumulation of the retinoid A2E, which disrupts metabolism in RPE cells, ultimately causing cell death (Sparrow *et al.* 2003; Sparrow *et al.* 2012; Saadat *et al.* 2014). While sodium iodate mimics certain facets of disease, no model recapitulates all aspects perfectly. Therefore, a second model, based on A2E, was employed to examine effects of NDI1 more thoroughly. A2E is extremely sensitive to light and becomes phototoxic after exposure to blue light (reviewed by Cougnard-Gregoire *et al.* 2023). It was applied to cells in darkness with only a small amount of dark red light available and then placed on a blue LED screen, which complicated optimisation of the protocols involving A2E. Cell morphology could not be checked with light microscopy until the end of the selected incubation period, as exposing it to light would degrade the chemical and render it inoperative. Additionally, as with the sodium iodate insult, the various assays require individual methods and doses, each of which had to be optimised individually.

The Seahorse analyser was employed again to examine oxygen consumption rates in cells insulted with A2E and treated with AAV2/8.CAG.ophNDI1. Both ARPE19 cells and primary porcine RPE cells were initially employed, but only pPRE cells yielded replicable results; these are presented in Figure 3.17. RPE cells were isolated from n=3 post-mortem pig eyes as described in M&M section 2.6.6. Cells were seeded in Seahorse analyser cell culture plates at a density of 1.375×10^4 cells per well and a minimum of 3 wells were transduced with AAV2/8.CAG.ophNDI1 the following day. After 48 hours a minimum of 20 wells, plus the transduced wells, were insulted with 30 μ M A2E in low-light conditions. The cells were then exposed to 3 hours of blue light before a mitochondrial stress test was carried out utilising the Seahorse Analyser. Cells were normalised as described (section 2.6.10). This assay was repeated 3 times, using cells from a new porcine eye each time. The oxygen consumption rate over time is presented in Figure 3.17, where thin dotted lines represent the mean measurements of each run and the continuous line represents the means of all assays. Figure 3.17b displays the various outcomes of this assay. The basal oxygen consumption rate was significantly higher in cells treated with AAV2/8.CAG.ophNDI1 compared to control cells and cells insulted with A2E (control cells 83.08 ± 23.8 vs A2E 88.18 ± 31.38 vs A2E+AAV 146.46 ± 49.9 pmol/min; $p < 0.0001$ for both). The Maximal OCR was significantly reduced in A2E-insulted cells (cells 181.6 ± 43.82 vs A2E 103.75 ± 18.06 pmol/min; $p < 0.0001$) and could be rescued by AAV2/8.CAG.ophNDI1 (A2E+AAV 216.65 ± 20.97 pmol/min; cells vs A2E+AAV $p = 0.0187$; A2E vs A2E+AAV $p < 0.0001$). The spare respiratory capacity, indicating the cells ability to deal with stress, is severely reduced after an insult with A2E (cells 104.65 ± 27.93 vs A2E 25.19 ± 26.65 pmol/min, $p < 0.0001$), but could also be rescued by AAV2/8.CAG.ophNDI1 (83.09 ± 48.84 pmol/min; A2E vs A2E+AAV $p < 0.0001$). The SRC was slightly lower in cells treated with A2E and AAV2/8.CAG.ophNDI1 than in control cells, but

this trend was not significant ($p=0.0652$). Finally, the OCR used for ATP production was significantly lowered in cells treated with A2E compared to control cells (cells 66.4 ± 15.91 vs A2E 29.02 ± 8.2 pmol/min; $p < 0.0001$), indicating that less ATP is being produced in these cells. Treatment with AAV2/8.CAG.ophNDI1 rescued the ATP production in cells that were insulted with A2E (63.38 ± 9.39 pmol/min; A2E vs A2E+AAV $p < 0.0001$). Figure 3.17a visualises the effects of rotenone and antimycin A on cells; as NDI1 is immune to rotenone, the difference between OCR post-rotenone injection and post-antimycin A injection can directly be attributed to successful delivery and functioning of NDI1.

Figure 3.17. Mitochondrial Stress Test on primary porcine RPE cells insulted with A2E and treated with AAV2/8.CAG.OPHNDI1. Primary RPE cells were isolated from post-mortem pig eyes ($n=3$), seeded at a density of 1.375×10^4 cells per well and transduced with AAV2/8.CAG.OPHNDI1 (M.O.I. 3.4×10^5) the following day. After 2 days cells were insulted with $30 \mu\text{M}$ of A2E and exposed to blue light for 3 hours before being analysed with the Seahorse XF analyser. This assay was performed thrice and cells from one pig were used in each repeat with multiple wells analysed per repeat ($n > 20$ for control cells, $n > 20$ for A2E-treated cells, and $n=3$ or $n=4$ for A2E+AAV). **A.** Oxygen Consumption Rate is represented over time following injections with various chemicals, which are indicated by vertical black dotted lines. The mean measurements per treatment are depicted in the heavy coloured lines, while individual runs are depicted by dotted lines (- · - · = run 1; - - = run 2; ··· = run 3). **B.** Bar charts represent the impact of A2E and AAV2/8.CAG.ophNDI1 on OCR after various chemical injections. Basal Oxygen Consumption rate was significantly higher in cells that received AAV2/8.CAG.ophNDI1 (cells 83.08 ± 23.8 vs A2E 88.18 ± 31.38 vs A2E+AAV 146.46 ± 49.9 pmol/min). The Maximal OCR was significantly decreased in cells insulted with A2E (cells 181.6 ± 43.82 vs A2E 103.75 ± 18.06 pmol/min; $p < 0.0001$) but rescued by AAV2/8.CAG.ophNDI1 (A2E+AAV 216.65 ± 20.97 pmol/min; cells vs A2E+AAV $p=0.0187$; A2E vs A2E+AAV $p < 0.0001$). Spare respiratory capacity (SRC) was significantly reduced in A2E insulted cells and rescued by AAV2/8.CAG.ophNDI1 (cells 104.65 ± 27.93 vs A2E 25.19 ± 26.65 vs A2E+AAV 83.09 ± 48.84 pmol/min; cells vs A2E $p < 0.0001$; A2E vs A2E+AAV $p < 0.0001$). Oxygen consumed for ATP production was significantly decreased in cells treated with A2E and rescued by AAV2/8.CAG.ophNDI1 (cells 66.4 ± 15.91 vs A2E 29.02 ± 8.2 vs A2E+AAV 63.38 ± 9.39 pmol/min; cells vs A2E $p < 0.0001$; A2E vs A2E+AAV $p < 0.0001$). Bars represent the mean with error bars indicating standard deviation. $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$; $p < 0.0001 = ****$

A**B**

3.5.6. Immunocytochemistry

Both ARPE19 and primary porcine RPE cells were transduced with NDI1 and treated with NaIO_3 before undergoing immunocytochemistry staining that allowed us to investigate potential benefit provided by the NDI1 therapy. ARPE19 cells were seeded with a density of 5×10^4 cells per well and transduced with 2.7×10^{10} viral genomes, corresponding to a MOI of 5.4×10^5 . The virus used on these cells was of serotype AAV2/8. After 48 hours the ARPE19 cells were treated with 5mM NaIO_3 for 24 hours. The cells were fixed in 4% PFA and stained with HOECHST (nucleus), Phalloidin-iFluor 647 (actin filaments), 8-OHdG-Alexa Fluor 488 (oxidative stress marker) and CPN60 (mitochondria). Cells treated only with NaIO_3 showed considerably higher levels of oxidative stress (8-OHdG) than untreated control cells and cells that received the therapeutic AAV (Figure 3.18). Actin filaments are only absent in NaIO_3 -insulted cells but not in cells transduced with AAV.CAG.ophNDI1. Furthermore, the mitochondrial staining was more punctuated and stronger, possibly indicating mitochondrial dysfunction, in the insulted cells but not in cells transduced with AAV.CAG.ophNDI1 before the insult with NaIO_3 . All together, these observations suggest that NaIO_3 causes these ARPE19 cells severe stress and cellular dysfunction. Critically, the data suggest that these effects can be rescued by transduction with AAV.CAG.ophNDI1. Indeed, we have seen that AAV.CAG.ophNDI1-transduced cells can stay alive for multiple days longer than untransduced cells after NaIO_3 treatment. Figure 3.19 depicts this as well. Here, the cells were treated in the same way as described here, but they were fixed and stained 48 hours after the NaIO_3 insult.

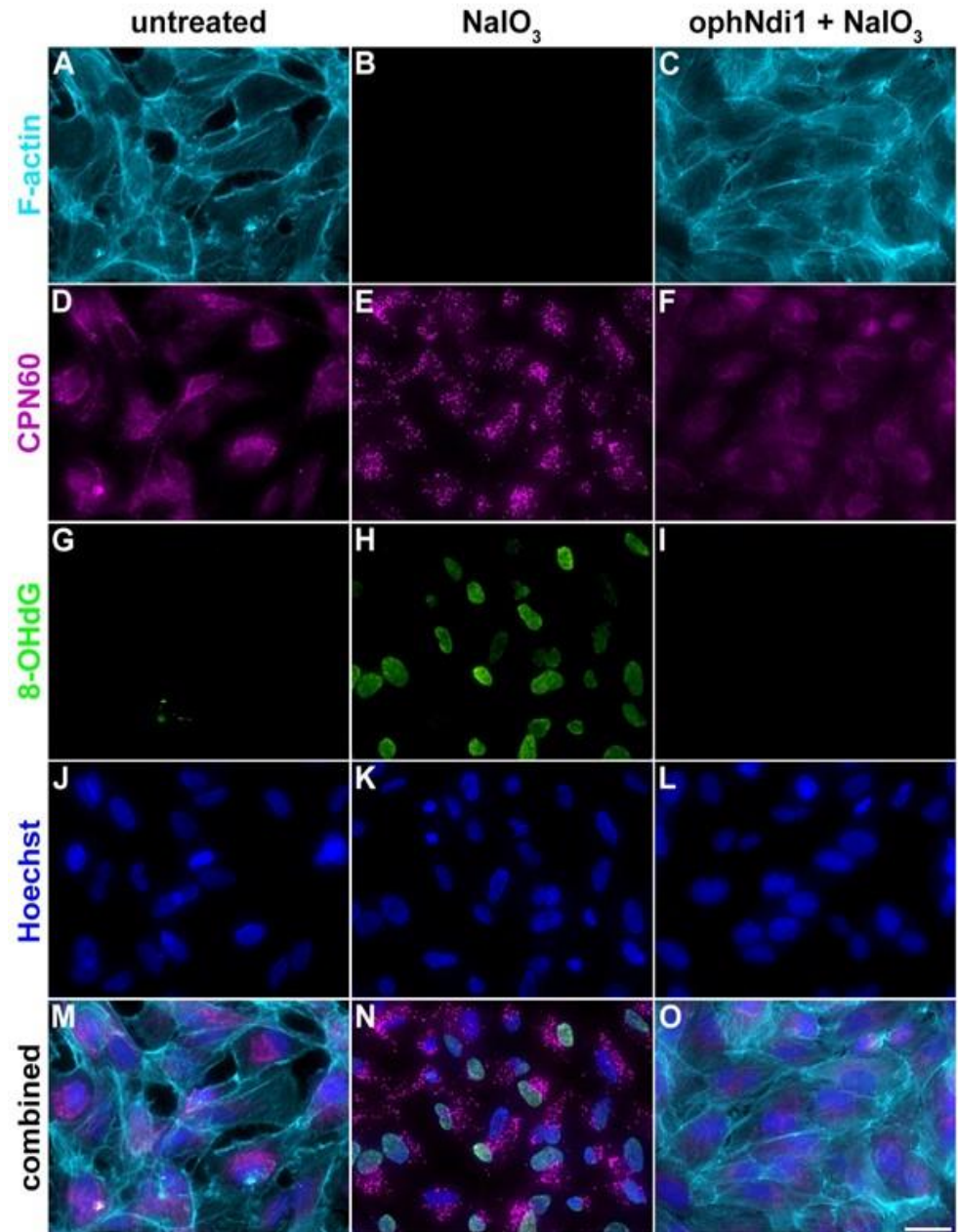


Figure 3.18. Rescue of ARPE19 cells 24 hours post NaIO₃ insult with AAV.CAG.ophNDI1. C, F, I, L, O: Cells were transduced with AAV2/8-CAG-NDI1 5 hours post-seeding at an MOI of 5.4×10^5 . B, C, E, F, H, I, K, L, N, O: Cells were insulted with 5mM NaIO₃ 28 hours post-transduction. All: Cells were fixed and stained with Phalloidin-iFluor 647 (F-actin, light blue), and CPN60 (magenta) and 8-OHdG-Alexa Flour 488 (green) immunocytochemistries; nuclei were counterstained with Hoechst (dark blue) 24 hours post-NaIO₃ treatment. Scale bar (O): 25 μ m

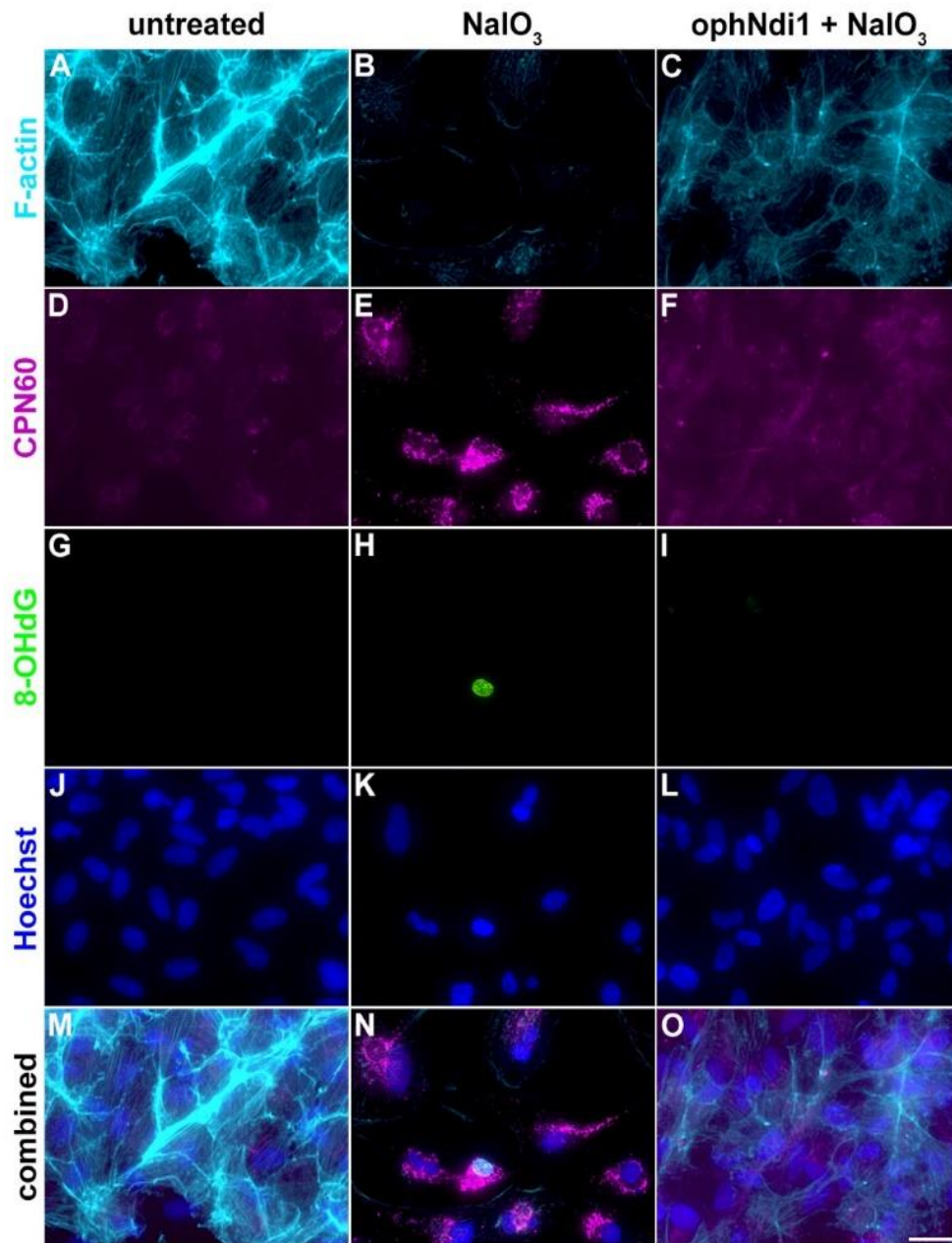


Figure 3.19. Rescue of ARPE19 cells 48 hours post insult with NaIO₃. **C, F, I, L, O:** Cells were transduced with AAV2/8.CAG.ophNDI1 5 hours post-seeding at an MOI of 5.4×10^5 . **B, C, E, F, H, I, K, L, N, O:** Cells were insulted with 5mM NaIO₃ 28 hours post-transduction. **All:** Cells were fixed and stained with Phalloidin-iFluor 647 (F-actin, light blue), and CPN60 (magenta) and 8-OHdG-Alexa Fluor 488 (green) immunocytochemistries; nuclei were counterstained with Hoechst (dark blue) 48 hours post-NaIO₃ treatment. Scale bar (**O**): 25 μ m

To further explore the therapeutic potential of AAV.CAG.ophNDI1, the experiment was also performed on primary porcine RPE cells (Figure 3.20). Primary pig RPE cells were seeded with a density of 7.5×10^4 in 8-well chamber slides as per section 2.8.1. These cells were transduced with AAV2/8 at a MOI of 5.4×10^4 and treated with 5mM of NaIO_3 after 28 hours. After 24 hours the slides were fixed and stained with Hoechst (nucleus), Phalloidin-iFluor 647 (actin filaments), 8-OHdG-Alexa Fluor 488 (oxidative stress marker) and CPN60 (mitochondria). The NaIO_3 insult caused higher oxidative stress levels, degradation of actin filaments and increased and punctuated mitochondrial staining as compared to control cells. Transduction with AAV2/8-NDI1 rescued these phenotypes. Repeat of the assay with a 48-hour time point after NaIO_3 -treatment showed that these protective effects prevail even after the untreated cells are completely gone (Figure 3.21).

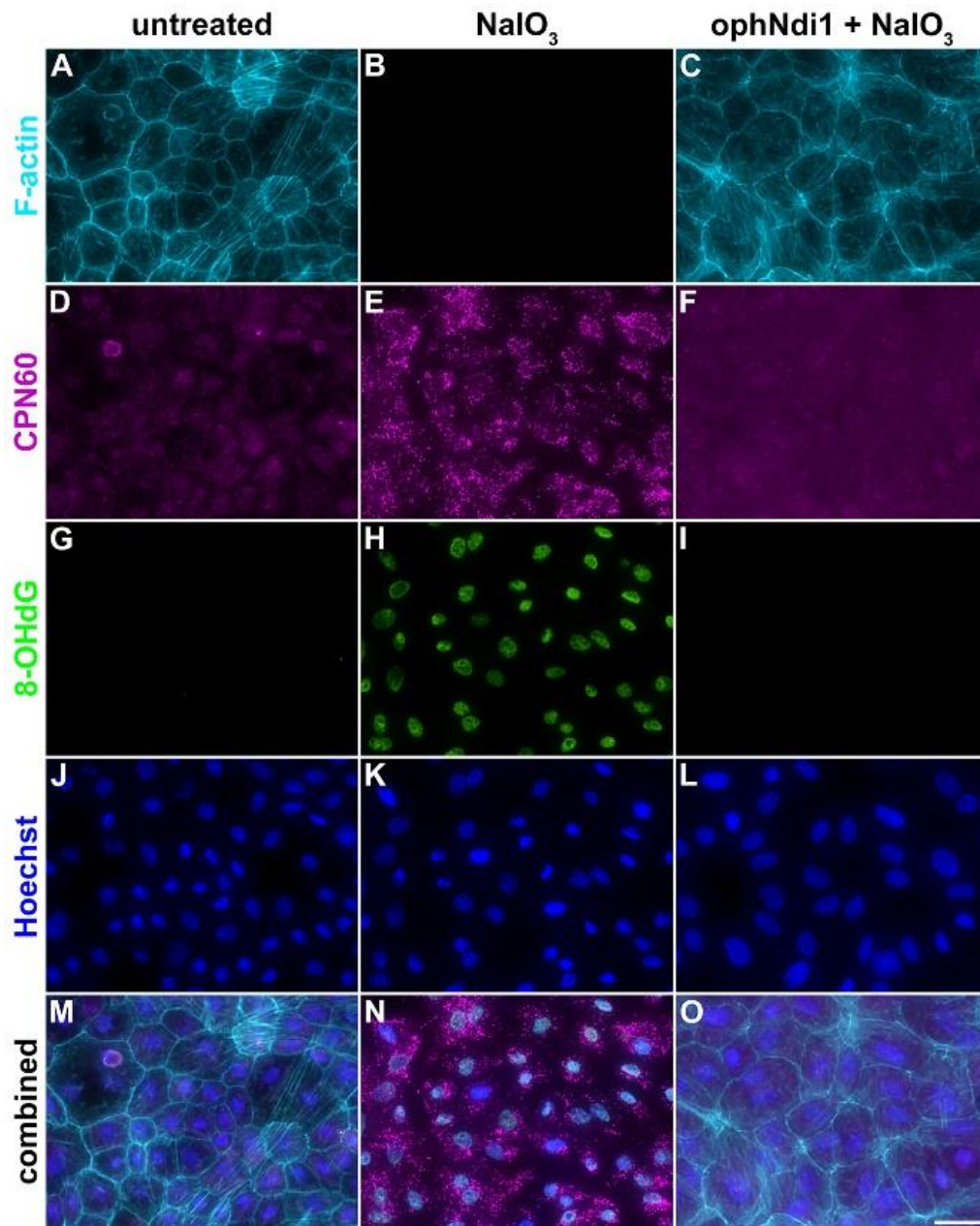


Figure 3.20. Primary pig RPE cells are rescued by treatment with AAV2/8.CAG.ophNDI1 24 hours post NaIO₃-insult C, F, I, L, O: Cells were transduced with AAV2/8-CAG-NDI1 5 hours post-seeding at an MOI of 5.4×10^5 . **B, C, E, F, H, I, K, L, N, O:** Cells were insulted with 5mM NaIO₃ 28 hours post-transduction. **All:** Cells were fixed and stained with Phalloidin-iFluor 647 (F-actin, light blue), and CPN60 (magenta) and 8-OHdG-Alexa Flour 488 (green) immunocytochemistries; nuclei were counterstained with Hoechst (dark blue) 24 hours post-NaIO₃ treatment. Scale bar (O): 25 μ m

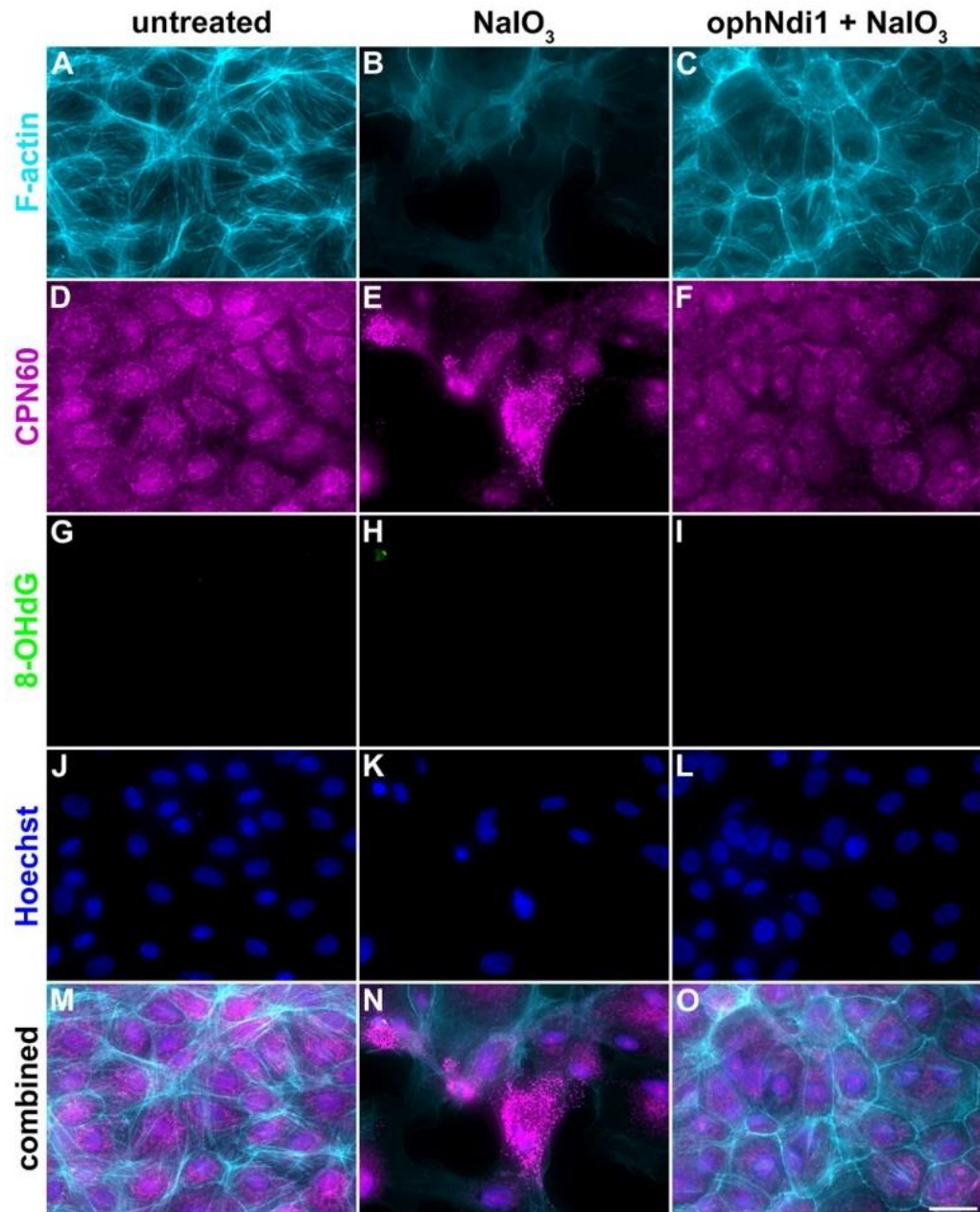


Figure 3.21. Primary pig RPE cells are rescued by treatment with AAV2/8.CAG.ophNDI1 48 hours post-NaIO₃ insult. C, F, I, L, O: Cells were transduced with AAV2/8-CAG-NDI1 5 hours post-seeding at an MOI of 5.4×10^5 . **B, C, E, F, H, I, K, L, N, O:** Cells were insulted with 5mM NaIO₃ 28 hours post-transduction. **All:** Cells were fixed and stained with Phalloidin-iFluor 647 (F-actin, light blue), and CPN60 (magenta) and 8-OHdG-Alexa Flour 488 (green) immunocytochemistries; nuclei were counterstained with Hoechst (dark blue) 24 hours post-NaIO₃ treatment. Scale bar (O): 25 μ m

3.5.7. AAV.CAG.ophNDI1 reduces cellular levels of Reactive Oxygen Species in cell models of disease

Furthermore, a flow cytometry-based assay was evaluated to examine oxidative stress in insulted and treated cells. ARPE19 cells and primary porcine RPE cells were seeded in 48-well plates at a density of 5×10^4 cells/well, respectively, and transduced with AAV.CAG.ophNDI1 24 hours later. After a 48-hour incubation period the cells were insulted with A2E and exposed to blue light before being incubated in total darkness at 37°C. Cells were then washed, trypsinised, and treated with CellRox™ as described above. However, this assay emerged to be complicated as the processing of the cells during this assay put physical strain on them, causing a significant number of insulted cells to die. As a result, only very low numbers of insulted cells remained, which skewed the outcome of the assay. An example of this is presented in figure 3.22. Here, pRPE cells (of n=1 pig) were insulted with 50 and 70µM A2E, respectively and exposed to blue light for 3 hours. After overnight recovery in total darkness, cellular ROS levels and cell viability were analysed. However, percentages of live cells were extremely low in insulted cells (Control $27.03 \pm 4.68\%$ vs 50µM A2E $11.63 \pm 4.87\%$ vs 70 µM A2E $0.54 \pm 0.34\%$), which makes the cellular ROS levels unreliable.

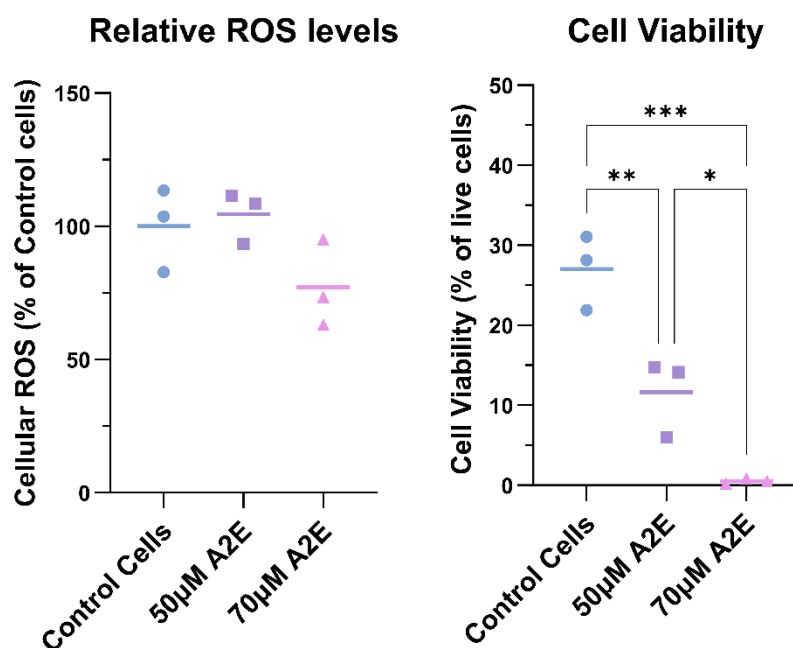


Figure 3.22. Overnight A2E exposure drastically reduces cell viability in primary RPE cells. Cells were seeded in 48-well plates (n=3 per treatment) and treated with A2E the following day and exposed to blue light. After 3 hours the light was switched off and cells were incubated overnight in full darkness, before being analysed with the CellRox-based FACS-assay. A. ROS levels were normalised to the mean ROS levels of control cells. No significant difference was seen between any of the groups. B. Cell viability was extremely reduced in cells insulted with A2E (Control 27.03 ± 4.68 vs 50µM A2E 11.63 ± 4.87 ; vs 70 µM A2E 0.54 ± 0.34). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Table 3.1 provides an overview of various adjustments that were made to this assay in our pursuit to optimise it. Each row represents a combination of A2E dose, time of blue light exposure, and recovery time that was tested. Ultimately, the exposure to blue light was reduced to 1 hour and immediate experimentation thereafter. This approach damaged the cells enough to mimic disease, but allowed for analysis of cells before cell death occurred. Cellular ROS levels were raised after treatment with 50 μ M of A2E (control $100.00 \pm 29.42\%$ vs A2E $159.13 \pm 13.95\%$) though this variability was not statistically significant ($p=0.11$). Treatment with AAV2/2.CAG.ophNDI1 at a MOI of 2.3×10^6 vg/cell significantly mitigated cellular ROS levels in cells insulted with A2E (129.52 ± 21.01 ; $p=0.0195$).

Table 3.1. Various optimisation steps tested for cellular ROS assays on cell models of disease

A2E dose (μ M)	Blue light exposure	Cell recovery time
15	30 minutes	16 hours
25	30 minutes	6 hours
25	3 hours	none
50	30 minutes	16 hours
50	30 minutes	6 hours
50	3 hours	none
50	3 hours	16 hours
50	6 hours	none
70	3 hours	16 hours

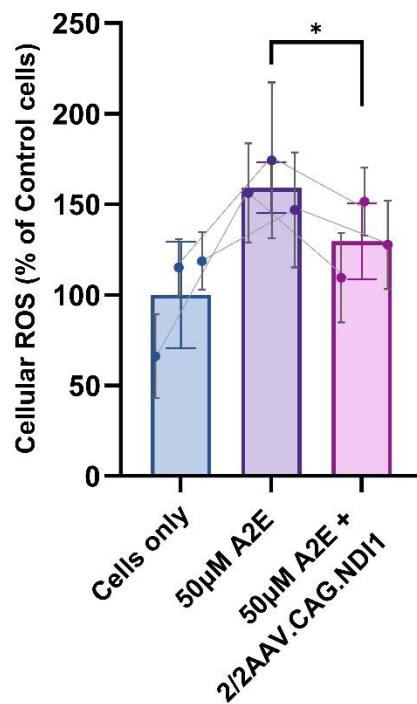


Figure 3.23. Cellular ROS levels are significantly reduced in cells treated with AAV2/2.CAG.OPHNDI1. Primary porcine RPE cells (from n=3 pigs) were seeded at a density of 1.35×10^4 cells per well and transduced with AAV2/2.CAG.OPHNDI1 at a MOI of 2.3×10^6 . After 48 hours cells were insulted with 50µM of A2E and exposed to blue light for 1 hour. CellRox Red was used to quantify cellular ROS levels, which were then relativised to control cells. Per pig eye, 5-6 replicates were used in each group and averaged out as one measurement, which are represented by the dots in each bar. Grey lines link data points of individual pigs between treatment groups. Cellular ROS levels in control cells were set at 100.00 and had a SD of 29.42. A2E-insulted cells showed an increase in cellular ROS-levels (159.13 ± 13.95) compared to control cells but this trend was not significant ($p=0.118$). Treatment with AAV2/2.CAG.OPHNDI1 significantly reduced cellular ROS numbers in cells insulted with A2E (129.52 ± 21.01 ; $p=0.0195$).

In summary, in this chapter the use of AAV vectors in cell models of Stargardt disease was optimised. Utilising AAV.EGFP vectors, it was established that both AAV2/2 and AAV2/8 robustly drive expression in the ARPE19 cell line and in primary RPE cells from post-mortem pig eyes. Plasmids carrying a humanised, codon-optimised version of NDI1 driven by the ubiquitous CAG-promoter were cloned and used to generate AAV.CAG.ophNDI1 serotypes AAV2/2 and AAV2/8. These therapeutic vectors proved to provide therapeutic benefit in various cell models of disease. Mitochondrial respiration rates were improved by AAV.CAG.ophNDI1 compared to insulted cells in 3 different models; i.e. ARPE19 cells insulted with NaIO_3 , pRPE cells insulted with NaIO_3 , and pRPE cells insulted with A2E. Additionally, immunocytochemistry showed that the structure of actin filaments, the mitochondrial morphology, and ROS levels were severely affected in cells insulted by NaIO_3 , and that AAV.CAG.ophNDI1 is able to rescue these parameters efficiently. Moreover, a different assay that quantifies relative ROS levels in cells demonstrated that A2E increases ROS levels significantly in as little as 1 hour and that AAV2/2.CAG.ophNDI1 significantly lowered this. Overall, it is notable that both histological and functional rescue by AAV.CAG.ophNDI1 was provided to a variety of cell models of Stargardt Disease.

3.5.8. Formal Acknowledgements of this chapter

In this paragraph I would like to highlight the division of labour of the results presented in this chapter.

Transduction efficiency assays were performed utilising AAVs carrying EGFP previously generated in our lab. The transduction, staining and imaging were all performed by me.

Cloning of pCAG.ophNDI1 was done by me, utilising plasmids previously generated in our lab. Generation of AAV.CAG.ophNDI1 was done by Dr Naomi Chadderton utilising pCAG.ophNDI1 and helper plasmids that would have been expanded by me.

Optimisation of the extraction of primary pig RPE cells in our lab was a joint effort between Dr Naomi Chadderton, Dr Sophia Millington-Ward and me, in which each person carried out 1/3 of the work. Dr Arpad Palfi would collect the eyes from the abattoir and pre-clean them. After optimisation, extracting cells from eyes remained a shared effort as this was a time-intensive yet time-sensitive process.

All mitochondrial stress tests (Seahorse analyser) were also a joint effort between Dr Naomi Chadderton, Dr Sophia Millington-Ward and me, in which each person carried out 1/3 of the work, mostly due to the fact that these assays were time sensitive and working together enabled us to perform multiple duplicate assays in succession, which yielded higher success.

For immunocytochemistry assays, cell seeding and transduction was alternatingly carried out by me and Dr Naomi Chadderton and Dr Sophia Millington-Ward and the immunocytochemistry staining and imaging were done by Dr Arpad Palfi.

The Flow Cytometry based assays to measure Reactive Oxygen species were fully done and optimised by me.

3.3. Discussion

AAVs were originally discovered as a contaminant of adenovirus preparations (Atchinson *et al.* 1965) but are now a leading gene therapy vector. In recent years, gene therapies utilising AAVs as a vehicle to transport transgenes into cells have been approved by the EMA and FDA. Examples include Glybera, a treatment for lipoprotein lipase deficiency (Gaudet *et al.* 2013), Zolgensma, a therapy for spinal muscular atrophy (Mendell *et al.* 2017; Groen *et al.* 2018), and Luxturna, a gene therapy for biallelic RPE65 inherited retinal degenerations (Maguire *et al.* 2008; Maguire *et al.* 2019). In this chapter the use of AAVs as a gene therapy for Stargardt Disease was explored in various cell models of disease. The optimisation of AAV transduction through the use of different serotypes to drive transgene expression in these cell models was the first step in this investigation. Two RPE-like cell lines, hTERT-RPE1 and ARPE19, and primary porcine RPE cells were transduced with various AAV.EGFP vectors. It was established that AAV2/2 and AAV2/8 were able to robustly drive expression of EGFP in both ARPE19 and pRPE cells, and these serotypes were therefore chosen for the design of therapeutic AAV vectors. Due to less efficient transduction and reduced proteomic and genomic similarities between hTERT-RPE1 and primary RPE cells compared to ARPE19 (Alge *et al.* 2006; Samuel *et al.* 2017), the decision to cease the use of hTERT-RPE1 and continue with only ARPE19 and primary RPE cells was made. ARPE19 and primary RPE cells from post-mortem pig eyes were treated with NaIO₃ and A2E, respectively, to mimic aspects of the disease in RPE cells.

It is important to note that in human eyes there appear to be differences between macular and peripheral RPE. Recent RNA-sequencing studies have shown that genes involved in oxidative stress responses are more highly expressed in the macular RPE, while in the peripheral RPE cells, genes involved in processes such as lipid metabolism are more highly expressed (Xu *et al.* 2021). However, it still remains unclear how this influences the origin and development of macular disorders such as AMD and Stargardt Disease, and further investigations remain necessary. Additionally, while pigs don't have a macula per se, they do have a cone-rich region that may serve similar purposes for increased visual acuity (reviewed by Middleton. 2010), which can influence the response of cells on potential therapeutic strategies.

Stargardt disease type 1 (STGD1) is estimated to affect 1 in 10000 people (Blacharski. 1988), therefore affecting many people worldwide. Dysfunction of ABCA4 causes accumulation of A2E and lipofuscin, which damages mitochondria, increases the generation of reactive oxygen species, and reduces ATP production (Saadat *et al.* 2014; Sivapathasuntharam *et al.* 2019). STGD1, being a monogenic disorder would be an ideal candidate for gene supplementation-based therapy, were it not for the size of the *ABCA4* gene. The cDNA of this gene is 6.8kb, which is too big for the commonly used gene therapy vector AAV, which has a maximal cargo capacity of

approximately 4.5kb (Wu *et al.* 2010b). Some groups are trying to bypass this issue with dual vector hybrid-systems, but side effects, such as the generation of unspecified recombinant DNA fragments with unknown consequences have not been fully eradicated yet (Dyka *et al.* 2019; McClements *et al.* 2020; Trapani 2018).

Antisense oligonucleotides (AONs) are also being explored to rescue splice defects in STGD1 (Albert *et al.* 2018; Garanto *et al.* 2019), but therapies like this are mutation specific and there are many different causative mutations in the *ABCA4* gene in STGD1. Over 2400 unique variants in *ABCA4* have been reported so far (LOVD v.3.0, assessed 17/07/2023). Due to the impact of A2E and lipofuscin accumulation on mitochondrial welfare and the size of the *ABCA4* gene, a different approach was undertaken in the current study. Common pathways of disease between STGD1 and other retinal disorders prompted the exploration of the therapeutic strategy evaluated in this study (Eells *et al.* 2019, Marie *et al.* 2018; Brown *et al.* 2019). In this chapter the use of a candidate therapy utilising the yeast complex 1 homolog NDI1 was evaluated as a means to improve cellular bioenergetics in cell models of Stargardt disease.

A vector driven by the CAG promoter and carrying an optimised and humanised variant of NDI1 (ophNDI1) was generated. The CAG promoter contains the early enhancer element of cytomegalovirus (CMV), the promoter, first exon and first intron of chicken beta-actin, and the splice acceptor of rabbit beta-globin (Miyazaki *et al.* 1989). This promoter is also employed in the FDA- and EMA-approved Zolgensma gene therapy vector (Mendell *et al.* 2017; Groen *et al.* 2018). NDI1 has been utilised to enhance mitochondrial function in models of other diseases such as Leber Hereditary Optic Neuropathy (Chadderton *et al.* 2013), Parkinson's Disease (Cronin-Furman *et al.* 2013; Marella *et al.* 2008), and Multiple Sclerosis (Talla *et al.* 2020), which are all disorders in which mitochondrial dysfunction plays a role in the mechanism of disease. Previously, our lab established that the optimised version of NDI1, ophNDI1, used in this study also shows benefit in murine models of Age-Related Macular Degeneration (Millington-Ward *et al.* 2022; 2023), a multifactorial disease that has a lot of phenotypic overlap with Stargardt disease, and in cell and murine models of retinal ganglion cell-related disorders, such as LHON (Chadderton *et al.* 2023).

In this study NaIO₃ was employed to induce oxidative stress, reactive oxygen species and mitochondrial dysfunction, which lowers cell viability and even leads to premature cell death, thereby creating a model of disease. Indeed, we found that cells treated with NaIO₃ deteriorated and showed high levels of oxidative stress (Figures 3.18 to 3.21). Actin filaments in RPE cells play an important role in maintaining cell structure, phagocytosis of photoreceptor outer segments (POS) and transport of melanosomes (Barral *et al.* 2004). Degradation of actin filaments has a

large impact on cellular welfare, and this can be used as a marker for the well-being of these cells. We have clearly shown that insult with NaIO_3 correlates with actin filament degradation, and that treatment with AAV2/8.CAG.ophNDI1 prevents this. Additionally, immunocytochemistry showed that mitochondrial morphology was abnormal and very punctuated in cells treated with NaIO_3 , and AAV.CAG.ophNDI1 was able to rescue this phenotype. Furthermore, 8-OHdG-Alexa Fluor 488, a marker for oxidative stress, was elevated in NaIO_3 cells but not in cells treated with NaIO_3 and AAV.CAG.ophNDI1 (Figures 3.18 to 3.21).

In addition, a cell model based on A2E, a bisretinoid known to accumulate and cause cytotoxicity in Stargardt disease, and blue light was established to verify potential benefits of AAV.CAG.ophNDI1. Photo-oxidation of A2E by blue light generates ROS, causing damage to retinal cells such as photoreceptors and RPE (Wu *et al.* 2010a; Yamamoto *et al.* 2012). In our study, primary pig RPE cells were insulted with A2E and exposed to blue light. Here, we found that AAV.CAG.ophNDI1 was able to significantly reduce cellular ROS levels (Figure 3.23).

Mitochondrial function was severely and significantly diminished in both ARPE19 cells and primary porcine RPE cells after insulted with either NaIO_3 or A2E (Figures 3.15 to 3.17). In this study, we found that mitochondrial dysfunction in cellular models of disease could be rescued with administration of NDI1 via AAV. Notably, the Oxygen Consumption Rate (OCR), which is an indicator of cellular health and mitochondrial function, the maximal OCR, and the ATP production were significantly increased in NaIO_3 -insulted cells that were transduced with AAV.CAG.ophNDI1 as compared to cells that only received NaIO_3 . This was consistently the case both in models based on RPE-like cell lines (ARPE19) and in primary porcine RPE cells. However, in the NaIO_3 -based model the Spare Respiratory Capacity (SRC) was significantly reduced in AAV.CAG.ophNDI1-treated cells as compared to control cells, both in the assays with ARPE19 and with primary RPE cells. The SRC was calculated as the difference between the maximal OCR and the basal OCR and it represents the metabolic energy reserves cells can use in case of a sudden increase in demand. In this case, AAV.CAG.ophNDI1 greatly increased the basal OCR, but the maximal OCR, though significantly higher than in NaIO_3 -insulted cells, was barely higher than the basal OCR in both cell lines, suggesting that cells rescued by NDI1 may be less stress resistant than control cells. Similarly, primary RPE cells insulted with A2E displayed reduced basal and maximal OCRs, SRC, and ATP production compared to control cells. Treatment with AAV.CAG.ophNDI1 rescued each of these parameters, indicating once more that NDI1 can provide functional benefit to cell models of disease.

In this chapter we used cell models to show that an AAV vector driving expression of NDI1 could be a suitable therapeutic strategy for retinal diseases such as Stargardt disease. Augmentation

of mitochondrial function can reduce oxidative stress and provide additional metabolic energy to cells. Following the *in vitro* experiments in this chapter, potential beneficial effects of the AAV.CAG.ophNDI1 vector were investigated in murine models of Stargardt disease, which is described in detail in the following chapter.

4. Investigation of therapeutic benefits of AAV.CAG.NDI1 on a mouse model of disease

4.1. Introduction

In vitro cell culture models are a great way to explore the effect of therapies on cells, particularly on specific organelles and structures. In the previous chapter of this thesis transduction efficacies of various AAV serotypes and the beneficial effects of our therapeutic AAV vectors were explored extensively in retinal cell lines, primary porcine RPE cells, and cell models of disease. However, the biological processes in the whole body are very complex and different cell types influence each other in many ways, as does the immunology of the target tissue and vasculature; such aspects are almost impossible to replicate in tissue culture models, even with the scientific advances of today such as induced pluripotent stem (iPS) cell-derived retinal organoids. Functionality, safety, and efficacy of a therapeutic agent might vary substantially between *in vitro* and *in vivo* models of disease. Therefore, it is important to explore therapies in *in vivo* models to gain a better understanding of their effects on disease mechanisms. In addition, only *in vivo* models enable functional benefit to be determined.

Upon review of the literature there are several *Abca4*^{-/-} animal models for Stargardt disease in various species, of which mice may be the most commonly used. *Abca4* knockout mouse models have been established on several backgrounds, including BALB/c, C57BL/6, and 129S. The BALB/c *Abca4*^{-/-} mouse, which is an albino mouse, presents with increased A2E and lipofuscin accumulation from 3 months of age onwards, an effect that can be magnified when these mice are fed a vitamin A-rich diet (Radu *et al.* 2008), and degeneration of photoreceptor cells is observed in one-year-old mice (Lenis *et al.* 2018). However, albinism is strongly associated with visual impairment even in the absence of the *Abca4*^{-/-} mutation (Summers *et al.* 2004; Braha *et al.* 2021), and even photoreceptor renewal varies between albino *Abca4*^{-/-} and pigmented *Abca4*^{-/-} mice (Lyu *et al.* 2023), so this model was not selected for this study. The *Abca4*^{-/-} mice that are available on C57BL/6 and 129S genetic backgrounds both carry the engineered *Abca4*^{tm1Ght} gene, in which the promoter and first exon of the *Abca4* gene have been replaced by an inverted neomycin resistance cassette, preventing translation of the gene into a protein (Weng *et al.* 1999). Immunoblotting revealed that no *Abca4* protein was present in the samples of homozygous *Abca4*^{-/-} mice (Weng *et al.* 1999). This model has been at the forefront of research looking into *Abca4*-related retinal degeneration, and it shows many disease features similar to humans, such as increased A2E and lipofuscin accumulation (Issa *et al.* 2013; Issa *et al.* 2015), photoreceptor degeneration (Bok, 2005) and delayed dark adaption after photo bleach (Weng *et al.* 1999). Another murine model with a different mutation, which was engineered

independently but also lacks exon 1, exhibits a similar phenotype, although the delay in dark adaption was less noticeable (Kong *et al.* 2008).

Two knock-in mouse lines have also been established, in which deleterious mutations found in human Stargardt patients have been introduced in mice. Mice homozygous for the p.Asn965Ser (N965S) Abca4 variant express only half the level of the Abca4 protein in photoreceptor cells compared to wild type mice and present with a 10-fold increase of A2E, elevated levels of lipofuscin, and increased fundus autofluorescence (Molday *et al.* 2018). However, functional deficits are not reported. Another knock-in model, Abca4^{PV/PV}, carries the complex allele Leu541Pro;Ala1038Val (L541P;A1038V), which in humans causes a severe phenotype when both of these mutations are present, but a mild phenotype if only A1038V is present. Abca4 RNA levels in the knock-in variant were similar to wild type, but misfolding of the protein significantly reduces protein levels, leading to accumulation of A2E and lipofuscin (Zhang *et al.* 2015). Again, no functional deficits were found in Abca4^{PV/PV} until 12 months of age, making this a slow model, which was therefore not selected for this study.

Mouse models in which multiple genes have been knocked out to exacerbate and accelerate the phenotype also exist. Examples of these include the widely used Abca4^{-/-}/Rdh8^{-/-} and Abca4^{-/-}/Rdh8^{-/-}/Nrl^{-/-} lines, but as both RDH8 and NRL are genes involved in the visual cycle and/or associated with retinal disease, the phenotype in these mice does not fully represent Stargardt disease (Maeda *et al.* 2008). Another mouse model was created by crossing the C57BL/6 background Abca4^{tm1Ght} mouse (described above) to a Bco2 knockout mouse. Bco2 is involved in the oxidation of beta-carotene, a precursor of vitamin A. However, since knockout of Bco2 suppresses A2E levels in RPE cells, this model was not considered for this study (Arunkumar *et al.* 2021).

Naturally occurring mutations (p.F1393Lfs*1395) in the Abca4 gene leading to a premature stop codon in exon 28 have been found in Labrador retriever dogs. These dogs presented with a phenotype similar to humans, including lipofuscin-induced autofluorescence in the retina, photoreceptor degeneration, and thinning of the outer nuclear layer (ONL) of the retina at ages between 10 and 12 years (Mäkeläinen *et al.* 2019). However, for practical and emotional reasons, dogs were not considered for the current study.

A novel rat model has also been created, in which CRISPR/Cas9 was utilised to excise exon 42 of the Abca4 gene leading to a loss of functional protein. A2E levels were increased three-fold in Abca4^{ex42-/-} rats compared to wild type rats and thinning of the ONL was observed at 12 months of age, although assessment through electroretinography did not reveal any changes (Cronin *et*

al. 2021). As rats are bigger than mice and their lifespans are longer, and in addition the phenotype of this model being very similar to mouse models, a mouse model was selected over a rat model.

In this study, the mouse model of choice is *Abca4*^{tm1Ght} on a 129S background (see above; Weng *et al.* 1999). This mouse model has been characterised previously by other groups doing work on therapies for Stargardt disease and AMD. Weng *et al.* (1999) noted that the *Abca4*^{-/-} mice displayed increased levels of all-trans-retinaldehyde (at-RAL), phosphatidylethanolamine (PE), and N-retinylidene- phosphatidylethanolamine (N-ret-PE) in the photoreceptor outer segments, and accumulation of A2E in the RPE. A2E is a known fluorophore that plays a large role in the formation and accumulation of the autofluorescent pigment lipofuscin. In human patients with Stargardt disease the accumulation of A2E and lipofuscin drastically reduces cell viability of photoreceptor and RPE cells (Zhou *et al.* 2006; Moiseyev *et al.* 2010; Sparrow *et al.* 2012; Saadat *et al.* 2014). A2E levels in these mice at 6 months old is 10 times higher than in age matched wild type controls and fundus autofluorescence (FAF) imaging showed that the intensity of autofluorescence from lipofuscin is doubled in the *Abca4*^{-/-} model (Issa *et al.* 2013). No ERG deficits were observed in this model. There are two variants of this mouse model, one carrying a methionine or leucine at position 450 of the Rpe65 gene. Mice with Leu450 have higher A2E levels in the RPE than mice with Met450 (Kim *et al.* 2004). Therefore, initially this model was evaluated for presence of the *Abca4*^{-/-} mutation and RPE65 variant as described below.

Once the *Abca4*^{tm1Ght} mice were received from the Jackson labs by our animal facility in TCD and an extensive breeding programme was initiated, additional characterisation of resulting offspring has been carried out on this *Abca4*^{-/-} murine model, which is presented in this chapter. ERGs, autofluorescence histology and ROS assays were performed on aged *Abca4*^{-/-} mice and compared to wild type controls. It was important to characterise the disease phenotype when housed in the conditions in our animal facility in TCD. Subsequently, the *Abca4*^{-/-} mice were treated with the candidate gene therapy AAV2/8.CAG.NDI1 via subretinal injection and results will be presented below.

4.2. Results

Initially, prior to treating the $Abca4^{-/-}$ mice with our gene therapy, the model was characterised further. First, it was important to establish that the mice we received were indeed $Abca4$ knock-out and also to confirm which polymorphic variant the mice carried at position 450 of Rpe65. Electrophysiology, histology and ROS levels of $Abca4^{-/-}$ mice were determined and compared to age-matched wild type controls in order to understand the model better. Following this, $Abca4^{-/-}$ were treated in one eye with our therapeutic vector and electrophysiology was performed on live mice.

4.2.1. Genetic characterisation of $Abca4^{-/-}$ mice:

The mouse model used in this study is $Abca4^{tm1Ght}$, which corresponds to JAX stock number #023725. The promoter and first exon of the $Abca4$ gene are replaced with a neomycin cassette, which fully knocks out this gene. Upon receipt of the mice, some genotyping was carried out to confirm the mice are indeed homozygous knockouts. The JAX lab recommends this and the primers they suggest for genotyping were used. The primers were named mouse_abca4_F, mouse_abca4_wt_R and mouse_abca4_mut_R and their corresponding sequences can be found in Supplemental Table 3. DNA was taken from ear clips of the original breeding pairs and isolated as described in section 2.4.7. PCRs were carried out and the PCR product was run out on an electrophoresis gel. The mutant product is 160bp and the wild type product 123bp. Heterozygous mice would yield both products. Figure 4.1 shows this gel and confirms that the $Abca4^{-/-}$ mice are homozygous carriers of the mutated $Abca4$ gene.

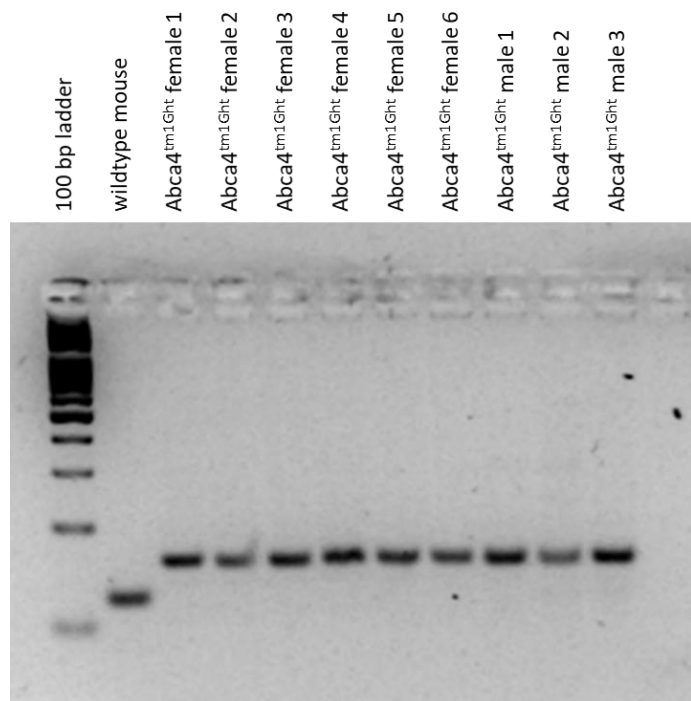


Figure 4.1. Gel electrophoresis showing PCR products confirming that the $Abca4^{tm1Ght}$ mice carry a mutated $Abca4$ promoter as compared to wild type. Wild type product is 123bp and the mutant product 160bp.

4.2.2. Interrogation of the RPE65 Met450Leu variant in *Abca4*^{-/-} mice

Additionally, a paper by Issa *et al.* (2013) noted that their *Abca4* knock out mice carried a mutation, Met450Leu in the *Rpe65* gene, with the Leu450 variant being associated with increased A2E accumulation in retinal cells. To verify whether our mouse model and controls also carried this mutation, tissue samples were taken from these mice via ear clipping. DNA was extracted as per M&M section 2.4.7. Primers as designed in the Issa *et al.* paper were used to amplify the region surrounding the suspected mutation via PCR. The size of the PCR product was verified via gel electrophoresis. The PCR product was then purified as described in section 2.4.4 before being sequenced as described in section 2.5.1. Figure 4.2 shows that both our wild type and our mutant models are homozygous carriers for the Met450Leu mutation.

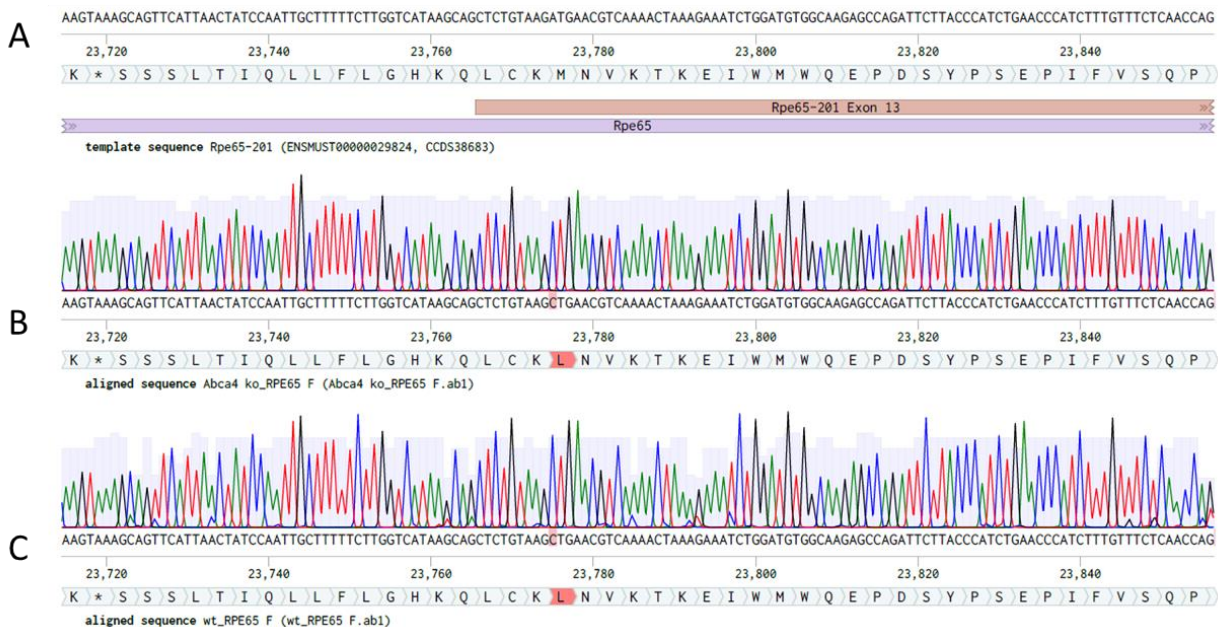


Figure 4.2. Sanger Sequencing shows that wild type 129 and *Abca4*^{-/-} both carry a Met450Leu mutation in the *Rpe65* gene. A. Standard sequence of mouse *Rpe65* according to GRCm38 (mm10, mus musculus). B. Sequence of *Abca4*^{-/-} 129s mouse. C. Sequence of wild type 129s mouse.

4.2.3. Evaluation of ERG over time in wild type and *Abca4*^{-/-} mice

The *Abca4*^{-/-} mouse model has been characterised before, however we wanted to characterise this murine model again when housed under the conditions in our animal facility. The retina is made up of neural cells that convert light into electrical synaptic currents are converted into vision by the human brain. Electroretinograms (ERGs) measure the electric activity in retinal cells; ERGs can therefore be used as a quantitative assay to evaluate and compare the functionality of retinal cells *in vivo*. At 4.5 months of age 7 wild type 129s mice and 25 *Abca4*^{-/-} mice were assessed via electroretinography according to materials and methods section 2.9.2. For this specific characterisation assay, both eyes were evaluated in each mouse and then the average

was taken and used as one measurement per mouse. The results are represented in figure 4.3. Male (n=9) and female (n=16) *Abca4*^{-/-} mice were also compared at this age to evaluate whether any variation between the sexes might be observed, hence the larger n-number in this group. However, no significant variation was found between males and females in rod-isolated responses (male 197.19±76.23µV vs female 197.52±68.09µV; p=ns; Figure 4.3a), mixed rod- and cone responses (male 353.52±127.22 µV vs female 346.09±109.27 µV; p=ns; Figure 4.3c), cone-isolated responses (male 40.05±11.23µV vs female 38.53±12.09µV, p=ns; Figure 4.3e), or flicker responses (male 20.52±6.30µV vs female 22.39±6.46µV; p=ns; Figure 4.3f). Therefore, the division based on sex was not utilised in further tests.

When comparing wild type mice to *Abca4*^{-/-} mice, mean ERG amplitudes are generally reduced in the mutants, however the reduction was only significant in the cone-isolated response at 4.5 months, which is in line with human Stargardt disease, where cone photoreceptors are thought to deteriorate first (Lois *et al.* 2001; Sajovic *et al.* 2022). The rod-isolated response was only very slightly reduced at 4.5 months (WT 211.07±63.69 µV vs *Abca4*^{-/-} 197.31±71.95 µV; p=ns; Figure 4.3b), but a stronger trend (WT 214.00±86.29 µV vs *Abca4*^{-/-} 171.84±66.55 µV; p=0.1297; Figure 4.3b) was indicated at 12 months; the rod-isolated response went down as *Abca4*^{-/-} mice aged, while it stayed constant in the wild type mice. The mixed cone-rod response showed a similar pattern, where the variation between mutant and wild type mice was minimal at 4.5 months (WT 387.14±114.50 µV vs *Abca4*^{-/-} 348.77±113.45 µV; p=ns; Figure 4.3d) but at 12 months the difference had grown (WT 382.07±137.91 µV vs *Abca4*^{-/-} 303.36±80.87 µV; p=0.068; Figure 4.3f). As described above, the cone-isolated response was significantly reduced in 4.5 month *Abca4*^{-/-} mice as compared to their wild type counterparts (WT 58.88±12.99µV vs *Abca4*^{-/-} 39.51±11.31µV; p=0.0003; Figure 4.3f). However, at 12 months, the difference between both genotypes has disappeared (WT 39.11±13.15µV vs *Abca4*^{-/-} 36.49±10.44µV; p=ns; Figure 4.3f). This can be explained by the significant reduction in old wild type mice as compared to young wild type mice (p=0.0005) to levels similarly found in *Abca4*^{-/-} mice. The response to flickering light did not vary between genotypes at 4.5 months (WT 24.20±6.28µV vs *Abca4*^{-/-} 21.19±6.29µV; Figure 4.3k) nor at 12 months (WT 21.66±7.99µV vs *Abca4*^{-/-} 21.09±5.83µV; (Figure 4.3l). All in all, these data suggest that ERG analyses might be used to evaluate the effect of our gene therapy on the functionality of cone photoreceptors in young mice, as cone function in *Abca4*^{-/-} appears to be reduced at 4.5 months. Additionally, ERGs will be used to verify the subretinal injection of AAV2/8.CAG.ophNDI1 did not do any physical damage to the retina, as is done by Issa *et al.* (2013; 2015). Further analysis of the vector will require utilisation of additional assays.

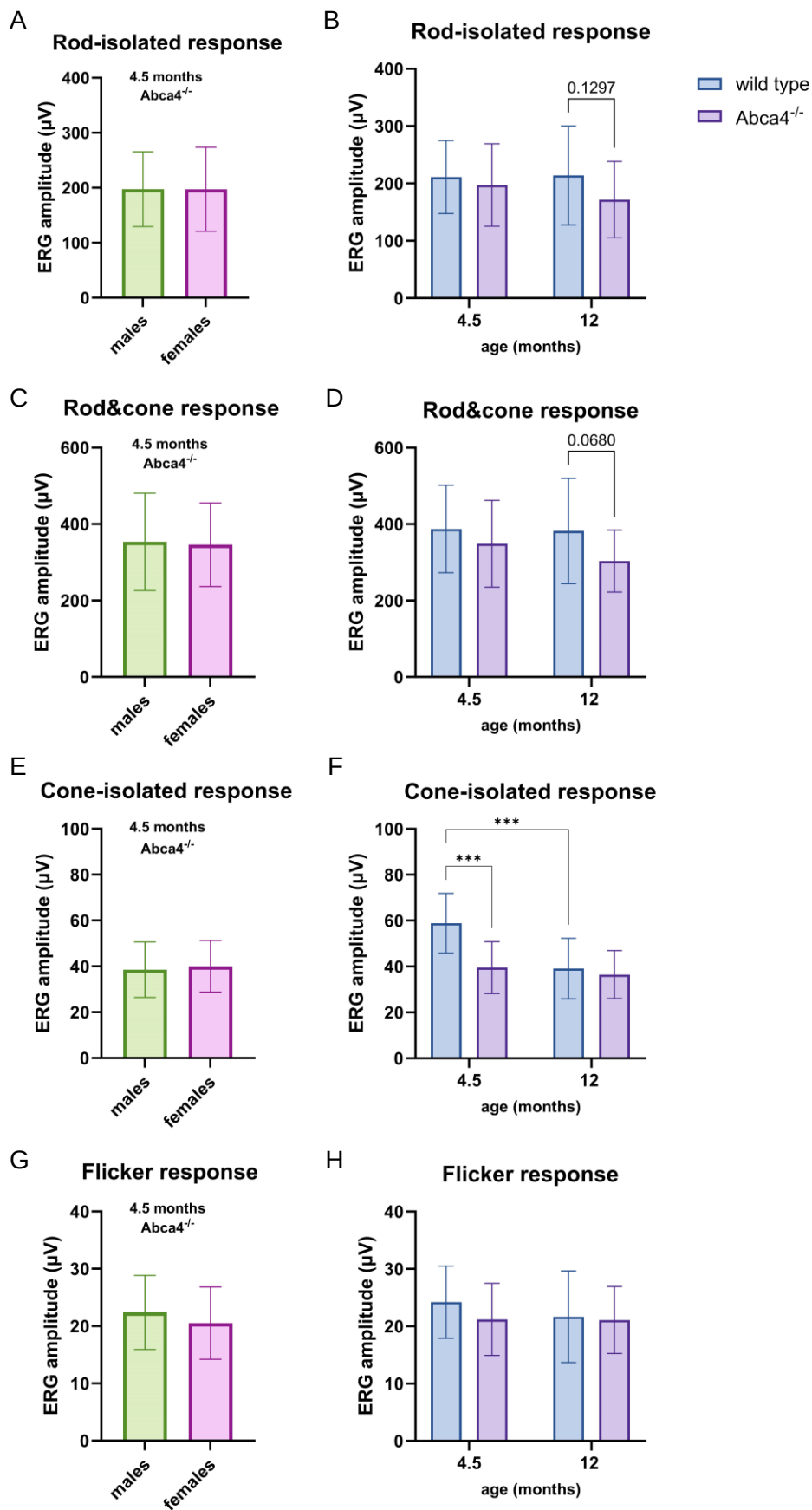


Figure 4.3. Electroretinograms of wild type and Abca4^{-/-} mice. ERGs were measured at 4.5 and 12 months of age (Abca4^{-/-} 4.5 months n=25 (9 males and 16 females), 12 months n=14; wt 4.5 months n=7, 12 months n=15). Each eye was measured separately and averaged out per mouse as one outcome. These box plots represent the mean of each sample with the range of sample measurements signified by the bars. **A.** No difference was observed in rod-isolated responses between male and female Abca4^{-/-} mice of 4.5 months old (male 197.19±76.23μV vs female 197.52±68.09μV). **B.** A slight but not significant trend was observed in rod-isolated responses in Abca4^{-/-} mice compared to age-matched controls at 4.5 months of age (wt 211.07±63.69 μV vs Abca4^{-/-} 197.31±71.95 μV) and at 12 months of age (wt 214.00±86.29 μV vs Abca4^{-/-} 171.84±66.55 μV; p=0.1297). Note that there is also a trend in the decrease of rod response over time in Abca4^{-/-} mice but not in wild type. **C.** No difference was observed in combined rod- and cone-responses between male and female Abca4^{-/-} mice at 4.5 months (male 353.52±127.22 μV vs female 346.09±109.27 μV). **D.** A slight but not significant trend was observed combined rod- and cone responses in Abca4^{-/-} mice compared to age-matched controls at 4.5 months of age (wt 387.14±114.50 μV vs Abca4^{-/-} 348.77±113.45 μV). At twelve months, the trend was nearly significant, mainly due to further decrease of rod and cone response in Abca4^{-/-} mice, but steady levels in wt. (wt 382.07±137.91 μV vs Abca4^{-/-} 303.36±80.87 μV; p=0.0680). **E.** The cone-isolated response does not differ between male and female Abca4^{-/-} mice of 4.5 months old (male 40.05±11.23μV vs female 38.53±12.09 μV). **F.** The cone-isolated response is significantly reduced in Abca4^{-/-} mice of 4.5 months when compared to wild type mice of the same age (wt 58.88±12.99μV vs Abca4^{-/-} 39.51±11.31μV; p=0.0003). At 12 months the difference between phenotypes is no longer present (wt 39.11±13.15μV vs Abca4^{-/-} 36.49±10.44μV). Cone response in wild type eyes at 12 months is significantly reduced compared to 4.5 months (4.5 months 58.88±12.99μV vs 12 months 39.11±13.15μV; p=0.0005) **G.** There is no difference in flicker response between male and female Abca4^{-/-} mice of 4.5 months old (male 20.52±6.30μV vs female 22.39±6.46μV). **H.** No difference was observed in the flicker response between wild type and Abca4^{-/-} mice at 4.5 months (wt 24.20±6.28μV vs Abca4^{-/-} 21.19±6.29μV), nor at 12 months (wt 21.66±7.99μV vs Abca4^{-/-} 21.09±5.83μV). *** = p<0.001

4.2.4. Evaluation of ERG in *Abca4*^{-/-} mice following subretinal injections with AAV2/8.CAG.opbNDI1

To verify whether AAV2/8.CAG.opbNDI1 would have a beneficial effect on retinal function in *Abca4*^{-/-} mice, these mice were subretinally injected with three different doses of this virus, namely a low dose (1×10^7 viral genomes per eye, n=18), an intermediate dose (5×10^7 viral genomes per eye, n=15), and a high dose (5×10^8 viral genomes per eye, n=11). These doses were chosen based on previous work in our lab with similar viruses in other mouse models (Millington-Ward *et al.* 2022; 2023). Each mice received an equal volume of PBS via subretinal injection in their left eye to serve as a control, while the AAV was injected in the right eye at 6-8 weeks of age. This time point was chosen because Stargardt disease, even though symptoms can first present as early as in childhood, is a disease of aging with a phenotype that progressively deteriorates over time.

Retinal function of all injected mice was analysed at approximately 6 months of age, 4 months after administration of the virus, via electrophysiology. The results of these ERGs are presented in Figure 4.4 and sorted per response. Thin lines between bars in these bar charts connect left and right eyes of the same mouse, as they do in all following figures comparing right vs left eye. Administration of AAV2/8.CAG.opbNDI1 in any dose did not alter the rod-isolated response as compared to control eyes (1×10^7 vg: PBS 226 ± 56.67 μ V vs AAV 209.69 ± 68.49 μ V; 5×10^7 vg: PBS 201.18 ± 93.73 μ V vs AAV 158.21 ± 70.52 μ V; 5×10^8 vg: PBS 156.88 ± 56.46 μ V vs AAV 173.39 ± 53.8 μ V). The maximal response, which is made up of mixed cone and rod responses, was not amended by AAV2/8.CAG.opbNDI1 (1×10^7 vg: PBS 319.44 ± 97.98 μ V vs AAV 312.13 ± 85.15 μ V; 5×10^7 vg: PBS 369.8 ± 136.35 μ V vs AAV 308 ± 109.38 μ V; 5×10^8 vg: PBS 292.33 ± 105.53 μ V vs AAV 315.92 ± 89.61 μ V), though paired t-tests revealed a negative trend in the intermediate dose with a p-value of 0.09. The cone-isolated response, which was significantly reduced in *Abca4*^{-/-} mice when compared to wild type controls at 4.5 months, was not affected by AAV2/8.CAG.opbNDI1 (1×10^7 vg: PBS 35.23 ± 12.18 μ V vs AAV 34.3 ± 9.82 μ V; 5×10^7 vg: PBS 48.04 ± 17.64 μ V vs AAV 42.43 ± 12.35 μ V; 5×10^8 vg: PBS 34.79 ± 8.27 μ V vs AAV 34.88 ± 9 μ V). When comparing these data to wild type and untreated *Abca4*^{-/-} mice it appears that the ERG amplitude reaches similar levels (4.5 month *Abca4*^{-/-} 39.51 ± 11.31 μ V, figure 4.4h). The flicker response was not significantly changed by the therapy either (1×10^7 vg: PBS 15.5 ± 5.63 μ V vs AAV 15.85 ± 6.28 μ V; 5×10^7 vg: PBS 25.18 ± 9.38 μ V vs AAV 20.66 ± 5.81 μ V; 5×10^8 vg: PBS 19.32 ± 4.52 μ V vs AAV 18.8 ± 5.96 μ V). A negative trend was seen in the intermediate dose (p=0.09), but when comparing the ERG amplitudes to those of 4.5-months-old *Abca4*^{-/-} 21.19 ± 6.29 μ V, there does not appear to be any adverse effect. Overall, the results of this assay indicate that subretinal injections with three doses of AAV2/8.CAG.opbNDI1 have no detrimental effect on physiological retinal function of *Abca4*^{-/-} mice up to 4 months post-injection.

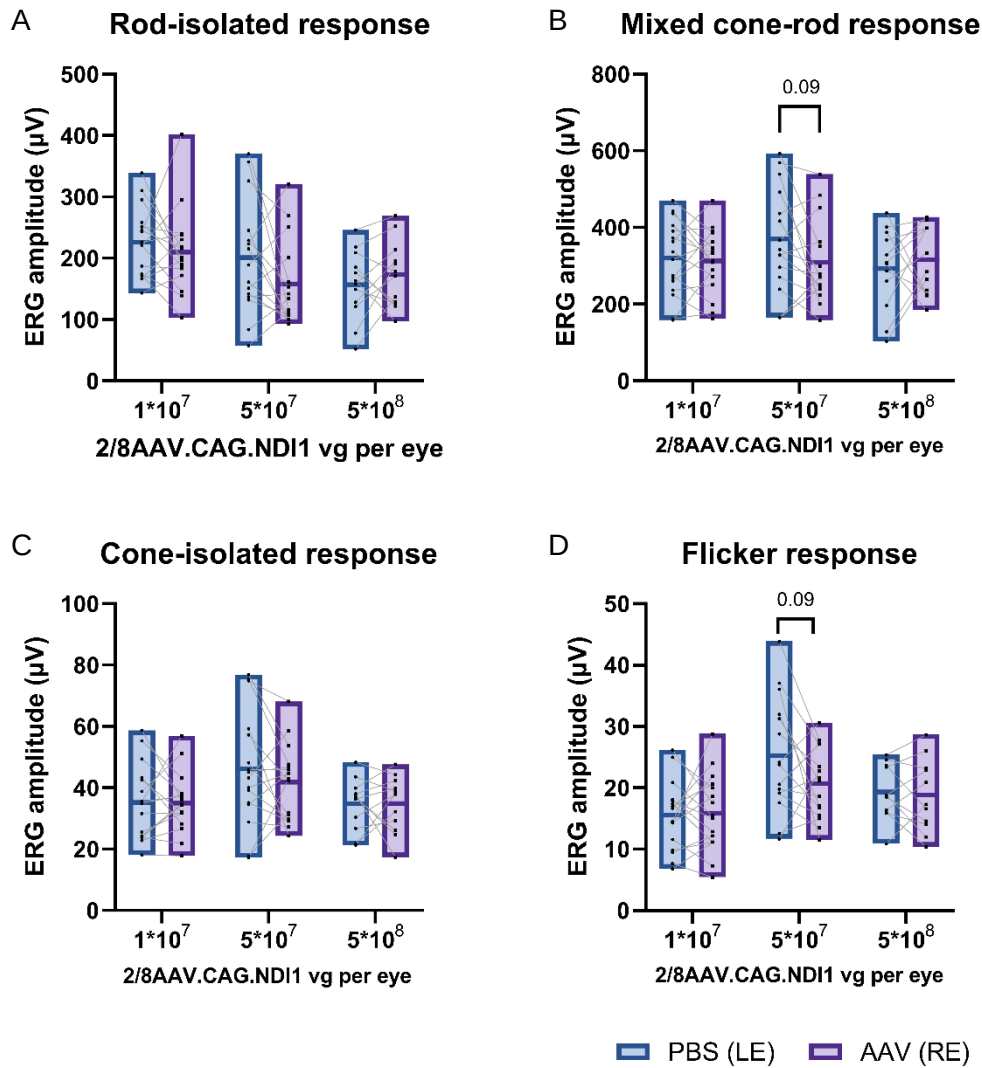
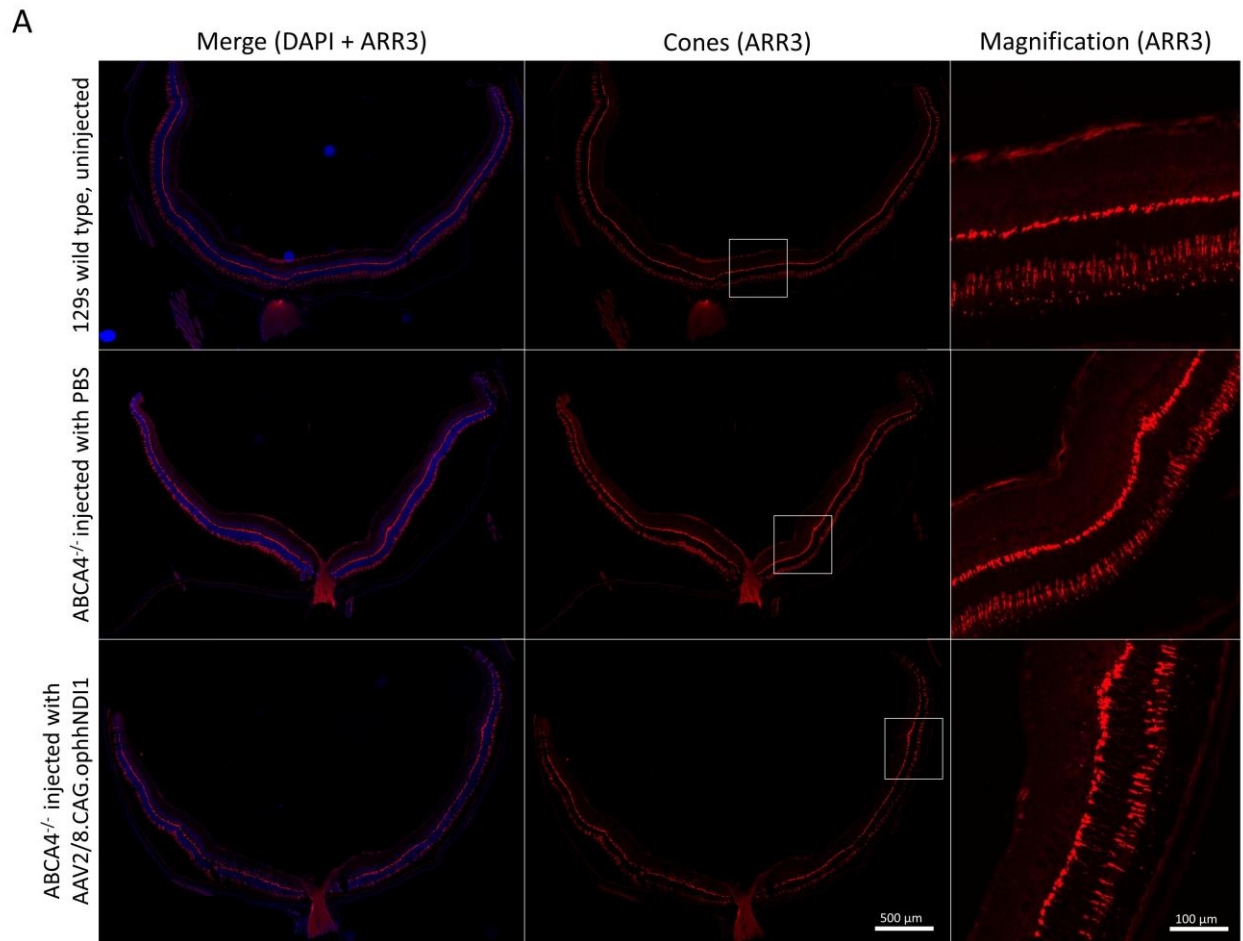


Figure 4.4. Electrophysiological responses of *Abca4*^{-/-} subretinally injected with low, intermediate, or high doses of AAV2/8.CAG.ophNDI1. *Abca4*^{-/-} mice received subretinal injections with PBS in their left eye (LE) or one of three doses of AAV2/8.CAG.ophNDI1 in their right eye (RE) at 6-8 weeks of age and ERGs were measured at 5-6 months. The box plots represent the mean and range of value, while dots represent individual values. The lines between these dots connect left and right eyes of the same mouse. Paired t-tests were carried out for statistical analysis and n-numbers were as follows: 1*10⁷ n=16; 5*10⁷ n=15; 5*10⁸ n=11. **A.** Rod-isolated responses do not differ between eyes injected with PBS and eyes injected with AAV2/8.CAG.ophNDI1 in any of the doses used (PBS 226±56.67 μV vs 1*10⁷ vg AAV 209.69±68.49 μV ; PBS 201.18±93.73 μV vs 5*10⁷ vg AAV 158.21±70.52 μV ; PBS 156.88±56.46 μV vs 5*10⁸ vg AAV 173.39±53.8 μV). **B.** The mixed cone-rod response was also not significantly affected by any viral dose when compared to PBS (PBS 319.44±97.98 μV vs 1*10⁷ vg AAV 312.13±85.15 μV ; PBS 369.8±136.35 μV vs 5*10⁷ vg AAV 308±109.38 μV ; PBS 292.33±105.53 μV vs 5*10⁸ vg AAV 315.92±89.61 μV), though a negative trend is seen at the intermediate dose (5*10⁷) with a p-value of 0.09. **C.** The cone-isolated response showed little to no difference after injection with AAV2/8.CAG.ophNDI1 at either of the doses used (PBS 35.23±12.18 μV vs 1*10⁷ vg AAV 34.3±9.82 μV ; PBS 48.04±17.64 μV vs 5*10⁷ vg AAV 42.43±12.35 μV ; PBS 34.79±8.27 μV vs 5*10⁸ vg AAV 34.88±9 μV). **D.** Response to flickering light is not affected by low or high doses of the therapy (PBS 15.5±5.63 μV vs 1*10⁷ vg AAV 15.85±6.28 μV ; PBS 19.32±4.52 μV vs 5*10⁸ vg AAV 18.8±5.96 μV), but a negative trend may be indicated in the intermediate dose (PBS 25.18±9.38 μV vs 5*10⁷ vg AAV 20.66±5.81 μV ; p=0.09).

4.2.5. Quantification of cone photoreceptor cell numbers in *Abca4*^{-/-} retinas

Reduced numbers of cone photoreceptor cells have been reported in mouse models of AMD and (Millington-Ward *et al*, 2022; 2023). In this project we therefore investigated if this parameter could be also used to evaluate any potential benefit to our Stargardt mouse model by AAV.CAG.ophNDI1. Eyes were taken from 129s wild type mice (n=4) or *ABCA4*^{-/-} mice subretinally injected with PBS in their left eye and 5×10^7 viral genomes of AAV2/8.CAG.ophNDI1 in their right eye (n=6). At 9 months these mice were sacrificed, their eyes enucleated and cryosectioned with a thickness of 12µm per section. These sections were then stained with DAPI for the cell nucleus and ARR3 (cone photoreceptor factor) according to M&M section 2.9.3 for histological analysis. Cones per section were manually counted according to M&M section 2.9.5. Figure 4.5 displays representations of these sections within one mouse (A) and a quantification of cone numbers per mm (B). No significant difference was found in *Abca4*^{-/-} mice between eyes injected with PBS or 5×10^7 vg AAV2/8.CAG.ophNDI1 (51.47 ± 3.27 cones/mm vs 48.54 ± 2.57 cones/mm; $p=0.22$). However, there was also no difference with wild type eyes (49.06 ± 5.47 cones/mm). Therefore, it was decided this parameter would not be used in further doses to evaluate AAV.CAG.ophNDI1 benefit.



B

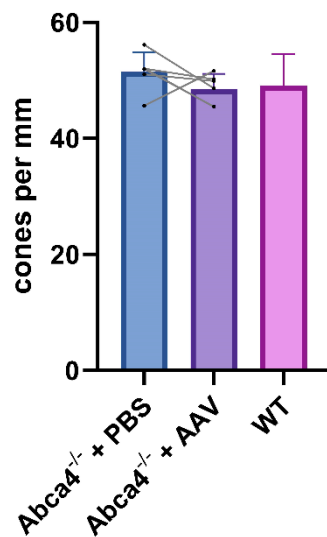


Figure 4.5. Quantification of cone photoreceptor density in wild type and *Abca4*^{-/-} mice. *Abca4*^{-/-} mice were subretinally injected with PBS in the left eye and 5×10^7 vg AAV2/8.CAG.opphNDI1 in the right eye. Wild type mice received no injection in this experiment. At 9 months of age, eyes were enucleated, fixed, cryosectioned, and stained with DAPI (blue) and ARR3 (cones, red). **A.** representative sections from wild type 129s mice (n=4) and *Abca4*^{-/-} mice (n=6). The first column shows both nuclear and cone stains, the second column only shows cone stain. The third column shows one of two magnified regions used to manually count cone photoreceptor cells. **B.** Two regions per section and a minimum of 4 sections per eye were selected and cones were manually counted on each of these, of which the averages per eye are represented in this bar chart, with error bars indicating standard deviation. No statistical difference was found between the PBS-injected (51.47 ± 3.27 cones/mm) and NDI1-treated eyes (48.54 ± 2.57 cones/mm) with a paired t-test ($p=0.22$). Neither of these showed significant differences from wild-type eyes (49.06 ± 5.47 cones/mm).

4.2.6. Evaluation of levels of autofluorescence in the *Abca4*^{-/-} mouse retina:

One of the main characteristics in Stargardt disease is build-up of A2E, which is a key component of the autofluorescent complex lipofuscin composed of oxidised proteins and lipids, among other components. Due to its fluorescent nature, accumulation of lipofuscin in mouse retinas can be observed with confocal scanning laser ophthalmoscopy (cSLO), which has also been used as an outcome to measure therapies in the *Abca4*^{-/-} mouse model by other groups (Issa *et al.* 2013; 2015). However, unfortunately, we do not have access to a cSLO and our pursuits to replicate this assay with a retinal imaging microscope were unsuccessful. In order to observe potential lipofuscin accumulation without a cSLO, initially we chose to section retinas from 18-month-old wild type (n=2 mice, 4 eyes) and *Abca4*^{-/-} mice (n=2 mice, 4 eyes). These sections were stained only with HOECHST, to visualise cell nuclei, while the green fluorescence is naturally present in lipofuscin. Figure 4.6 shows that the RPE and photoreceptor outer segments in the *Abca4*^{-/-} retina are enveloped in green fluorescence, suggesting they have accumulated significant levels of lipofuscin. Retinal sections of aged wild type mice display very low levels of fluorescence, indicating low lipofuscin levels. This assay was then repeated in 9-month-old *Abca4*^{-/-} mice (n=4 mice, 8 eyes), although DAPI was used to stain cell nuclei instead of HOECHST, which is also presented in Figure 4.6. These assays were only performed to characterise the model and as they showed such a large difference and because quantification of fluorescence intensity is incredibly time-consuming, it was decided not to quantify these sections. However, based on these data it

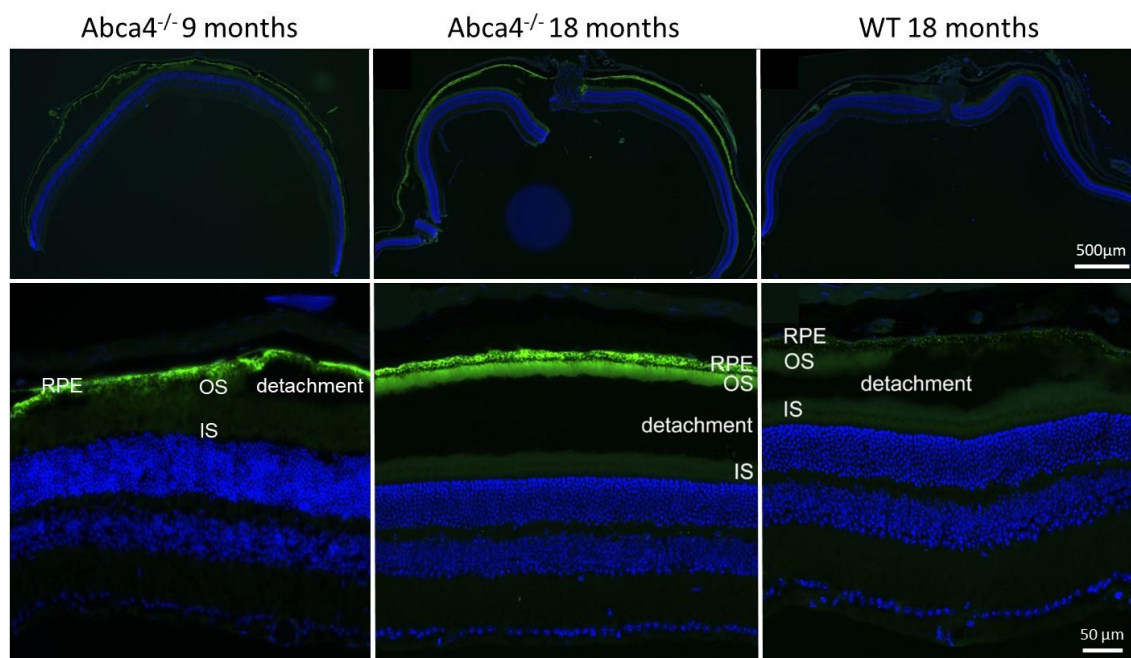


Figure 4.6. Autofluorescence caused by A2E/lipofuscin accumulation in murine retinal sections. Sections were stained with DAPI (9 months *Abca4*^{-/-}) or HOECHST (18 months *Abca4*^{-/-} and WT) to show cell nuclei in blue. Lipofuscin was elucidated through the 488nm filter on the microscope as it is naturally fluorescent green. Representative images are presented here. RPE, Outer Segments (OS) and Inner segments (IS) of photoreceptors, and detachment due to processing, are indicated in this figure.

was established that this assay could be used to highlight potential differences in lipofuscin

accumulation between *Abca4*^{-/-} eyes treated with our therapeutic AAV versus control eyes injected with PBS.

Abca4^{-/-} mice were then injected with various doses of AAV2/8.CAG.ophNDI1 as described before. After these mice were analysed with ERGs, some were taken for autofluorescence quantification. At 9 months eyes were enucleated and fixed in 4% PFA before being dissected, cryoprotected, frozen, and cryosectioned (12 μ m) as described in M&M section 2.9.3. These sections were then stained with DAPI and imaged. The autofluorescence was quantified by measuring the intensity with ImageJ in 4 regions per section and 4-6 sections per eye, as described in M&M 2.9.6. Representative images are presented in Figure 4.7 and Figure 4.8, representing retinal sections of mice subretinally injected with 1×10^7 and 5×10^7 viral genomes, respectively. The results were then normalised to the intensity of autofluorescence found in $n=4$ wild type retinas (100.00 ± 7.7) and summarised in Figure 4.9. On average, the low dose of AAV2/8.CAG.ophNDI1 (1×10^7 vg/eye; $n=9$) decreased autofluorescence intensity compared to control eyes, but this trend was not statistically significant (PBS 394.59 ± 80.2 vs AAV 333.37 ± 60.0 ; $p=0.1312$). The intermediate dose (5×10^7 vg/eye; $n=6$), however, significantly reduced the autofluorescence intensity compared to control eyes (PBS 359.46 ± 64.4 vs AAV 260.58 ± 22.7 , $p=0.0189$). These data indicate that augmentation of mitochondrial function may be able to improve the capacity of retinal cells to remove lipofuscin, or to reduce the formation of lipofuscin. These findings also suggest that the dose of the therapy could be an important factor in its benefits.

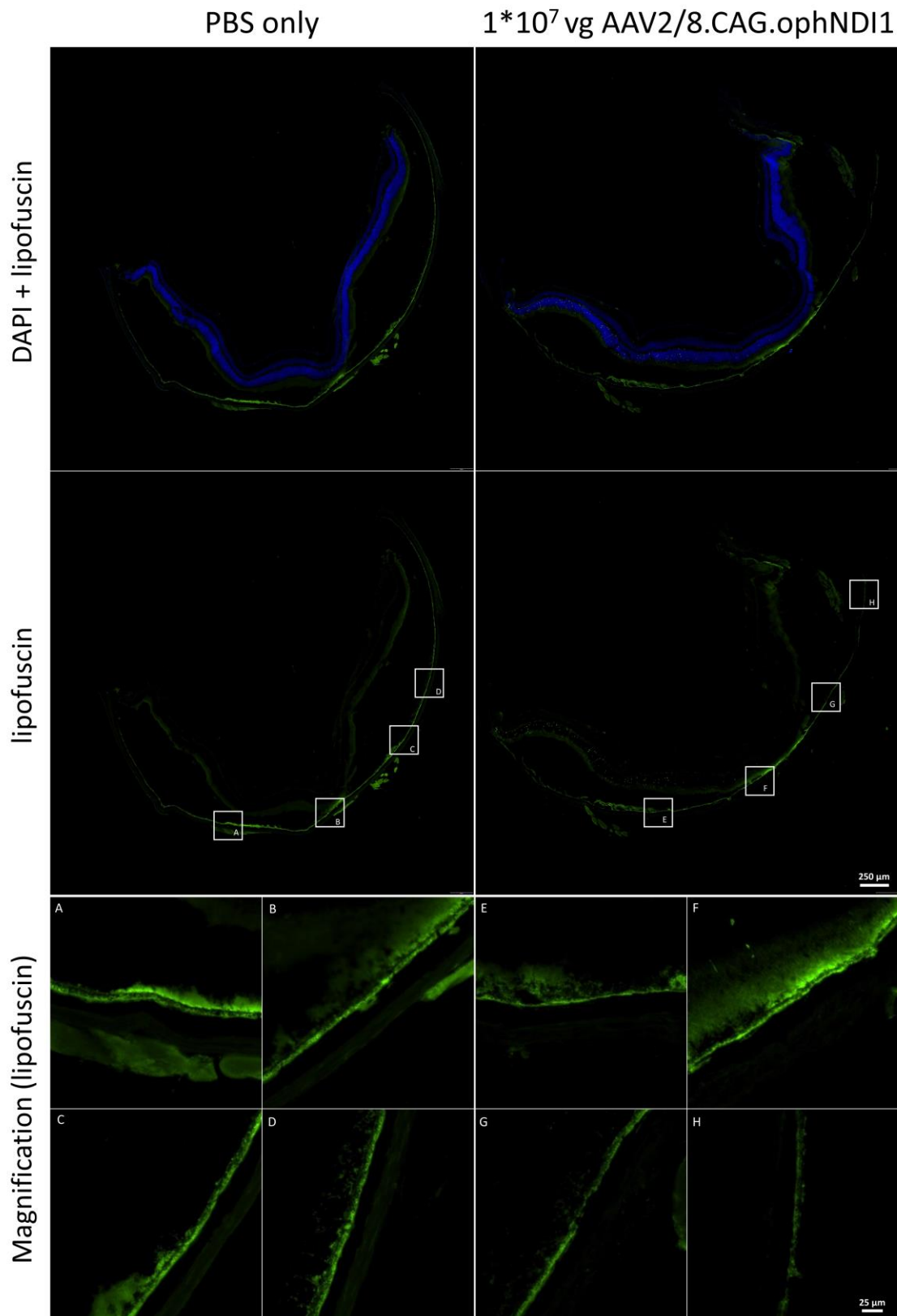


Figure 4.7. Autofluorescence in *Abca4*^{-/-} mice following low dose of AAV2/8.CAG.ophNDI1. Mice received PBS in one eye (left) and 1×10^7 vg AAV2/8.CAG.ophNDI1 in the other (right) eye via subretinal injection at 6 weeks. At approximately a year, eyes were enucleated, fixed, and dissected. The eye cups were cryoprotected and sectioned (12µm), and then stained with DAPI (blue) to visualise cell nuclei. The autofluorescence (green) was imaged through the 488nm filter. This figure shows representative sections of both eyes of one mouse. Four representative regions were taken from each section and used for analysis of autofluorescence intensity.

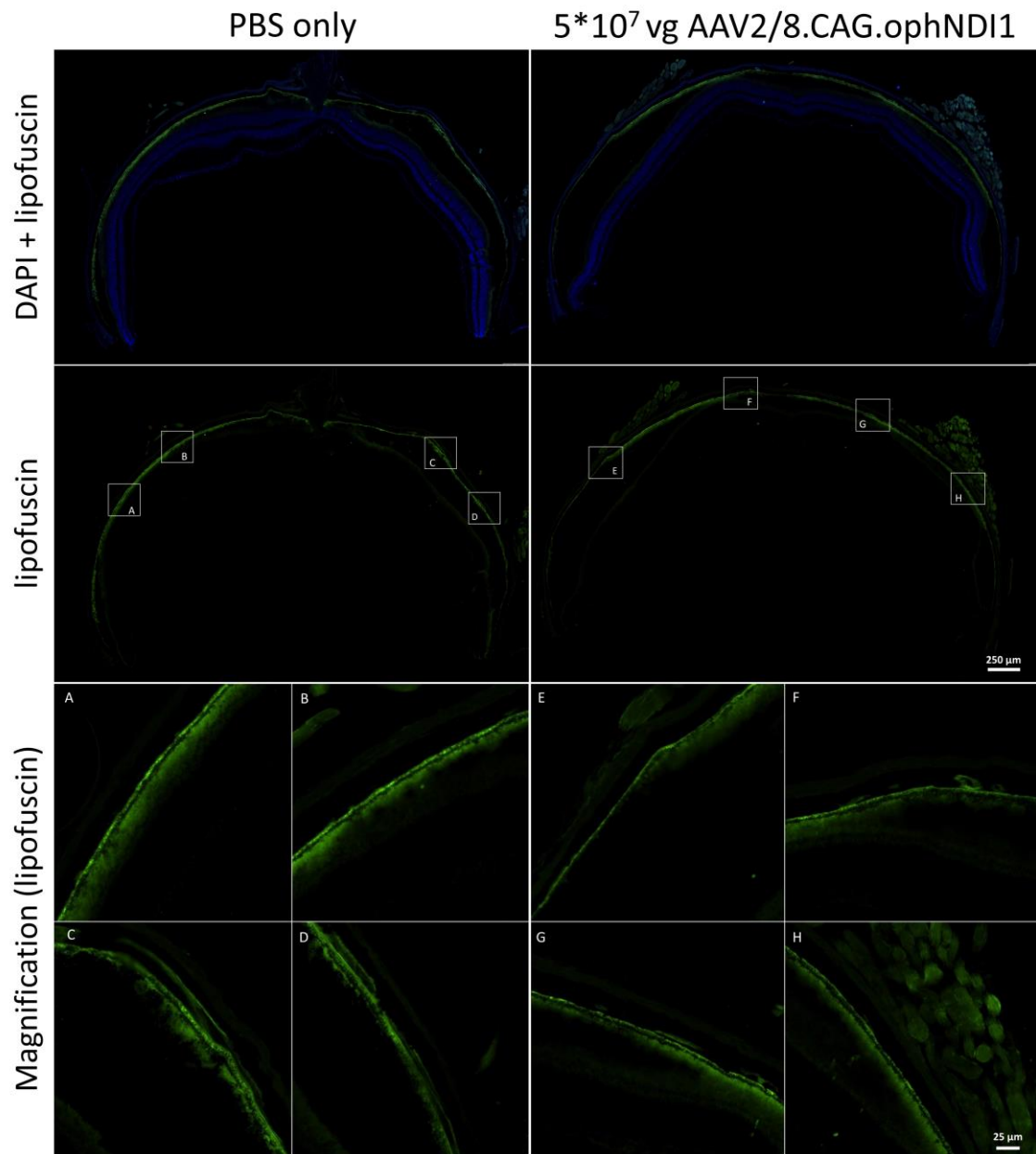


Figure 4.8. Autofluorescence in *Abca4*^{-/-} mice following low dose of AAV2/8.CAG.ophNDI1. Mice received PBS in one eye (left) and 5×10^7 vg AAV2/8.CAG.ophNDI1 in the other (right) eye via subretinal injection at 6 weeks. At approximately a year, eyes were enucleated, fixed, and dissected. The eye cups were cryoprotected and sectioned (12µm), and then stained with DAPI (blue) to visualise cell nuclei. The autofluorescence (green) was imaged through the 488nm filter. This figure shows representative sections of both eyes of one mouse. Four representative regions were taken from each section and used for analysis of autofluorescence intensity.

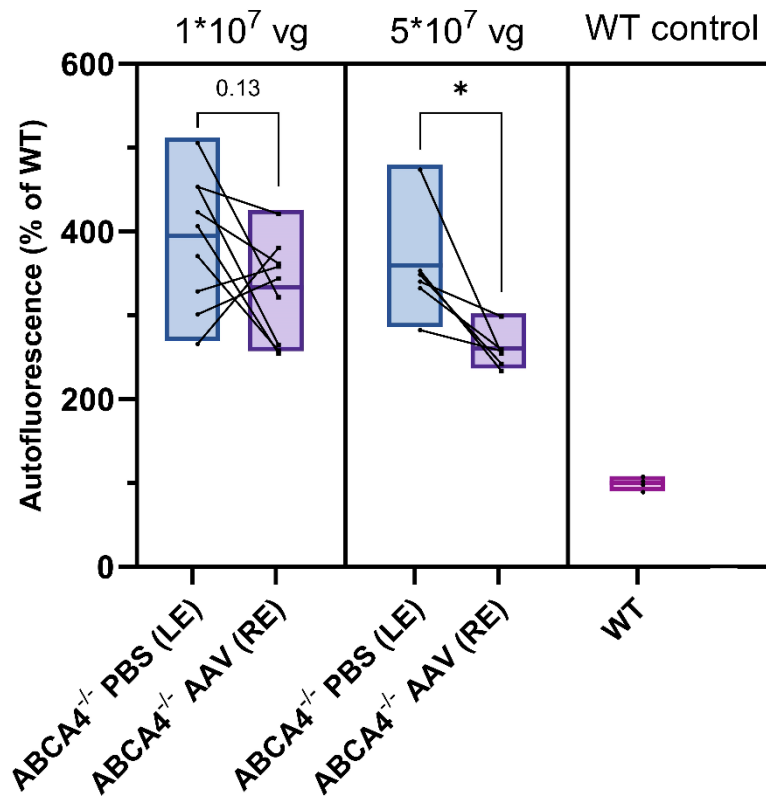


Figure 4.9. Analysis of autofluorescence intensity in *Abca4*^{-/-} mice following low and intermediate doses of AAV2/8.CAG.ophNDI1. Mice received 1*10⁷ (n=9) or 5*10⁷ vg (n=6) AAV2/8.CAG.ophNDI1 in one eye via subretinal injection at 6 weeks while the other eye, injected with PBS, served as control. At 12 months, eyes were enucleated, fixed, and dissected. The eye cups were cryoprotected and sectioned (12µm), and then stained with DAPI (blue) to visualise cell nuclei. The autofluorescence (green) was imaged through the 488nm filter and the intensity was quantified with ImageJ and then normalised to levels of autofluorescence in wild type retinas (n=4). Administration with the lower dose of AAV2/8.CAG.ophNDI1 led to a non-significant decline in autofluorescence (PBS 394.59±80.2 vs AAV 333.37±60.0; p=0.1312). The intermediate dose resulted in a significant reduction of autofluorescence levels (PBS 359.46±64.4 vs AAV 260.58±22.7, p=0.0189). Box plots represent the mean and the bars represent the variance. Measures per mouse are indicated and connected with fine lines. *=p<0.05

4.2.7. Evaluation of levels of ROS in Abca4^{-/-} mouse retinas:

Accumulation of A2E in RPE cell lines leads to mitochondrial dysfunction and ROS production (Saadat *et al.* 2014). In addition to evaluating the Abca4^{-/-} disease model via electrophysiology, we also investigated if the level of reactive oxygen species (ROS) differs between Abca4^{-/-} mice and wild type mice and therefore if this could be used as a possible outcome measure when evaluating candidate therapies such as NDI1. CellROX™ is a cell-permeable, fluorescent dye that, when oxidated by ROS, binds to DNA and exhibits a stable green, fluorescent light. With fluorescence activated cell sorting (FACS) the cellular ROS can be quantified as described in M&M section 2.9.8. To investigate whether FACS-assisted ROS analysis can be a potential outcome measure for our future gene therapy studies, we analysed retinas taken from wild type and Abca4^{-/-} mice at three different time points (3, 6, and 12 months). Because the quantified fluorescence intensities representing cellular ROS levels vary between individual assays based on factors such as incubation time and temperature, in all instances these were normalised to and are presented as percentages of wild type ROS levels (Figure 4.10). At 3 and 6 months of age, ROS levels in Abca4^{-/-} mice were significantly increased (3mo: WT (n=8) 100.00±14.47% vs Abca4^{-/-} (n=7) 132.01±10.84%; p=0.000356; 6mo: WT (n=5) 100.00±10.98% vs Abca4^{-/-} (n=5) 120.27±13.71%; p=0.0326). At 12 months the increase in ROS levels in Abca4^{-/-} ceased to be significant, but a trend was still present (WT (n=8) 100.00±23.79% vs Abca4^{-/-} (n=5) 123.41±24.08%; p=0.11). From these data we concluded that it is important to analyse cellular ROS levels before twelve months; though keeping in mind that Stargardt is a disease that progresses with time, it is also important to know if a therapy is functional long-term.

Abca4^{-/-} mice were then subretinally injected with low (1*10⁷ vg/eye), intermediate (5*10⁷ vg/eye), and high (5*10⁸ vg/eye) doses of AAV2/8.CAG.ophNDI1. Approximately 6 months post-administration of the virus, cellular ROS levels were investigated post ERG (Figure 4.11). Unfortunately, due to separate technical issues, the results of the intermediate and high dose retinas could not be reliably interpreted and are therefore not included in this thesis. The low dose group (n=6), however, presented a significant decrease in cellular ROS levels in photoreceptor cells (PBS 100.00±14.98% vs AAV 77.92±9.88%; p=0.0059).

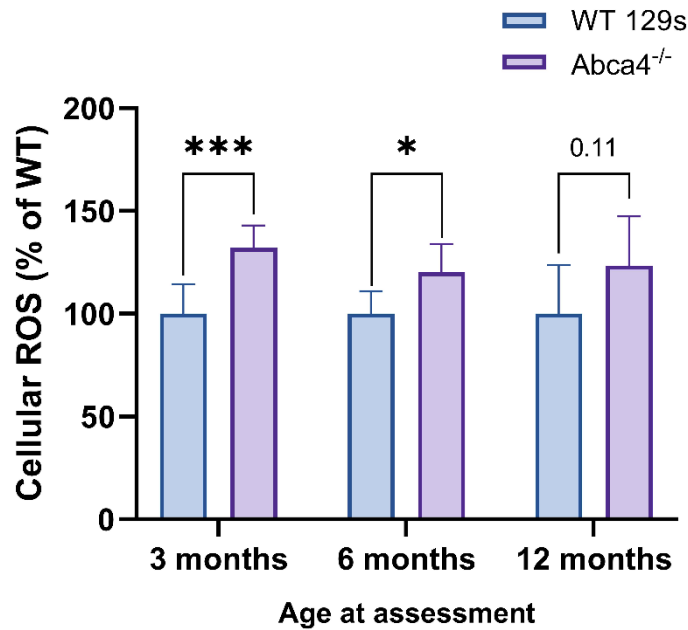


Figure 4.10. Analysis of ROS levels in photoreceptor cells from wild type and Abca4^{-/-} mice at various ages. Relative cellular reactive oxygen species levels in photoreceptor cells in wild type and Abca4^{-/-} mice were assayed at 3 months, 6 months, and 12 months with CellRox Green fluorescent dye and flow cytometry. Due to the nature of this assay, average ROS levels in wild type were set at 100% for each age. Cells were isolated from post-mortem mouse retina's. ROS levels are significantly increased in Abca4^{-/-} at 3 months compared to wild type mice (WT n = 8, Abca4^{-/-} n = 7; WT 100.00±14.47% vs Abca4^{-/-} 132.01±10.84%; p=0.000356) and at 6 months (WT n=5, Abca4^{-/-} n=5; WT 100.00±10.98% vs Abca4^{-/-} 120.27±13.71%; p=0.0326). At 12 months the increased ROS levels in Abca4^{-/-} were no longer significant, but a trend was still present (WT n=8, Abca4^{-/-} n=5; WT 100.00±23.79% vs Abca4^{-/-} 123.41±24.08%, p=0.11). Bar charts represent the mean fluorescence intensity and the error bars portray the standard deviation. *** = p<0.001; * = p<0.05

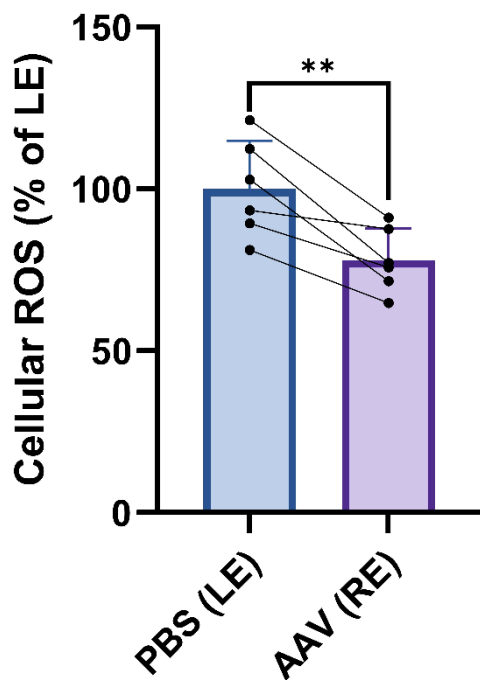


Figure 4.11. Effect of low dose AAV2/8.CAG.ophNDI1 on photoreceptor cell ROS levels. Abca4^{-/-} mice (n=6) were subretinally injected with PBS in their left eye (LE) and 1*10⁷ vg of AAV2/8.CAG.ophNDI1 in their right eye (RE) at 6-8 weeks of age. At approximately 11 months old cellular ROS levels were assessed. Cells were isolated from post-mortem mouse retinas. Cellular ROS levels in photoreceptor cells were significantly lower in eyes that received the therapy as compared to eyes that received PBS (PBS 100.00±14.98% vs AAV 77.92±9.88%; p=0.0059). Bars portray the mean and error bars represent standard deviation. ** = p<0.01

In summary, we characterised the Abca4^{-/-} mouse model under the conditions at our own facilities and using our own equipment. Using electrophysiology it was shown that subretinal injections, with therapeutic AAV vector or PBS, did not cause any harm to electrophysiological functionality of photoreceptor cells of Abca4^{-/-} compared to control mice. However, the therapeutic vector also did not improve the electrophysical light-response in Abca4^{-/-} eyes. Additionally, cone photoreceptor numbers in histological sections of Abca4^{-/-} retinas did not differ from those in wild type retinas. Lipofuscin deposits were quantified using autofluorescent qualities of this pigment complex and it was shown that AAV2/8.CAG.ophNDI1 can lower the autofluorescence with the intermediate dose utilised in this study.. Furthermore, it was established that cellular ROS levels are significantly increased in Abca4^{-/-} retinas than in WT retinas, but that they can be reduced with a low dose of AAV2/8.CAG.ophNDI1. These results indicate that AAV2/8.CAG.ophNDI1 may be beneficial in Abca4^{-/-} mice , but some assays should be repeated with other quantities to find the optimal dosage for maximal benefit.

4.2.8. Formal Acknowledgements of this chapter

In this paragraph I would like to highlight the division of labour of the results presented in this chapter.

Genetic characterisation of the mouse model was carried out by me.

Subretinal injections were performed by Dr Paul Kenna.

All ERGs were performed by Dr Sophia Millington-Ward, but all data was extracted from the ERG-program and analysed by me.

Dr Arpad Palfi taught me how to use the cryostat to section mouse retinas, how to stain sections, and how to image these sections on the microscope, and then this knowledge was used by me to quantify lipofuscin-induced autofluorescence in retinal sections and to quantify cone numbers in retinal sections.

Flow cytometry-based ROS assays were done by me (with supervision by Dr Sophia Millington-Ward or Dr Naomi Chadderton during mouse euthanasia).

4.3. Discussion

Stargardt disease is the most common inherited retinal disorder caused by mutations in a single gene. Several studies have been performed on *Abca4*-linked disease models when evaluating candidate treatments for Stargardt disease, such as dual vector AAV-ABCA4 strategies (Dyka *et al.* 2019; Li *et al.* 2023; McClements *et al.* 2019; Tripani *et al.* 2015), nano particle mediated non-viral delivery of *ABCA4* (Han *et al.* 2012), and administration of compounds that prevent the formation of A2E (Issa *et al.* 2015; Arunkumar *et al.* 2021; Zhang *et al.* 2021). Additionally, a therapy based on delivering an *ABCA4* transgene via a lentiviral vector has been tested on 22 patients in a phase I/II clinical trial, and though adverse effects related to the vector were described in only one case, little to no benefit was seen in any of 22 patients (Parker *et al.* 2022). As lentivirus requires cell division to enter, this vector might not be an efficient option for the retina. Other candidate therapies include, but are not limited to, retinylamine-derived compounds to reduce accumulation of all-trans-retinal (atRAL) (Yu *et al.* 2021) and norbixin, an antioxidant derived from the achiote shrub, that reduces A2E accumulation and neuroprotects RPE and photoreceptor cells against blue light-induced oxidative stress (Fontaine *et al.* 2016; 2020).

Due to metabolic demands and light-exposure in retinal cells, high levels of reactive oxygen species (ROS) and mitochondrial dysfunction are very common deficits in retinal degenerations, and this is known to contribute to photoreceptor and RPE cell death (Rozanowska *et al.* 1995; Okawa *et al.* 2008; Sivapathasuntharam *et al.* 2019). Bisretinoids that accumulate in Stargardt disease, such as A2E, can also create additional ROS (reviewed by Kim *et al.* 2021a). Elevated ROS levels have been reported in cellular models of disease, such as the 661W photoreceptor cell line insulted with atRAL (Chen *et al.* 2021) and increased mRNA and protein levels of various oxidative stress genes were reported in two different models of Stargardt disease; the albino *Abca4*^{-/-} mouse and the *Abca4*^{-/-}/*Rdh8*^{-/-} mouse (Radu *et al.* 2011; Chen *et al.* 2012). Increased ROS levels were also reported in retinal cells affected in other retinal degenerations, such as AMD, LHON and retinitis pigmentosa (Millington-Ward *et al.* 2022,; Carelli *et al.* 2004; Chan *et al.* 2020). Indeed, in this study we demonstrated that cellular ROS levels in photoreceptors are significantly increased in *Abca4*^{-/-} mice compared to wild type mice (figure 4.10). High levels of ROS are linked to inflammation, reduced respiration, and damage to mitochondrial DNA and various organelles, including mitochondria (Brown *et al.* 2018; Eells *et al.* 2019; Tan *et al.* 2020). Mitochondrial damage may potentially lead to aberrant autophagy and eventually result in apoptosis of the entire cell. Specifically, deficiencies in the mammalian mitochondrial complex 1 have been found in photoreceptor and RPE cell models of macular degenerations (Eells *et al.* 2019, Marie *et al.* 2018; Brown *et al.* 2019), indicating supplementation of this complex may represent a potential

therapy. In this study we utilised NDI1, a yeast homolog of this complex, in order to modulate mitochondrial function, targeting photoreceptors and RPE, to drive a therapeutic benefit in the mouse retina. Indeed, yeast NDI1 is a single nuclear gene that in principle can substitute for the mammalian complex I comprising 45 subunits encoded by 45 genes, 7 of which are found in the mitochondrial genomes and the rest in the nuclear gene (Marres *et al.* 1991; Sharma *et al.* 2009; Zhu *et al.* 2016). Therefore, NDI1 was explored as a candidate gene therapy in this *Abca4*^{-/-} murine model of Stargardt disease.

In humans, ERG analysis is used to group patients with Stargardt disease into three categories based on severity of photoreceptor dysfunction. In group 1, the damage is limited to photoreceptors in the macula, a feature lacking in mice, so the comparison between the species here is complicated. Group 2 is characterised by cone photoreceptor damage, and in group 3 both cones and rods are impacted (Lois *et al.* 2001; Sajovic *et al.* 2022). In the current study significant ERG deficits were established in the cone-isolated response in *Abca4*^{-/-} mice at 4.5 of age compared to wild type 129 mice of the same age, but not in rods. We also found a trend in reduced rod-isolated and combined cone-rod responses at 12 months of age, which is in line with the findings in humans. Interestingly, ERG deficits have not been observed in many other studies on *Abca4*^{-/-} mice at the age range we used, though Issa *et al.* (2013) found a significant decline in rod function only in 18-month-old *Abca4*^{-/-} mice with the same background as ours, which supports the trend we saw at 12 months. Indeed, some groups use electroretinography in younger mice to confirm that their therapies do not do any damage to the visual function of these mice (Issa *et al.* 2015; McClements *et al.* 2020). So while our gene therapy did not rescue the electrophysiological response of photoreceptor cells in *Abca4*^{-/-} mice, our results reflect that AAV2/8.CAG.ophNDI1 did not cause damage in doses up to 5×10^8 vg.

Furthermore, this study found that administration of 1×10^7 vg of AAV2/8.CAG.ophNDI1 can significantly reduce cellular ROS levels at least 6 months post-administration (Figure 4.11). Due to technical issues and time limitations, no data is currently available on mice injected with higher doses, but it would be extremely valuable to repeat this assay with the other doses in the near future.

Accumulation of bisretinoids leads to the formation of lipofuscin deposits in the RPE. These autofluorescent pigment complexes can be quantified with fundus autofluorescence imaging, both in mouse models and in human patients (Issa *et al.* 2013, 2015; Birtel *et al.* 2023; Varela *et al.* 2023). This non-invasive procedure allows for relatively easy monitoring of disease progression, and it would be a useful method to observe the ideal time point for ex vivo experiments. Since we did not have access to a cSLO capable of taking these images, we utilised

histology and fluorescence microscopy to quantify autofluorescence. Adopting this methodology, we showed that administration of 5×10^7 viral genomes of AAV2/8.CAG.ophNDI1 was able to significantly reduce accumulation of lipofuscin granules in *Abca4*^{-/-} mice at least 10 months after this dose was injected (Figure 4.9). A lower dose, 1×10^7 vg, was less efficient and therefore did not cause a significant effect, though a trend was still implicated.

Others have shown before that targeting lipofuscin accumulation is a possible therapeutic strategy for Stargardt Disease. A variety of compounds, ranging from small molecules (Shin *et al.* 2023) and carotenoids (Arunkumar 2021; 2023), to vitamin A-analogues (Zhang *et al.* 2021), have been shown to reduce lipofuscin in mouse models. Another strategy by Lyu *et al.* injects superoxide generators into the vitreous, which led to a 30% reduction of lipofuscin, though long term effects of this therapy were not investigated as measures were taken within 48 hours of administration and the mechanisms behind this result remain elusive (Lyu *et al.* 2023). In the current study we showed for the first time that manipulation of mitochondrial function can reduce lipofuscin levels long term. A possible explanation is that autophagy, which is predicted to play a role in lipofuscin clear up (Höhn *et al.* 2019; Li *et al.* 2021), can be augmented when mitochondrial function is enhanced. In line with these results, HPLC-based A2E quantification would also be a valuable future experiment, as it can give a deeper insight into the accumulation of A2E and bisretinoids that give rise to lipofuscin.

In Stargardt disease, it is believed that while both photoreceptors and RPE are affected, it may be RPE dysfunction that causes subsequent photoreceptor cell loss, even though both cell types express *Abca4* and are affected in Stargardt disease (Lenis *et al.* 2018). In the current study AAV2/8 was used to deliver NDI1 as this serotype is known to transduce both photoreceptors and RPE efficiently when injected subretinally (Allocca *et al.* 2007; Leberherz *et al.* 2008). However, the application of subretinal injections requires great expertise and it is a considerably invasive procedure. Therefore, it may be valuable to investigate the use of other AAV serotypes that are able to transduce photoreceptors and RPE through intravitreal injections. For example, our team showed that a capsid engineered AAV serotype PHP.eB was able to extremely efficiently transduce photoreceptor and RPE cells following an intravitreal injection with AAV-PHP.eB-CMV-EGFP (Palfi *et al.* 2023). The CAG promoter used in the potential NDI1 gene therapy drives gene expression in both cell types, that is RPE and photoreceptors (McClements *et al.* 2022; Millington-Ward *et al.* 2022). In future studies, it would be interesting to interrogate if the benefit observed with AAV2/8.CAG.ophNDI1 is due to modulation of mitochondrial function using NDI1 in the RPE or in the photoreceptor cells, or in both cell types. Our team explored the use of the VMD2 promoter, which expresses only in RPE (Esumi *et al.* 2004; Wang *et al.* 2019), in cellular and murine models of dry AMD. However, it was established that, though AAV2/8.VMD2.NDI1

was able to transduce the RPE and provided some benefit, this approach was less effective than utilising an ubiquitous promoter (Millington-Ward *et al.* 2022; 2023). Another possible approach is the use of a photoreceptor-specific promoter, such as the rhodopsin kinase promoter (Khani *et al.* 2007) or the rhodopsin promoter (Palfi *et al.* 2010), to drive NDI1 expression. The use of tissue-specific promoters would limit expression of the transgene to the target tissue, possibly preventing unwanted side-effects.

One of the biggest limitations in this study was the aging models, which drastically reduces the number of experiments that can be performed. Waiting for experimental outcomes is essential for the optimisation of subsequent experiments, but 6- to 12-month intervals between administration of a therapy and analysis of the assay severely affects the amount of data that can be gathered in a set time. These long intervals also mean that if an experiment fails, for example due to technical issues or mistakes, or when it was performed under sub-optimal circumstances, (e.g. another timing may have yielded stronger results) repeating them within the time frame set for this PhD thesis was not always possible; especially as the work presented in this thesis was already affected by the Covid-19 pandemic due to complete closures of the labs for almost 4 months in 2020, and subsequent access restrictions to comply with social distancing.

All in all, the data generated in this chapter provided evidence that modulation of mitochondrial function employing a transkingdom complex I gene therapy (NDI1) can provide therapeutic benefit in a murine model of Stargardt disease, a devastating early onset macular degeneration. Further experiments may be carried out to further optimise dosage and transduction efficiencies, which would be necessary to establish if this therapy approach would be suitable for the clinic.

5. Genetic Architecture of Stargardt Disease in Ireland

5.1. Introduction

This chapter of this PhD thesis encompasses an exploration of the genetic background of Stargardt disease in Ireland.

5.1.1. Inherited Retinal Degenerations

Inherited retinal degenerations (IRDs) are the most prevalent cause of vision loss in young people, affecting an estimated 5.5 million people globally (Hanany *et al.* 2020). IRDs comprise an extremely heterogeneous group of rare genetic disorders, encompassing a great variety of clinical phenotypes and inheritance patterns. One of the features these disorders have in common is progressive deterioration of the retina. At the moment of writing, RetNet, a global retinal information network, recognises 281 identified genes and an additional 36 genetic loci to be implicated in one or more IRDs (<https://sph.uth.edu/retnet/>). The great variety of genes involved in IRD pathology is directly linked to the physiology of the retina, which contains many protein cascades and pathways involved in visual transduction; these features have been more described in more detail in chapter 1.

With all the exciting developments in the field of gene therapy, it is increasingly important to correctly diagnose the genetic cause of vision loss in patients. For example, the mechanism of Luxturna is based on supplementation of functional copies of RPE65; patients are only eligible to receive this therapy if biallelic mutations in RPE65 have been confirmed (Russell *et al.* 2017). However, some cases of Leber Congenital Amaurosis (LCA) and retinitis pigmentosa (RP) can be attributed to dominant negative mutations of RPE65, which would require a different therapeutic approach, such as ‘suppression-and-replacement’ of the faulty gene. Additionally, mutation-specific therapies such as AONs are being investigated as genuine treatment options for retinal diseases (reviewed in Ferencsik *et al.* 2021); evidently, it is vital that causative variants have been confirmed before these types of treatments can be applied. Another reason it is important to determine the exact genetic cause of a phenotype is to help with genetic counselling. People may choose to make certain life choices based on this information. Psychological or medical decisions, such as reproduction, for the present and future can have greatly variable outcomes depending on the genetic background of a disease.

Gene defects can be caused by a wide range of mutations. While most variants are single nucleotide polymorphisms, they can have a variety of effects. While a nonsense variant, leading to the formation of a premature stop codon, causes truncation of the protein, missense variants

have less predictable effects. Misfolding of the protein due to amino acid changes can have greatly varying consequences on protein function, as described in depth by Curtis *et al.* 2020.

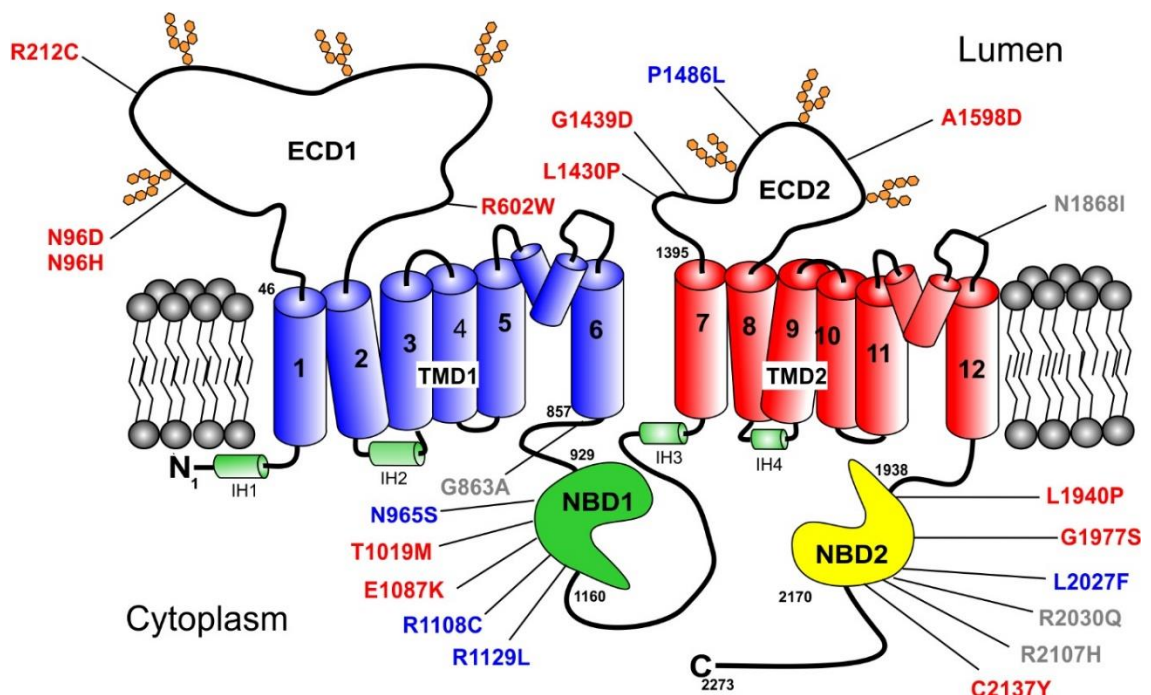


Figure 5.1. Model of the ABCA4 protein and the location of various missense variants. Colours of the variants, annotated by protein change, indicate the severity of their effect on protein function with red being severe, blue being moderate, and grey being mild. TMD = Transmembrane Domain; NBD = Nuclear Binding Domain; IH = (proposed) intracellular α -helix; ECD = extracellular domain. Source: Curtis *et al.* 2020

5.1.2. Splicing

Approximately 1.5% of the human genome has a protein coding function. These regions, exons, are separated from noncoding introns by the spliceosome through a process called splicing. The spliceosome recognises specific nucleotide sequences and assembles various subunits 'on site'. The pre-mRNA at the intronic 5' end splice site is cleaved first, after which the spliceosome will bind to the complementary strand (Clancy, 2008; Will and Lührmann, 2011). The nucleotides at the start and end of each exon are extremely conserved, which is represented in Figure 5.2. The size of each nucleotide in this image directly relates to the frequency of that nucleotide on that position (Brent & Guigo, 2004). Variants in these regions can have major effects on regulation of splicing and indeed lead to exon skipping, exon elongation, intron retention, or combinations of these events, resulting in misfolded proteins, frameshifts, and truncations. Increasing evidence shows that deep-intronic variants can also cause alternative splicing events, causing genetic disease such as Stargardt, highlighting the importance of full gene sequencing (Runhart *et al.* 2019; Khan *et al.* 2019; Qian *et al.* 2021).

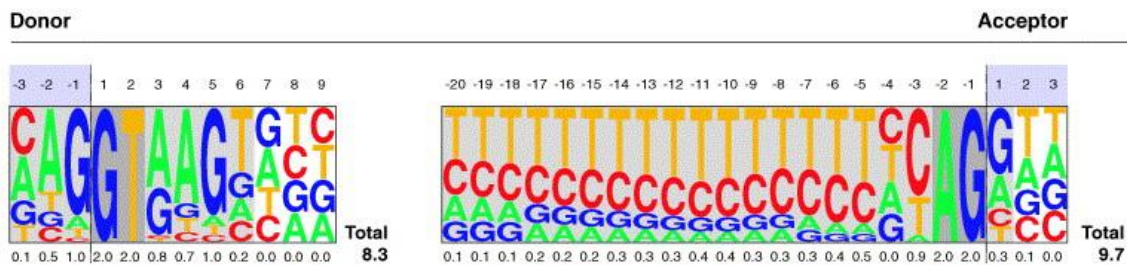


Figure 5.2 Consensus sequences of human donor and acceptor splice sites. The size of the letter (corresponding to a nucleotide) is directly related to the frequency of that nucleotide on that position. Source: Brent and Guigo, 2004

5.1.3. Clinical hallmarks of Stargardt disease

Earlier chapters of this thesis focused on the consequences of ABCA4 dysfunction on a cellular or molecular level. Here, the phenotypic presentation of lack-of-function mutations in humans will be summarised. Though the age of onset can greatly vary depending on severity of mutations, the typical presentation of central vision loss due to ABCA4 dysfunction appears somewhere between adolescence and young adulthood (Gerber et al 1995; Runhart et al 2018; Zernant et al 2017; 2018).

Progressive degeneration of photoreceptor cells in the macula may well be the most common aspect of Stargardt disease, leading to increasing deterioration of functional vision over time (Sparrow *et al.* 2012; Lenis *et al.* 2018). Following this, deterioration of other retinal cell types and nuclear layers may present in later stages or severe phenotypes (Bertelsen *et al.* 2014; Muller *et al.* 2017). However, some patients with Stargardt disease display preservation of central vision due to foveal sparing (Bax *et al.* 2019b; Runhart *et al.* 2019). The fovea is a small region of the macula with a very high visual acuity, so functional preservation of this region has significant benefits for visual function. Interestingly, the retinal region surrounding the optic nerve is also routinely spared from deterioration at earlier stages of disease (Cideciyan *et al.* 2005). Furthermore, accumulation of autofluorescent lipofuscin in the RPE, both evenly dispersed throughout the retina, and condensed into ‘flecks’, are also associated with ABCA4 dysfunction and can be visualised with Fundus Autofluorescence imaging. These flecks can present themselves within in a wide morphological spectrum, as is reviewed by Cremers *et al.* 2020, but clear links between fleck patterns, their contribution to disease, and how genotypes affect these patterns have not been elucidated yet.

Interestingly, in approximately 20% of patients with Stargardt disease, the phenotype presents itself through Bull’s Eye Maculopathy (BEM). The name of this phenotype is directly related to the likeness of fundus images to a dartboard’s bull’s eye; beginning atrophy is confined to a circular region in the central macula. It is important to note, however, that BEM-like phenotypes

are not unique to Stargardt disease, but can be caused by many inherited macular diseases, viral infections, or even medication such as chloroquine and hydroxychloroquine.

Quantification of autofluorescence levels in the RPE, due to lipofuscin accumulation, is a helpful tool in distinguishing *ABCA4*-associated

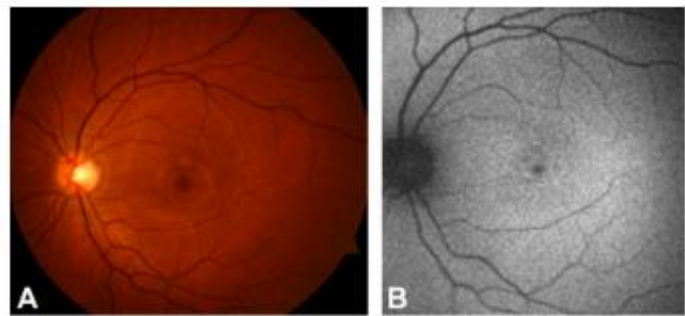


Figure 5.3. Bull's Eye Maculopathy in a patient with a homozygous mutation in *ABCA4* (c.5882G>A). A fundus image. B. Fundus Autofluorescence Source: adapted from Cella *et al.* 2009

BEM from other types. (Cella *et al.* 2009; Duncker *et al.* 2015b). However, people don't visit a specialist because they know they're accumulating lipofuscin. Functional problems with vision, such as night blindness or a reduction in visual acuity are often a first indication that the retina is deteriorating. Assays such as electroretinograms or visual acuity tests are useful tools in establishing how much visual function a person living with an IRD has left. Visual acuity is usually measured utilising a Snellen chart, as depicted in Figure 5.4, which indicates what the test subject can see at 6 meters compared to the distance at which a healthy control would be able to see that same image or letter.



Figure 5.4. Example of Snellen chart used to quantify visual acuity. Standardised charts are viewed from a distance of 6 meters and the smallest letters a person can read represent their visual acuity as a fraction of the distance a healthy person would be able to read that letter. Figure adapted from <https://medschool.co/signs/reduced-visual-acuity>

5.1.4. Target 5000

Target 5000 is a project set up by Fighting Blindness and the Farrar lab that aims to genetically diagnose all people with sight loss due to IRDs in Ireland. Furthermore, it provides clinical services and genetic counselling to people living with IRDs. Based on IRD prevalence world-wide (Hanany *et al.* 2020), it is estimated that approximately 5000 people in Ireland would have a form of IRD. However, because of the great variety in genotypes and phenotypes, it can be hard to diagnose everyone correctly. Additionally, there seems to be an overlap between IRDs and other multifactorial ocular degenerations such as Age-Related Macular Degeneration (AMD), highlighting again the importance of genetic diagnosis. Indeed, one of Target 5000's most recent projects focuses on identifying genes that may be implicated in AMD by comparing 1831 genomes from inherited macular degeneration (iMD) patients, 1123 genomes from AMD patients, and 847 genomes from healthy control individuals (Hitti-Malin *et al.* 2022).

Due to the collaborative effort of everyone involved in the Target 5000 project, from clinicians, to patients, to researchers, a national registry incorporating genetic and clinical data of people affected by IRDs is being set up. This would facilitate easier diagnosis and be useful to identify patients eligible for clinical trials and, in the (hopefully near) future, approved therapies to ameliorate symptoms. This thesis chapter focuses on the collation of genetic and clinical data of people living with Stargardt disease or Stargardt-like phenotypes, providing an overview of the genetic landscape of this IRD in Ireland. Many of the data investigated here were collated in previous studies by the Farrar lab and collaborators as part of the Target 5000 project. This section will summarise the next generation sequencing techniques that were used previously to gather and interpret genomic data from IRD patients.

5.2. Materials and Methods

5.2.1. Patient recruitment

Patients were assessed by clinicians and, based on a clinical diagnosis, referred to the Target 5000 study if their phenotype indicated a genetic cause of disease. Informed consent was requested prior to participation in these studies. Ethical approval was awarded to all hospitals and research labs that participated in this study.

5.2.2. Next generation sequencing

The extreme decrease in cost and the great progress being made to improve techniques, has made next generation sequencing available to many people living with inherited conditions. The rise of NGS has enabled sequencing of large gene panels, whole exomes, and whole genomes, which drastically improved the diagnostic rate in genetic disorders. Additionally, NGS provides new insights in the function of introns, exons, and the interaction of genes. Because of the great progress being made on a yearly basis, several NGS techniques were used since the start of the Target 5000 project.

The first method of NGS used in these studies is referred to as Target Capture Sequencing (TCS). This is a panel-based sequencing method of, mostly, exonic regions of 210 and 254 genes, respectively. Patient DNA was fragmented into 200-250 bp fragments, and then captured with the Nimblegen SeqCap EZ kit (Roche Ireland Ltd., Dublin, Ireland). Subsequently, the captured DNA was multiplexed into 24-sample or 96-sample pools, respectively, and sequenced using the Illumina MiSeq at the TCD Genomics Core facility. These methods can be found in greater detail in our published works (Carrigan *et al.*, 2016; Dockery *et al.*, 2017; Whelan *et al.*, 2020a). A list of the genes of which exonic regions were sequenced using this method can be found in supplementary table 5.

For some monogenic disorders, such as Stargardt disease, whole gene sequencing is a cost-effective option to thoroughly investigate potential candidate variants. Our collaborators in the human genetics department of RadboudUMC, (Nijmegen, the Netherlands), have developed double-tiled single molecule Molecular Inversion Probes (smMIPs) to sequence the complete *ABCA4* gene. Other than uncovering variants in exonic regions of the gene, this method enables the identification of deep-intronic variants that are causative of the disease. Additionally, coding regions of the *PRPH2* gene were also included, as pathogenic mutations in this gene can present with phenotypes that are very similar to Stargardt disease. The smMIPs-based approach does not require the generation of a sequencing library and can be easily scaled up for faster analysis (Khan *et al.* 2019; 2020).

A similar smMIPs-based method was then used by the team in Nijmegen to set up a Macular Degeneration panel to identify variants in 105 genes associated with iMD and AMD. This approach focuses on exonic or coding regions of these genes, but 68 previously documented pathogenic deep-intronic variants were also included, covering a total of over 450kb nucleotides. This panel utilises 17,394 smMIPs and is able to achieve high sequencing coverage, which aids in the detection of copy number variants. This method is described in depth by Hitti-Malin *et al.* (2022; 2023) and a list of genes can be found in supplemental table 7.

A number of probands were sequenced using whole exome or whole genome sequencing (WES or WGS, respectively) approaches if panel-based sequencing didn't yield a diagnosis. The benefit of these approaches is the availability of data on all genes in the genomes, but these methods are over 20 times more expensive than smMIPs-based approaches. WES was performed at BGI-Europe, utilising the SureSelectXT Human all Exon V5 (Agilent Technologies) kit for library preparation and a Illumina HiSeq 4000 (Illumina Inc.) for sequencing. Reads were aligned to the hg19 version of the human reference genome and analysed with the Genome Analysis Toolkit as described previously (Haer-Wigman *et al.* 2017; Verbakel *et al.* 2019).

As WGS is a costly approach, only unsolved cases that were previously screened with TCS or WES were subsequently referred for WGS (as described in detail in Fadaie *et al.* 2021). A total of 38 Irish probands (with any clinical IRD phenotype) who remained unsolved following TCS underwent WGS, which led to the further diagnosis of 3 Irish patients with biallelic pathogenic *ABCA4* variants (Fadaie *et al.*, 2021). Additionally, because the deep-intronic variant c.4539+2028C>T in *ABCA4* was found in a complex allele with a benign near-intronic variant covered in the TCS panel, retrospective analysis of this variant was carried out on a cohort of probands who harboured c.302+68C>T (Whelan *et al.* 2023).

5.2.3. Formal acknowledgements of this chapter

This chapter is the results of a lot of collaborative efforts and in this paragraph I will describe my efforts.

The majority of individuals described in this chapter received a genetic diagnosis before I got involved in the Target5000 project as described in the section above (5.2.2.). I then collated all this data from various different files, programs and databases and analysed and interpreted these data to find patterns in the genetic architecture of Stargardt disease.

I also carried out cascade analysis for 136 individuals to confirm their initial diagnosis before writing them up. This included the isolation of DNA from blood of patients and family members (though for some people this had already been done previously), primer design and PCR of

regions/genes of interest and interpretation of sanger sequencing data. This also led to the genetic diagnosis of 16 additional individuals.

Then I liaised with clinicians in order to collect and understand clinical data of various individuals and I matched it to specific mutations.

Based on the data I collated, I was then also involved in the decisions of what Stargardt patients would be recommended to undergo new rounds of Next Generation Sequencing.

5.3. Results

The Target 5000 project has collected genetic and clinical information of Irish people affected by IRDs. For this PhD chapter, the Target 5000 cohort was used for an in-depth study on the occurrence of the Stargardt phenotype and the genetic landscape of *ABCA4* in Ireland. First, several data sets were organised and sorted through and combined into one large file. Samples were considered for the Target 5000 Stargardt cohort if either their clinical or genetic diagnosis was Stargardt disease, or if they were affected and other affected family members were clinically or genetically diagnosed with Stargardt disease. Genetic and clinical data was added to this file based on availability. A total of 327 affected people, of 225 pedigrees, fit these criteria. These numbers also indicate that Stargardt disease, or Stargardt-like phenotypes, is a highly prevalent IRD, as the entire Target 5000 cohort is comprised of 1460 pedigrees. This is in line with findings from Heath Jeffery et al (2021) who established that approximately 12% of registered IRDs can be attributed to STGD1. Out of 327 affected individuals, we have managed to genetically diagnose 212 people with biallelic *ABCA4*-related Stargardt disease, and 16 were found to have causative mutations in other IRD genes. In 34 cases only one pathogenic variant was found in *ABCA4* after NGS. For 65 probands, no causative variants have been discovered, though it should be noted that 28 of these are currently still being analysed as part of the MD smMIPs study.

Sequencing outcomes in Target 5000 Stargardt cohort

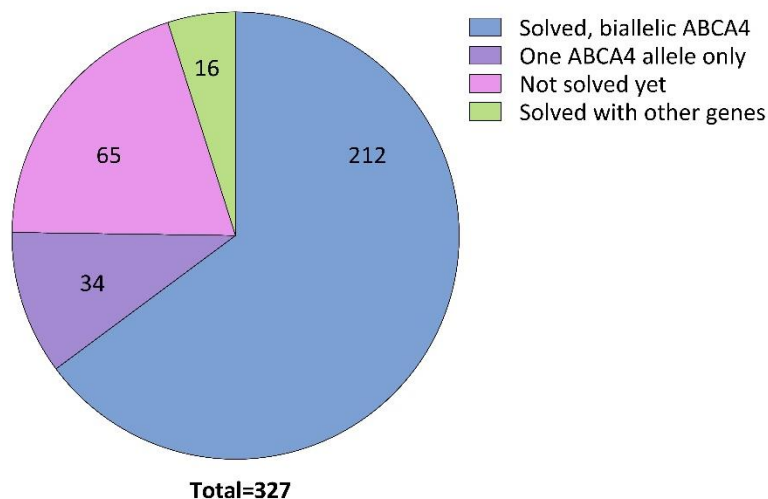


Figure 5.5. Sequencing outcomes of Target 5000 Stargardt cohort. Biallelic candidate variants in *ABCA4* were detected in 212 out of 327 probands in our Stargardt cohort and 16 people were solved with variants in other genes. Only one allele in *ABCA4* was found in 34 people and 65 people were not solved yet.

5.3.1. An overview of the genetic Stargardt landscape in Ireland

Utilising the data collected through NGS as described in the section above, an extensive overview of all Stargardt cases in the Target 5000 cohort was created. Hereafter, I carried out cascade analysis of 136 individuals in 67 pedigrees to verify NGS results and segregation of the variants with disease as follows. DNA was isolated from blood as described in M&M section 2.4.8.. Primers were designed according to the following guidelines: GC content between 40-60%, preferably ending and starting with G or C, length between 18-24 nucleotides, melting temperature between 55 and 65 °C with a maximal difference of 1.5°C between matching forward and reverse primers. These primers were then used to amplify specific gene regions via polymerase chain reaction as described in section 2.4.11. (enzyme used was either Q5 High-Fidelity, NEB or Firepol®, Solis Biodyne). Sanger sequencing was performed through Eurofins Tubeseq services as described in section 2.5.

The collaborative effort between the research team and the clinical teams also allowed for cost-effective diagnosis of some affected family members. When pathogenic variants had been indicated in a proband, so far unsolved, affected siblings or other family members who presented with the same, or similar, phenotypes would be investigated through sanger sequencing of specific genetic loci. This approach led to the genetic diagnosis of an additional 16 individuals in 10 pedigrees. Interestingly, in 6 pedigrees within the Stargardt cohort, more than two *ABCA4*-variants were implicated, leading to a variety of allele-combinations.

In our cohort, a total of 120 unique variants were identified in *ABCA4* in the solved patients cohort. Table 5.1 gives an overview of the protein effect of these variants. This shows that, in line with findings in literature as summarised by Cremers et al (2020), missense variants are most common, though in our cohort both the percentage of unique missense variants and the total missense variants is higher than in the global population (70.83% and 65.10%, respectively, versus 50% and 61%). Interestingly, while only 3 unique variants cause a missense effect and disrupt splicing, they make up almost 1/8 of total variants in our cohort. Part of this is due to enrichment of the c.161G>A variant in this cohort, which will be described in more detail in section 5.3.2. below. An overview of all probands with biallelic pathogenic mutations in *ABCA4* is given in Table 5.3. Pedigree numbers are given when multiple affected family members from one pedigree participated in the study.

Table 5.1. Overview of variant types in *ABCA4*

Protein effect	Unique variants	Total alleles
Missense	85 / 70.83%	347 / 65.10%
Nonsense	12 / 10%	23 / 4.32%
Splice-altering	14 / 11.67%	67 / 12.57%
Frameshift	5 / 4.17%	5 / 0.94%
Missense and Splice effect	3 / 2.50%	66 / 12.38%
Unknown effect	1 / 0.83%	25 / 4.69 %
Total	120	533

Allele frequencies can greatly vary between populations, especially on islands such as the Republic of Ireland and Northern Ireland. Figure 5.6 depicts a graphical presentation of allele prevalence, with the block size indicating allele counts. For clarity, unique variants that were only identified once or twice were grouped, but can be found in Supplementary table 7. Table 5.2 gives an overview of the 16 most common variants found in *ABCA4* in our STGD1 cohort and compares it to global and non-Finnish European allele frequencies. This table shows that the two most common variants in the Target 5000 Stargardt cohort, c.161G>A and c.4539+2028C>T are much more prevalent in the all-Ireland cohort than elsewhere. Section 5.3.2. will discuss the c.161G>A variant in more detail. Another interesting missense mutation is c.5603A>T, a hypomorphic allele that is highly prevalent in the entire general population, with estimated allele frequencies between 4 and 10 percent (Zernant *et al.* 2017; Runhart *et al.* 2018). As this allele is so common, it is found in various complex alleles. Indeed, while the c.5603A>T was identified 66 times in solved individuals in the Target 5000 cohort, in only 21 incidents this variant stood on its own. Complex alleles with c.2588G>C, c.5461-10T>C, and 2827C>T were identified 18, 12, and 6 times, respectively. Another 5 complex alleles with c.5603A>T were identified a total of 9 times in our cohort.

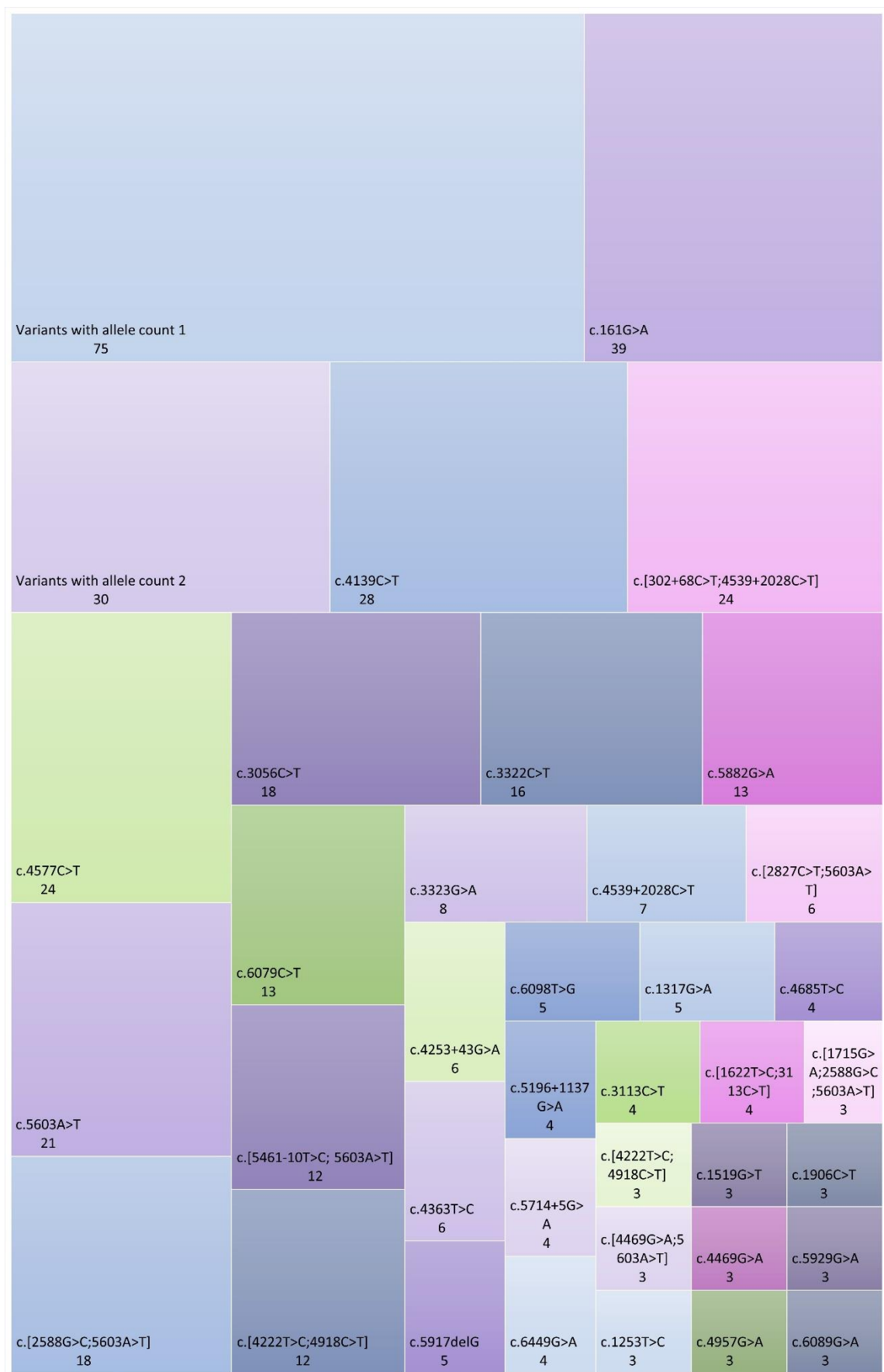


Table 5.2. The allele frequencies of all pathogenic *ABCA4* variants identified at least 6 times in all solved STGD1 cases of Target 5000. Global and European allele frequencies in gnomAD are given, along with allele count in gnomAD, the number of variants reported in LOVD, and the severity of the variant. AF = Allele Frequency, AC = Allele Count, AN = total Allele Number, N/A = not available, # as reported by Cornelis *et al.* 2017, [†] data was not available for complex alleles, so counts per variant are noted, however this does not mean these counts occur together but is meant to indicate frequency.

Variant (DNA)	Variant (protein)	Allele Count	Homo-zygotes	gnomAD AF (Global)	gnomAD AF (European, non-Finnish)	gnomAD AC/AN (European, non-Finnish)	LOVD count	Severity [#]
c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	40	5	0.00003943	0.0000735	5/68030	145	moderate
c.4539+2028C>T	p.[=,Arg1514Leufs*36]	31	2	0.00003943	0.00008819	6/68036	45	moderate
c.4139C>T	p.Pro1380Le	28	1	0.0002431	0.0004263	29/68032	513	moderate
c.4577C>T	p.Thr1526Met	24	0	0.00008542	0.0001764	12/68032	172	moderate
c.5603A>T	p.Asn1868Ile	21	3	0.04042	0.06636	4512/67988	1475	mild
c.3056C>T	p.Ala1038Val	18	0	0.00002628	0.0000294	2/68032	95	severe
c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]	18	0	0.001951305	0.003980625	226/56775	936; 1475 [†]	mild; mild
c.3322C>T	p.Arg1108Cys	16	0	0.0001905	0.0003527	24/68042	286	moderate
c.6079C>T	p.Leu2027Phe	13	1	0.0001774	0.0002352	16/68032	382	moderate
c.5882G>A	p.Gly1961Glu	13	2	0.00311	0.003249	221/68028	2339	mild
c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6; Asn1868Ile]	12	1	N/A	N/A	N/A	940; 1475 [†]	severe, mild
c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]	12	0	0.000031836	0.000052794	3/56825	86; 172 [†]	mild, severe
c.3323G>A	p.Arg1108His	8	1	0.00002629	0.0000147	1/68036	63	mild
c.4363T>C	p.Cys1455Arg	6	0	0.00001314	0.00002939	2/68042	42	severe
c.4253+43G>A	p.[=,Ile1377Hisfs*3]	6	0	0.004701	0.005498	374/68022	165	mild
c.[2827C>T;5603A>T]	p.[Arg943Trp; Asn1868Ile]	6	0	0.000039793	0.000052807	3/56811	15; 1475 [†]	mild, mild

Table 5.3. Overview of all individuals with biallelic pathogenic mutations in *ABCA4*. Pedigree numbers are given for those related to one another. Known complex alleles are indicated between square brackets, p.? indicates that the effect on protein structure is not known, p.= indicates no protein change.

Patient ID	Pedigree	variant 1 (DNA change)	variant 1 (protein change)	variant 2 (DNA change)	variant 2 (protein change)
BF014	RP014	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)
BF141		c.1317G>A	p.Trp439*	c.3352C>G; c.5603A>T	p.His1118Asp; p.Asn1868Ile
BF155		c.768G>T	p.Leu257Valfs*17	c.6079C>T	p.Leu2027Phe
BF161		c.4577C>T	p.Thr1526Met	c.6089G>A	p.Arg2030Gln
BF234		c.[4222T>C; 4918C>T]	p.[Trp1408Arg; Arg1640Trp]	c.6089G>A	p.Arg2030Gln
BF246		c.1906C>T	p.Gln636*	c.4363T>C	p.Cys1455Arg
BF420		c.4469G>A	p.Cys1490Tyr	c.5196+1137G>A; c.5603A>T	p.(=, Met1733Glufs*78); p.Asn1868Ile
BF431		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)
BF440		c.1804C>T; c.2311A>T	p.Arg602Trp; p.Thr771Ser	c.2894A>G; c.4283C>T	p.Asn965Ser; p.Thr1428Met
BF448		c.[2588G>C; 5603A>T]	p.[Gly863Ala, Gly863del; Asn1868Ile]	c.4139C>T	p.Pro1380Leu
BF456		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4685T>C	p.Ile1562Thr
BF458	RP014	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)
BF466		c.1317G>A	p.Trp439*	c.[2588G>C; 5603A>T]	p.[Gly863Ala, Gly863del; Asn1868Ile]
BF473		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)
BF474		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.3322C>T	p.Arg1108Cys
BF479		c.1317G>A	p.Trp439*	c.[2588G>C; 5603A>T]	p.[Gly863Ala, Gly863del; Asn1868Ile]
BF482		c.[4469G>A; 5603A>T]	p.[Cys1490Tyr; Asn1868Ile]	c.2617T>C	p.Phe873Leu
BF492		c.1906C>T	p.Gln636*	c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
BF501		c.1317G>A	p.Trp439*	c.4577C>T; c.5908C>T	p.Thr1526Met; p.Leu1970Phe
BF505		c.1817G>A	p.Gly606Asp	c.4577C>T	p.Thr1526Met
BF511		c.6079C>T	p.Leu2027Phe	c.6079C>T	p.Leu2027Phe
BF513		c.[1715G>A; 2588G>C; 5603A>T]	p.[Arg572Gln; Gly863Ala, Gly863del; Asn1868Ile]	c.4577C>T	p.Thr1526Met
BF514		c.1715G>A	p.Arg572Gln	c.[2588G>C; 5603A>T]	p.[Gly863Ala, Gly863del; Asn1868Ile]
BF521		c.655A>T	p.Arg219*	c.5603A>T	p.Asn1868Ile
BF522		c.[1715G>A; 2588G>C; 5603A>T]	p.[Arg572Gln; Gly863Ala, Gly863del; Asn1868Ile]	c.5312+3A>T	p.(Asn1734Glyfs*14)
BF528	RP528	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.[1715G>A; 2588G>C; 5603A>T]	p.[Arg572Gln; Gly863Ala, Gly863del; Asn1868Ile]

BF539		c.4139C>T	p.Pro1380Leu	c.5381C>A	p.Ala1794Asp
BF541		c.1519G>T	p.Asp507Tyr	c.5312+3A>T	p.(Asn1734Glyfs*14)
BF542		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.6089G>A	p.Arg2030Gln
BF559		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6];Asn1868Ile]
BF563		c.3323G>A	p.Arg1108His	c.6416G>C	p.Arg2139Pro
BF565		c.2041C>T	p.Arg681*	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]
BF566		c.4577C>T	p.Thr1526Met	c.5603A>T	p.Asn1868Ile
BF594		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.4469G>A	p.Cys1490Tyr
BF621		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.3323G>A	p.Arg1108His
BF622		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.3323G>A	p.Arg1108His
BF637	RP637	c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.4254-5T>A	p.(Ser1418_Pro1451delinsArg,Ser1418Argfs*13,Ser1418_Cys1488del)
BF638	RP637	c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.4254-5T>A	p.(Ser1418_Pro1451delinsArg,Ser1418Argfs*13,Ser1418_Cys1488del)
BF639		c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	c.[3758C>T;5882G>A]	p.[Thr1253Met;Asn1868Ile]
EEA49	A49	c.[302+68C>T;4539+2028C>T]	p.[?;=,Arg1514Leufs*36]	c.[4222T>C;4918C>T]	p.[Trp1408Arg;Arg1640Trp]
EEB24	B24	c.1253T>C	p.Phe418Ser	c.3056C>T	p.Thr1019Met
EEB33	B33	c.1018T>C	p.Tyr340His	c.4139C>T	p.Pro1380Leu
EEB37		c.[302+68C>T;4539+2028C>T]	p.[?;=,Arg1514Leufs*36]	c.[302+68C>T;4539+2028C>T]	p.[?;=,Arg1514Leufs*36]
EEC10	C11	c.4919G>A	p.Arg1640Gln	c.5714+5G>A	p.(=,Glu1863Leufs*33)
EEC4	C4	c.[2827C>T;5603A>T]	p.[Arg943Trp;Asn1868Ile]	c.4577C>T	p.Thr1526Met
EEC7	C7	c.4577C>T	p.Thr1526Met	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6];Asn1868Ile]
EECA16		c.4139C>T	p.Pro1380Leu	c.[302+68C>T;4539+2028C>T]	p.[?;=,Arg1514Leufs*36]
EECA23	CA23	c.3056C>T	p.Thr1019Met	c.6472A>G	p.Lys2158Glu
EECA5	CA5	c.3323G>A	p.Arg1108His	c.3323G>A	p.Arg1108His
EECA62	CA62	c.5594dupA	p.His1865Glnfs*47	c.5773A>G	p.Arg1925Gly
EECB12	CB12	c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6];Asn1868Ile]

EECB27		c.[466A>G;477 1G>A]	p.[Ile156Val; Gly1591Arg]	c.[4469G>A;56 03A>T]	p.[Cys1490Tyr; Asn1868Ile]
EECB31	CB31	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.3322C>T	p.Arg1108Cys
EECB32	CB31	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.3322C>T	p.Arg1108Cys
EECB45	G22	c.3056C>T	p.Thr1019Met	c.6079C>T	p.Leu2027Phe
EECB60	CB60	c.4139C>T	p.Pro1380Leu	c.4577C>T	p.Thr1526Met
EECB73		c.4685T>C	p.Ile1562Thr	c.4957G>A	p.Gly1653Arg
EECB77	G22	c.3056C>T	p.Thr1019Met	c.6079C>T	p.Leu2027Phe
EECB80	CB80	c.1933G>A	p.Asp645Asn	c.6098T>G	p.Leu2033Arg
EECC17	CC17	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.4539+2028C >T	p.(=, Arg1514Leufs*36)
EECC18	CC17	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.4539+2028C >T	p.(=, Arg1514Leufs*36)
EECC22	F65	c.[2588G>C;56 03A>T]	p.[Gly863Ala,Gly8 63del; Asn1868Ile]	c.4577C>T	p.Thr1526Met
EECC23	CC23	c.3056C>T	p.Thr1019Met	c.4326C>A	p.Asn1442Lys
EECC38		c.2041C>T	p.Arg681*	c.5882G>A	p.Gly1961Glu
EECC40	J21	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;49 18C>T]	p.[Trp1408Arg; Arg1640Trp]
EECC41	J21	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;49 18C>T]	p.[Trp1408Arg; Arg1640Trp]
EECC44		c.[185C>T;560 3A>T]	p.[Pro62Leu; Asn1868Ile]	c.6098T>G	p.Leu2033Arg
EECD26		c.3322C>T	p.Arg1108Cys	c.4139C>T	p.Pro1380Leu
EECD3		c.1906C>T	p.Gln636*	c.3113C>T	p.Ala1038Val
EECD44	CD44	c.5113C>T	p.Arg1705Trp	c.5929G>A	p.Gly1977Ser
EECD59		c.3056C>T	p.Thr1019Met	c.5714+5G>A	p.(=,Glu1863Leufs*3 3)
EECD78		c.3113C>T	p.Ala1038Val	c.[4222T>C;49 18C>T]	p.[Trp1408Arg; Arg1640Trp]
EECD79	CD79	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.6449G>A	p.Cys2150Tyr
EECE23	CC23	c.3056C>T	p.Thr1019Met	c.4326C>A	p.Asn1442Lys
EECE4		c.4253+43G>A	p.[=,Ile1377Hisfs* 3]	c.4861A>T	p.Asn1621Tyr
EECE66		c.3056C>T	p.Thr1019Met	c.3322C>T	p.Arg1108Cys
EECE75		c.3322C>T	p.Arg1108Cys	c.4139C>T	p.Pro1380Leu
EECF37	CB60	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.2483C>T	p.Pro828Leu
EECF37	CF37	c.4139C>T	p.Pro1380Leu	c.4577C>T	p.Thr1526Met
EECF45	CF45	c.2382+1dup	p.?	c.5603A>T	p.Asn1868Ile
EECF65		c.466A>G	p.Ile156Val	c.4253+43G>A	p.(=,Ile1377Hisfs*3)
EECF74		c.4957G>A	p.Gly1653Arg	c.4957G>A	p.Gly1653Arg
EECG9		c.3137T>G	p.Leu1046Trp	c.3322C>T	p.Arg1108Cys

EECH32		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4577C>T	p.Thr1526Met
EECH55		c.5917delG	p.Val1973*	c.5917delG	p.Val1973*
EECL2	G34	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]	c.6286G>A	p.Glu2096Lys
EED19	C7	c.4577C>T	p.Thr1526Met	c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
EED2	C4	c.[2827C>T;5603A>T]	p.[Arg943Trp; Asn1868Ile]	c.4577C>T	p.Thr1526Met
EED21	D21	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4225A>G	p.Ile1409Val
EED23		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]	c.3056C>T	p.Thr1019Met
EED29	D29	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4539+2028C>T	p.(=, Arg1514Leufs*36)
EED32	D29	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4139C>T	p.Pro1380Leu
EED43		c.[1622T>C;3113C>T]	p.[Leu541Pro; Ala1038Val]	c.5882G>A	p.Gly1961Glu
EEE2	A49	c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
EEE23		c.6079C>T	p.Leu2027Phe	c.5196+1137G>A	p.(=,Met1733Glufs*78)
EEE37	E37	c.[4222T>C; 4918C>T]	p.[Trp1408Arg; Arg1640Trp]	c.3056C>T	p.Thr1019Met
EEE45		c.2588G>C	p.[Gly863Ala,Gly863del]	c.4363T>C; c.5693G>A	p.Cys1455Arg; p.Arg1898His
EEE47	E37	c.3056C>T	p.Thr1019Met	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
EEE48	E37	c.3056C>T	p.Thr1019Met	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
EEF29		c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.6743T>C	p.Phe2248Ser
EEF3		c.4139C>T	p.Pro1380Leu	c.6449G>A	p.Cys2150Tyr
EEF30		c.3323G>A	p.Arg1108His	c.6098T>G	p.Leu2033Arg
EEF41		c.4577C>T	p.Thr1526Met	c.869G>A	p.Arg290Gln
EEF45	D29	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.3113C>T	p.Ala1038Val
EEF54	F54	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.1805G>A	p.Arg602Gln
EEF57		c.[5113C>T;5882G>A]	p.[Arg1705Trp; Gly1961Glu]	c.5882G>A	p.Gly1961Glu
EEF65	F65	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]	c.4577C>T	p.Thr1526Met
EEF83		c.286A>C	p.Asn96His	c.5603A>T	p.Asn1868Ile
EEG22	G22	c.3056C>T	p.Thr1019Met	c.6079C>T	p.Leu2027Phe
EEG24		c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.3212C>A	p.Ser1071*

EEG34	G34	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.6286G>A	p.Glu2096Lys
EEG42		c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6];Asn1868Ile]	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6];Asn1868Ile]
EEG46		c.4253+43G>A; c.5603A>T	p.[=,Ile1377Hisfs*3]; p.Asn1868Ile	c.5603A>T	p.Asn1868Ile
EEG49		c.3305A>T	p.Asp1102Val	c.4468T>C	p.Cys1490Arg
EEG56	G56	c.735T>G	p.Tyr245*	c.5603A>T	p.Asn1868Ile
EEG66		c.4363T>C	p.Cys1455Arg	c.5882G>A	p.Gly1961Glu
EEH12		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del;Asn1868Ile]	c.3329-1G>A	p.?
EEH17	H17	c.4577C>T	p.Thr1526Met	c.4685T>C	p.Ile1562Thr
EEH31		c.2701A>G	p.Thr901Ala	c.5603A>T	p.Asn1868Ile
EEH62		c.4320del; c.5603A>T	p.Phe1440Leufs*6; p.Asn1868Ile	c.455G>A	p.Arg152Gln
EEH8	H8	c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.4225A>G	p.Ile1409Val
EEI1	C4	c.[2827C>T;5603A>T]	p.[Arg943Trp;Asn1868Ile]	c.4577C>T	p.Thr1526Met
EEI16	H17	c.4577C>T	p.Thr1526Met	c.4685T>C	p.Ile1562Thr
EEI18		c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.3056C>T	p.Thr1019Met
EEI36		c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	c.5603A>T	p.Asn1868Ile
EEI66		c.4139C>T	p.Pro1380Leu	c.4539+2028C>T	p.(=,Arg1514Leufs*36)
EEJ11		c.4861A>T	p.Asn1621Tyr	c.5196+1G>A	p.?
EEJ13	J13	c.223T>G	p.Cys75Gly	c.[302+68C>T;4539+2028C>T]	p.[?; =,Arg1514Leufs*36]
EEJ17	J17	c.1804C>T	p.Arg602Trp	c.5603A>T	p.Asn1868Ile
EEJ21	J21	c.[302+68C>T;4539+2028C>T]	p.[?; =,Arg1514Leufs*36]	c.[302+68C>T;4539+2028C>T]	p.[?; =,Arg1514Leufs*36]
EEJ25		c.3386G>T	p.Arg1129Leu	c.6098T>G	p.Leu2033Arg
EEJ43		c.[2827C>T;5603A>T]	p.[Arg943Trp;Asn1868Ile]	c.4577C>T	p.Thr1526Met
EEK18		c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)
EEK40		c.3322C>T	p.Arg1108Cys	c.4577C>T	p.Thr1526Met
EEK7		c.161G>A	p.(Cys54Serfs*14,Cys54Tyr)	c.4577C>T	p.Thr1526Met
EEK8		c.2894A>G; c.5603A>T	p.Asn965Ser; p.Asn1868Ile	c.4577C>T	p.Thr1526Met
EEL19	J13	c.223T>G; c.302+68C>T	p.Cys75Gly; p.?	c.[302+68C>T;4539+2028C>T]	p.[?; =,Arg1514Leufs*36]
EEN3		c.3322C>T	p.Arg1108Cys	c.3329-1G>A	p.?
EEO27	O27	c.1519G>T	p.Asp507Tyr	c.3056C>T	p.Thr1019Met
EEO41		c.3113C>T	p.Ala1038Val	c.3322C>T	p.Arg1108Cys

EEP11	B24	c.1253T>C	p.Phe418Ser	c.[2827C>T;5603A>T]	p.[Arg943Trp;Asn1868Ile]
EEP12	P12	c.1037A>C	p.Lys346Thr	c.5329A>G	p.Met1777Val
EEP20		c.4577C>T	p.Thr1526Met	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
EER11		c.5351T>C	p.Leu1784Arg	c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
EER13		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.1865G>A	p.Ser622Asn
EER44		c.5644A>G	p.Met1882Val	c.5929G>A	p.Gly1977Ser
EER53		c.3364G>A	p.Glu1122Lys	c.[4222T>C;4918C>T]	p.[Trp1408Arg;Arg1640Trp]
EES15	B33	c.1018T>C	p.Tyr340His	c.4139C>T	p.Pro1380Leu
EES17		c.5348T>C	p.Phe1783Ser	c.5917delG	p.Val1973*
EES44		c.4139C>T	p.Pro1380Leu	c.[302+68C>T;4539+2028C>T]	p.[?; =, Arg1514Leufs*36]
EES5		c.4577C>T	p.Thr1526Met	c.5603A>T	p.Asn1868Ile
EES51		c.[5461-10T>C;5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]	c.5929G>A	p.Gly1977Ser
EES77	S77	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.3056C>T	p.Thr1019Met
EET15	S77	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]
EET4	A49	c.[302+68C>T;4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;4918C>T]	p.[Trp1408Arg;Arg1640Trp]
EET6		c.[4469G>A;5603A>T]	p.[Cys1490Tyr;Asn1868Ile]	c.5603A>T	p.Asn1868Ile
EET8		c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863del; Asn1868Ile]	c.5714+5G>A	p.(=,Glu1863Leufs*33)
EEU5		c.3323G>A	p.Arg1108His	c.4139C>T	p.Pro1380Leu
EEU7		c.587C>T	p.Pro196Leu	c.5714+5G>A; c.5882G>A	p.(=,Glu1863Leufs*33); p.Gly1961Glu
EEX1		c.[1622T>C;3113C>T]	p.[Leu541Pro;Ala1038Val]	c.6448T>C	p.Cys2150Arg
MH1322		c.6079C>T	p.Leu2027Phe	c.6449G>A	p.Cys2150Tyr
MH1326		c.3323G>A	p.Arg1108His	c.5351T>C	p.Leu1784Arg
MH1328		c.4139C>T	p.Pro1380Leu	c.5921A>G	p.Asn1974Ser
MH1332		c.[3481C>A;5603A>T]	p.[Arg1161Ser;Asn1868Ile]	c.5882G>A	p.Gly1961Glu
MH1336		c.4139C>T	p.Pro1380Leu	c.5921A>G	p.Asn1974Ser
MH1337		c.2564G>A	p.Trp855*	c.6079C>T	p.Leu2027Phe
MH1341		c.5312+1G>A	p.?	c.5882G>A	p.Gly1961Glu
MH1347		c.1532G>A;c.5882G>A	p.Arg511His; p.Gly1961Glu	c.[2852T>C;5603A>T]	p.[Ile951Thr;Asn1868Ile]
MH1360		c.4577del	p.Thr1526Argfs*10	c.5882G>A	p.Gly1961Glu
MH1361		c.4253+43G>A	p.[=,Ile1377Hisfs*3]	c.223T>C	p.Cys75Arg

MH1365		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.1317G>A; c.5908C>T	p.Trp439*; p.Leu1970Phe
MH1382		c.5882G>A	p.Gly1961Glu	c.6221G>A	p.Gly2074Asp
MH1383		c.868C>T	p.Arg290Trp	c.5917delG	p.Val1973*
MH1406		c.4363T>C	p.Cys1455Arg	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)
MH1407		c.752delT	p.Phe251Serfs*11	c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
MH1408		c.4139C>T	p.Pro1380Leu	c.4139C>T	p.Pro1380Leu
MH1417		c.4229A>G; c.4925G>T	p.Tyr1410Cys; p.Ser1642Ile	c.66G>T; c.618C>G	p.Lys22Asn; p.Ser206Arg
MH1418		c.5603A>T	p.Asn1868Ile	c.1222C>T	p.Arg408*
MH1433		c.[2827C>T;5603A>T]	p.[Arg943Trp; Asn1868Ile]	c.1253T>C	p.Phe418Ser
MH1435		c.4539+2028C>T	p.Arg1514Leufs*36	c.2160+1G>C	p.?
MH1445		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4139C>T	p.Pro1380Leu
MH1446		c.4234C>T	p.Gln1412*	c.5882G>A	p.Gly1961Glu
MH1456		c.4139C>T	p.Pro1380Leu	c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]; Asn1868Ile]
MH1462		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
MH1465		c.6079C>T	p.Leu2027Phe	c.4537dup	p.Gln1513Profs*42
MH1471	1471	c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
MH1472	1471	c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]
MH1473	1471	c.[4222T>C; 4918C>T]	p.[Trp1408Arg; Arg1640Trp]	c.4539+2028C>T	p.(=, Arg1514Leufs*36)
MH1479		c.3322C>T	p.Arg1108Cys	c.4139C>T	p.Pro1380Leu
MH1491		c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.3322C>T	p.Arg1108Cys
MH1502		c.4139C>T	p.Pro1380Leu	c.5882G>A	p.Gly1961Glu
MH1506		c.2549A>G	p.Tyr850Cys	c.5882G>A; c.6098T>G	p.Gly1961Glu; p.Leu2033Arg
MH1519		c.[302+68C>T; 4539+2028C>T]	p.[?; =, Arg1514Leufs*36]	c.6472A>G	p.Lys2158Glu
MH1525		c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.5898+5G>A	p.(=, Cys1967Valfs*24)
MH1529		c.4253+43G>A	p.[=, Ile1377Hisfs*3]	c.4363T>C	p.Cys1455Arg
MH1532	1532	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.5196+1137G>A	p.(=, Met1733Glufs*78)
MH1533	1532	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.5196+1137G>A	p.(=, Met1733Glufs*78)
MH1536		c.191C>T	p.Ala64Val	c.3322C>T; c.5603A>T	p.Arg1108Cys; p.Asn1868Ile
MH1558		c.3056C>T	p.Thr1019Met	c.6079C>T	p.Leu2027Phe

MH1561	c.4139C>T	p.Pro1380Leu	c.6449G>A	p.Cys2150Tyr
MH1585	c.731T>C	p.Leu244Pro	c.3056C>T	p.Thr1019Met
MH1587	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]	c.2453G>A	p.Gly818Glu
MH1593	c.4793C>A	p.Ala1598Asp	c.3322C>T	p.Arg1108Cys
MH1595	c.3322C>T	p.Arg1108Cys	c.[302+68C>T; 4539+2028C> T]	p.[?; =, Arg1514Leufs*36]
MH1624	c.4139C>T	p.Pro1380Leu	c.6079C>T	p.Leu2027Phe
MH1636	c.3322C>T	p.Arg1108Cys	c.4139C>T	p.Pro1380Leu
MH1661	c.6658C>T	p.Gln2220*	c.550- 551TC>CG	p.Ser184Arg
MH1677	c.3754G>T; c.5603A>T	p.Glu1252*; p.Asn1868Ile	c.4539+2028C >T	p.(=, Arg1514Leufs*36)
MH1678	c.1519G>T	p.Asp507Tyr	c.4139C>T	p.Pro1380Leu
MH1679	c.4253+43G>A	p.[=,Ile1377Hisfs* 3]	c.5917delG	p.Val1973*
MH1685	c.4363T>C	p.Cys1455Arg	c.6079C>T	p.Leu2027Phe
MH1688	c.161G>A	p.(Cys54Serfs*14, Cys54Tyr)	c.4139C>T	p.Pro1380Leu
MH1693	c.4139C>T	p.Pro1380Leu	c.4469G>A; c.5603A>T	p.Cys1490Tyr; p.Asn1868Ile

ABCA4 is a very large gene with over 2000 different variants reported so far (Cornelis *et al.* 2020; LOVD database). While considerable effort has been put into the assessment and interpretation of all these variants over the 25 years since this gene was discovered, more comprehensive studies remain necessary. Recent findings indicate that synonymous variants or previously considered benign missense variants can also have deleterious effects on protein expression, for example through the generation of defective splice products (Fadaie *et al.* 2019; Kaltak *et al.* 2023). Indeed, the synonymous c.768G>T variant found in one of our patients (BF155) has been shown to cause exon elongation, leading to a 35 nucleotide insertion and frameshift (Sangermano *et al.* 2018). Similarly, our group found that a synonymous variant in *PROM1* is responsible for disrupted splicing, leading to recessive early onset retinal degeneration (Whelan *et al.*, in preparation).

As written above, splice altering variants make up a total of 24.95% of the total alleles in Irish biallelic *ABCA4* probands. Table 5.4 summarises the *in silico* prediction scores for 6 non-canonical splice site variants found in our cohort that are predicted to affect splicing, but have not been assessed *in vitro*. As the prediction scores in these variants are extremely high, researchers might choose to use their resources to instead investigate variants whose effect on splicing is less predictable. Table 5.5 displays splice-altering variants identified in our cohort that have been assessed in *in vitro* splice assays, either through mini- or midgenes, or through the use of patient-derived photoreceptor precursor cells.

Table 5.4. Overview of putative splice altering variants and in silico prediction scores

DNA change	number in cohort	SpliceAI (0-1)	dbscSNV Ada (0-1)
c.2160+1G>C	1	1	1
c.2382+1dup	1	0.9	N/A
c.3329-1G>A	2	0.93	1
c.5196+1G>A	1	0.99	1
c.5312+1G>A	1	0.96	1
c.6148G>C	1	0	1

Table 5.5. Overview of splice-altering variants in *ABCA4* found in Target 5000 Stargardt cohort that were assessed in vitro.

DNA change	Alleles in cohort	RNA change	Protein effect	WT RNA (%)	Severity of splice defect	References
c.161G>A	40	r.[161_302del,=]	p.[Cys54Serfs*14,Cys54Tyr]	44	Moderate	Fadaie <i>et al.</i> 2019
c.2588G>C	30	r.[2588G>C,2588_2590del]	p.[Gly863Ala,Gly863del]	60	Mild/Moderate	Maugeri <i>et al.</i> (1999); Sangermano <i>et al.</i> 2018
c.4253+43G>A	7	r.[=, 4129_4253del(125	p.[=, Ile1377Hisfs*3	64	Moderate	Sangermano <i>et al.</i> 2019; Khan <i>et al.</i> 2019, 2020; Bauwens <i>et al.</i> 2019
c.4254-5T>A	2	r.[4254_4352del,4254_4539del,4254_4466del]	p.[Ser1418_Pro1451delinsArg,Ser1418Argfs*13,Ser1418_Cys1488del]	2	Severe	Corradi <i>et al.</i> in preparation
c.4539+2028C>T	29	r.[=,4539_4540ins4539+1891_4540-2162]	p.[=,Arg1514Leufs*36]	70	Moderate	Braun <i>et al.</i> 2013; Zernant <i>et al.</i> 2014; Albert <i>et al.</i> 2018; Khan <i>et al.</i> 2019; 2020
c.5196+1137G>A	4	r.[=,5196_5197ins5196+1140_5196+1212]	p.[Met1733Gluufs*78, =]	55	Moderate	Braun <i>et al.</i> 2013; Zernant <i>et al.</i> 2014; Sangermano <i>et al.</i> 2019; Khan <i>et al.</i> 2019
c.5312+3A>T	2	r.5197_5312del	p.(Asn1734Glyfs*14)	0	Deleterious	Sangermano <i>et al.</i> 2018
c.5461-10T>C	12	r.[5461_5714del,5461_5584del]	p.[Thr1821Valfs*13, Thr1821Aspfs*6]	0	Deleterious	Sangermano <i>et al.</i> 2016; 2018
c.5714+5G>A	4	r.[=, 5585_5714del]	p.[=,Glu1863Leufs*33]	40	Moderate	Sangermano <i>et al.</i> 2018
c.5898+5G>A	1	r.[5898_5899ins_5899+1_5890-1,5898_5899ins5899+1_5899+170,=]	p.[Cys1967Valfs*24,=]	48	Moderate	Khan <i>et al.</i> 2020
c.768G>T	1	r.768_769ins769+1_769+30	p.(Leu257Valfs*17)	0	Deleterious	Sangermano <i>et al.</i> 2018

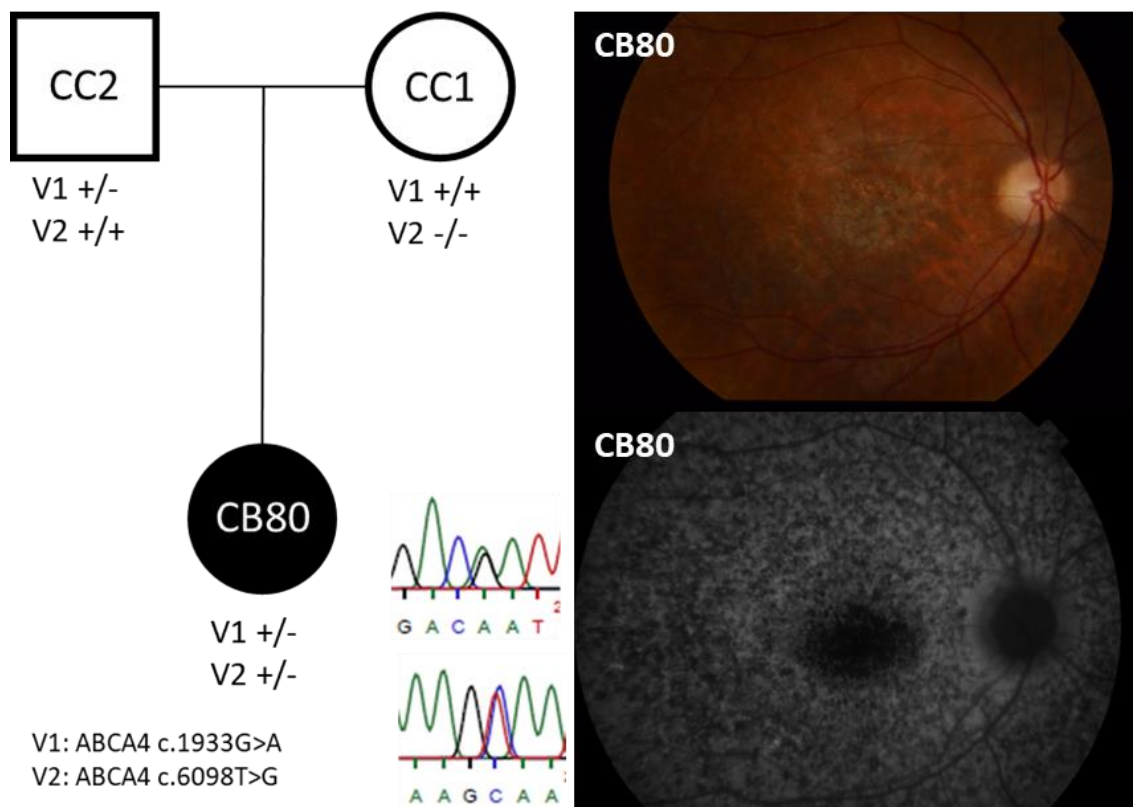


Figure 5.7. Pedigree and fundus images of proband with two mild/moderate variants. Proband CB80 is compound heterozygous for ABCA4 c.1933G>A and c.6098T>G. Fundus images indicate moderate peripheral atrophy and mild chorioretinal atrophy.

The CB80 pedigree presented in figure 5.7 is another interesting case possibly indicating the presence of modifier variants. The proband is compound heterozygous for two mild/moderate variants in *ABCA4*, c.1933G>A and c.6098T>G, respectively, and presented with symptoms at age 10. At age 11, ERGs indicated both rod and cone dystrophy and visual acuity is 6/60 in both eyes. Fundus images reveal retinal atrophy extending to the mid-periphery and mild chorioretinal atrophy. However, what is extremely interesting is that during cascade analysis, sanger sequencing indicated that the mother of the proband is homozygous for the c.6098T>G variant, but to our knowledge, remains unaffected. This variant has been reported 14 times in LOVD and assessment utilising ACMG guidelines implies this variant is likely pathogenic; the prevalence of this variant is significantly increased in affected individuals (PS4), while the allele frequency in gnomAD is extremely low (PM2), it is a non-synonymous variant in a mutational hotspot where other mutations have also been found to cause disease and benign missense mutation are not common (PM1 and PP2). Additionally, various *in silico* tools, such as MetaLR (0.97/1) and Revel (0.97) predict this missense variant to be deleterious (PP3). While the presence of unidentified modifier loci or incomplete penetrance of the variant may explain the lack of phenotype, another possibility is unconscious ignorance when self-reporting phenotypic conditions.

ABCA4 c.5348T>C (p.Phe1783Ser) is a variant of unknown significance that to our knowledge has not been reported before. This novel variant was identified in a proband (S17) alongside a severe frameshift allele. Both variants were confirmed with Sanger sequencing, but as this patient was adopted and has no children, segregation analysis could not be performed. The c.5348T>C variant has not been reported in any population genome databases such as gnomAD or ExAC, nor in clinical mutation databases such as ClinVar and LOVD. When utilising ACMG guidelines (Richards *et al.* 2015) the following assessments were made: PM1, PP2 (supporting) and PM2. The effect on protein function could not be predicted as no *in silico* or *in vitro* assessments have been generated for this variant before. Here, we compare the clinical phenotype of this patient to another patient that is homozygous for the same null allele (CH55). Figure 5.8 shows autofluorescence and coloured fundus images of both patients. Peripheral retinal atrophy is moderate in both individuals, meaning it extends to mid-periphery and chorioretinal atrophy is restricted to macular area. Visual acuity is poor in both individuals. ERGs indicated rod and cone dystrophy in the homozygote, while the compound heterozygous individual displayed a near-normal ERG. These data can also be compared to individual CA5, who homozygously carries two mild variants. While ERG responses indicate cone and rod dystrophy in this individual and visual acuity is poor, retinal atrophy remains mild (figure 5.9) and the age of onset is much higher than in the patients with more severe variants. Though caution should be taken when interpreting clinical data from such small numbers of individuals, these data provide some insight in the effect of c.5348T>C on the phenotype.

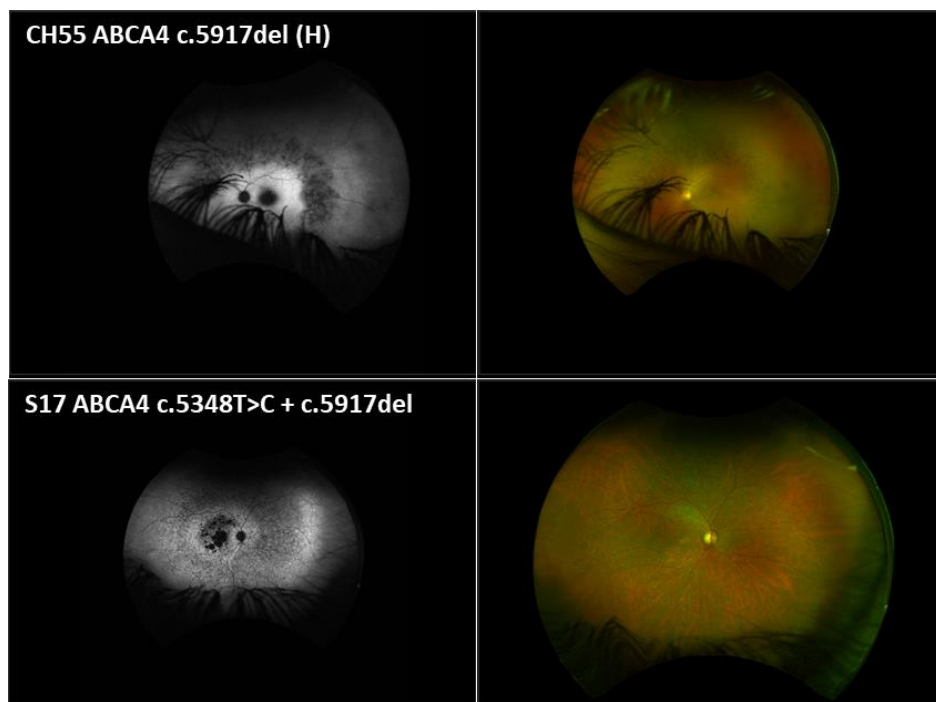


Figure 5.8. Autofluorescence and coloured fundus images of a patient carrying the null allele c.5917del homozygously compared to a patient carrying that variant heterozygously in combination with novel variant c.5348T>C in *ABCA4*.

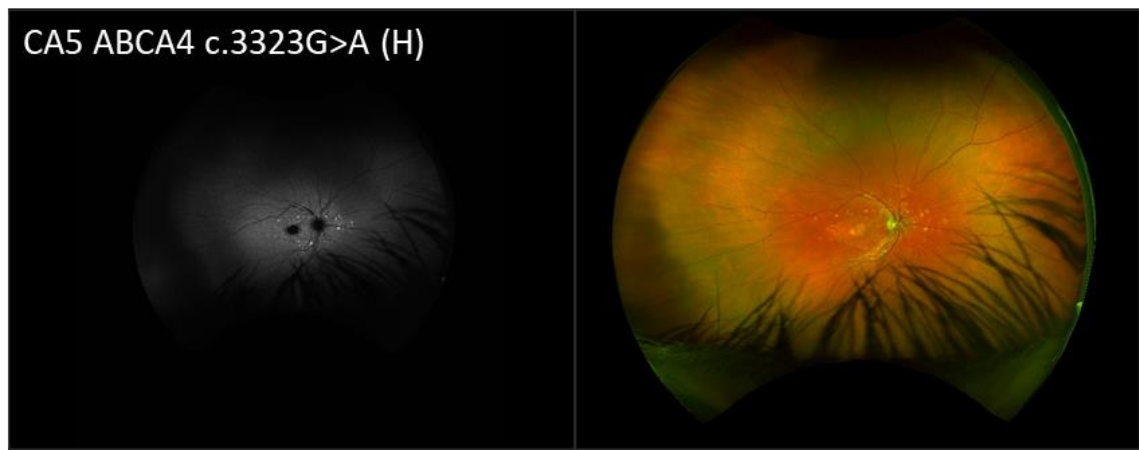


Figure 5.9. Fundus images of proband homozygous for mild/moderate variant c.3323G>A in ABCA4.

Table 5.6. Clinical information on 3 probands with different allele combinations

ID	YOB	Severity of variants	Age at Onset	BCVA RE	BCVA LE	ERG	Peripheral Retinal Atrophy	Chorioretinal Atrophy
CH55	1999	Null/null	6	6/60	6/60	Rod/Cone Dystrophy	Moderate	Mild
S17	1990	Null/VUS	7	1/24	1/24	Borderline normal	Moderate	Mild
CA5	1983	Mild/mild	23	6/60	6/60	Rod/Cone Dystrophy	Mild	Mild

5.3.2. Enrichment of *ABCA4* c.161G>A in the Irish population

Previously our lab described a deep-intronic variant in *ABCA4* that is highly enriched in the Irish population (Whelan *et al.* 2023). The c.4539+2028C>T variant (p.[=,Arg1514Leufs*36]) causes pseudoexon inclusion leading to a frameshift and premature truncation of the protein, and appears to have a moderate effect on protein function (Albert *et al.* 2018; Cornelis *et al.* 2017). To date, 31 alleles in 29 affected individuals have been identified in our Stargardt cohort, whereas globally only 15 other cases have been reported (Braun *et al.* 2013; Zernant *et al.* 2014; Schultz *et al.* 2017; Khan *et al.* 2019; 2020), indicating a possible founder effect. Here, we present another variant that affects splicing of the *ABCA4* gene that appears to be enriched in the Irish population; c.161G>A (p.(Cys54Serfs*14,Cys54Tyr)).

In the Target 5000 Stargardt cohort, 40 alleles of the c.161G>A variant were found, of which 10 were present in 5 homozygotes, which made this the most common allele in our cohort. Though this variant has been described before (Zhang *et al.* 1999; Fadaie *et al.* 2019), it appears to be rare in the healthy population. ClinVAR estimates the global AF to be 0.000089, and gnomAD predicts the global AF to be 0.00003943, with there only being 6 alleles in their world-wide database, 5 of which are from European donors. LOVD, however, counts 136 incidences of this allele, highlighting its role in disease. Interestingly, this means 29% of the alleles identified globally were present in the Irish population of less than 7 million, which may be suggestive of a founder effect. In our own database, the only non-affected individuals harbouring this variant were those who were tested in cascade analysis for confirmation of an affected family member. Additionally, this variant was not found in a cohort of 408 whole genome sequenced individuals of an ALS study by the Russell McLaughlin lab at TCD. Table 5.7 gives an overview of the affected individuals enrolled in the Target 5000 project that were found to harbour this gene, their clinical diagnosis, the second variant found in *ABCA4* in this individual, and the predicted severity of this second variant as assessed through *in silico* meta-analysis by Cornelis *et al.* 2017.

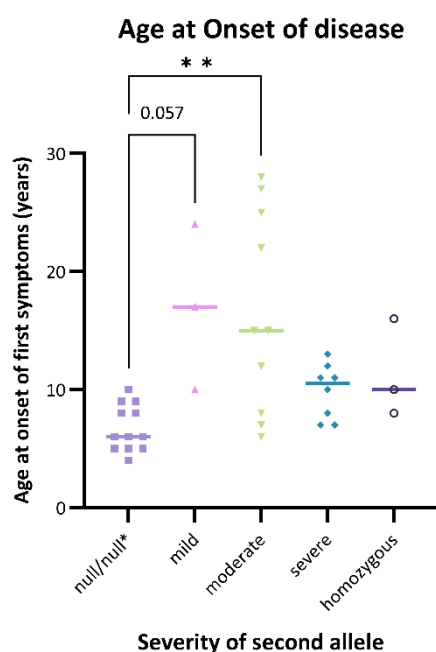
Table 5.7. Overview of all solved individuals who carry the Cys54Tyr variant in *ABCA4*, their second variant, and what NGS method was used to identify the variants. V1 = first allele; V2 = second allele; CRD = Cone-Rod Dystrophy; STGD1 = Stargardt disease type 1; RP = Retinitis Pigmentosa; MD = Macular Degeneration; N/A = not available to us; *severity as estimated by Cornelis et al 2017

Patient ID	Pedigree	Clinical Diagnosis	V1	V2 (DNA Change)	V2 (Protein Change)	Severity V2*	Sequencing Type
BF014	RP014	Cone Rod Dystrophy	c.161G>A	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	moderate	<i>ABCA4</i> smMIPs
BF374		STGD1	c.161G>A	N/A	N/A	N/A	blueprint IRD panel
BF375		STGD1	c.161G>A	N/A	N/A	N/A	blueprint IRD panel
BF431		RP	c.161G>A	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	moderate	WES
BF456		STGD1	c.161G>A	c.4685T>C	p.Ile1562Thr	benign/mild	<i>ABCA4</i> smMIPs
BF458	RP014	Rod-Cone Dystrophy	c.161G>A	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	moderate	WES
BF473		STGD1	c.161G>A	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	moderate	Target Capture
BF474		STGD1	c.161G>A	c.3322C>T	p.Arg1108Cys	moderate	Target Capture
BF528		STGD1	c.161G>A	c.[1715G>A;2588G>C;5603A>T]	p.[Arg572Gln; Gly863Ala,Gly863de; Asn1868Ile]	severe	<i>ABCA4</i> smMIPs
BF637	RP637	STGD1	c.161G>A	c.4254-5T>A	splice defect	severe	<i>ABCA4</i> smMIPs
BF638	RP637	STGD1	c.161G>A	c.4254-5T>A	splice defect	severe	<i>ABCA4</i> smMIPs
EECB12		STGD1	c.161G>A	c.[5461-10T>C; 5603A>T]	p.[Thr1821Valfs*13,Thr1821Aspfs*6; Asn1868Ile]	severe	<i>ABCA4</i> smMIPs
EECB31	CB31	STGD1	c.161G>A	c.3322C>T	p.Arg1108Cys	moderate	Target Capture
EECB32	CB31	STGD1	c.161G>A	c.3322C>T	p.Arg1108Cys	moderate	Sanger
EECF37		STGD1	c.161G>A	c.2483C>T	p.Pro828Leu	N/A	<i>ABCA4</i> smMIPs
EECH32		STGD1	c.161G>A	c.4577C>T	p.Thr1526Met	moderate	MD smmips

EED21		STGD1	c.161G>A	c.4225A>G	p.Ile1409Val	mild/moderate	Target Capture
EED29	D29	STGD1	c.161G>A	c.4539+2028C>T	p.[=,Arg1514Leufs*36]	moderate	Target Capture
EED32	D29	STGD1	c.161G>A	c.4139C>T	p.Pro1380Leu	moderate	Target Capture
EEF45		STGD1	c.161G>A	c.3113C>T	p.Ala1038Val	mild	Target Capture
EEF54	D29	STGD1	c.161G>A	c.1805G>A	p.Arg602Gln	moderate	Target Capture
EEH8		Rod-Cone Dystrophy	c.161G>A	c.4225A>G	p.Ile1409Val	mild/moderate	Target Capture
EEI18		Choroideremia	c.161G>A	c.3056C>T	p.Thr1019Met	severe	Target Capture
EEK18		STGD1	c.161G>A	c.161G>A	p.[Cys54Serfs*14,Cys54Tyr]	moderate	Target Capture
EEK7		STGD1	c.161G>A	c.4577C>T	p.Thr1526Met	moderate	Target Capture
EER13		STGD1	c.161G>A	c.1865G>A	p.Ser622Asn	N/A	Target Capture
EES77	S77	STGD1	c.161G>A	c.3056C>T	p.Thr1019Met	severe	Target Capture
EET15	S77	STGD1	c.161G>A	c.[2588G>C;5603A>T]	p.[Gly863Ala,Gly863de; Asn1868Ile]	mild	Target Capture
MH1365		STGD1	c.161G>A	c.1317G>A	p.Trp439*	severe	Target Capture
MH1406		STGD1	c.161G>A	c.4363T>C	p.Cys1455Arg	severe	Target Capture
MH1445		CRD	c.161G>A	c.4139C>T	p.Pro1380Leu	moderate	Target Capture
MH1462		Best disease	c.161G>A	c.[4222T>C;4918C>T]	p.[Trp1408Arg; Arg1640Trp]	severe	MD smmips
MH1525		CRD	c.161G>A	c.5898+5G>A	p.[=,Cys1967Valfs*24]	moderate	<i>ABCA4</i> smMIPs
MH1532	MH1532	STGD1	c.161G>A	c.5196+1137G>A	p.[=,Met1733Glufs*78]	moderate	Sanger
MH1533	MH1532	STGD1	c.161G>A	c.5196+1137G>A	p.[=,Met1733Glufs*78]	moderate	<i>ABCA4</i> smMIPs
MH1688		MD	c.161G>A	c.4139C>T	p.Pro1380Leu	moderate	MD smmips

Interestingly, the c.161G>A variant not only causes a missense effect on protein translation, it also causes skipping of exon 3 in 56% of transcripts (Fadaie et al 2019). This bimodal effect appears to have a moderately severe impact on protein function, as age of onset of first symptoms in probands carrying this specific variant is relatively low with a median age of 11 years (range 6-28, n=26). It is important to note, however, that due to time limits and busy schedules, clinical data was not available for all patients harbouring the Cys54Tyr variant at the moment of writing this thesis. The following section describing the effects of this variant on phenotype, therefore, is based on patients whose data was attainable. Table 5.6 presents clinical data for each proband harbouring at least one c.161G>A variant in *ABCA4*.

The severity of the second variant is used to group patients in order to compare phenotypic data. These data were compared to genotype-phenotype correlations previously reported by Fakin *et al.* (2016). That study recruited 20 patients with two null or null-like alleles and a further 82 patients who harboured one of 15 specific *ABCA4* variants in trans with a null allele. There, the median age of onset in the group with two null alleles was 6 years (range = 4-10, n=12), which we then compared to the median ages of onset in our c.161G>A cohort. When the second allele was a mild variant, the median age of onset was 17 (range 10-24, n=3), and a Kruskal-Wallis test showed there was a strong trend when compared to double null alleles, likely due to the low n-



number. The second allele having a moderate effect on protein function is linked to a median age at onset of 15 (range 6-28, n=10), which was significantly higher than the age at onset with null/null alleles (Kruskal-Wallis test, p=0.0053). The median age of onset in people harbouring homozygous c.161G>A variants was 10 (range 8-16, n=3), and in patients with the second allele being severe 10.5 (range 7-13, n=8). Neither of these were significantly later than the age at onset in the double null patients described by Fakin *et al.* (2016).

Figure 5.10. Age at the onset of first symptoms of disease, grouped by the severity of the second allele. Age at onset of first symptoms of disease when the second allele is a mild variant is 17 (median, range 10-24, n=3). The median age of onset for patients harbouring moderate second alleles was 15 (range 6-28, n=10), and for those with a severe variant the age of onset was 10.5 (range 7-13, n=8). Age at onset data was available for 3 individuals homozygous for the Cys54Tyr variant, leading to a median age at onset of 10 (range 8-16). Median age of onset with two null alleles is 6 (range 4-10), these data were reported previously (Fakin *et al.* 2016). ** = p<0.01

Table 5.8. Overview of clinical data of patients harbouring c.161G>A variants in ABCA4, arranged by severity of variant 2 (V2). YOB = Year of Birth, BCVA = Best Corrected Visual Acuity, RE = right eye, LE = left eye, ERG = electroretinogram, [#] = homozygous for c.161G>A, CF = counting fingers, HM = hand movement, // = no data available. Extent of peripheral retinal atrophy and chorioretinal atrophy was categorised into mild (atrophy confined to retinal arches), moderate (atrophy extends to mid-periphery), or extensive (atrophy extends to far periphery). ERG results were also categorised into groups; group 1 indicates reduced macular function, group 2 indicates cone dystrophy, and group 3 indicates cone-rod dystrophy

Patient ID	YOB	Severity V2	Age at onset	Age at last assessment	BCVA RE	BCVA LE	Extent peripheral retinal atrophy	Extent chorioretinal atrophy	Drusenoid deposits	Retinal Fibrosis	ERG	Age at last ERG
BF456	1976	mild	24	//	6/7.5	6/7.5	//	//	//	//	//	//
EED21	1993	mild	17	27	6/48	6/48	Moderate	Mild	Yes	No	Group 3	27
EEF45	1975	mild	//	//	6/48	6/48	Mild	Mild	//	//	//	//
EEH8	1963	mild	10	48	3/60	CF	Extensive	Moderate	Yes	No	Group 3	48
EET15	1957	mild	//	65	CF	6/10	Extensive	Mild	No	No	//	//
BF474	1986	moderate	15	//	6/60	6/60	//	//	//	//	//	//
EECB31	2005	moderate	8	8	//	//	Mild	Mild	No	No	//	//
EECB32	2006	moderate	//	//	//	//	//	//	//	//	//	//
EECH32	2002	moderate	7	20	6/60	6/60	Moderate	Mild	Yes	No	Group 2	20
EED29	1978	moderate	6	37	CF	6/60	Moderate	Moderate	No	No	Group 3	37
EED32	1993	moderate	22	22	//	//	Moderate	Mild	No	No	//	//
EEF54	1983	moderate	27	38	6/60	6/60	Moderate	Mild	Yes	No	Group 1	32
EEK7	1987	moderate	12	35	6/60	CF	Extensive	Moderate	Yes	Yes	//	//
MH1445	N/A	moderate	//	//	//	//	//	//	//	//	//	//
MH1525	1986	moderate	15	//	//	//	Moderate	Moderate	//	//	//	//
MH1532	1966	moderate	25	//	//	//	//	//	//	//	//	//
MH1533	1963	moderate	28	//	//	//	Moderate	//	//	//	//	//
MH1688	1998	moderate	//	//	//	//	//	//	//	//	//	//
BF014 [#]	1976	moderate [#]	10	//	6/60	6/60	//	//	//	//	//	//

BF431#	1950	moderate#	//	//	//	//	//	//	//	//	//	//	//
BF458#	1977	moderate#	16	//	2/60	HM	//	//	//	//	//	//	//
BF473#	1980	moderate#	8	//	//	//	//	//	//	//	//	//	//
EEK18#	1988	moderate#	//	34	//	//	Extensive	Moderate	Yes	No	//	//	//
BF374	//	//	//	//	//	//	//	//	//	//	//	//	//
BF375	//	//	//	//	//	//	//	//	//	//	//	//	//
EECF37	2000	//	11	18	6/60	6/60	Moderate	Mild	No	No	Group 1	15	
EER13	1995	//	8	19	6/12	6/9	Mild	Mild	No	No	Group 1	18	
BF528	1958	severe	10	//	1/150	6/240	//	//	//	//	//	//	//
BF637	1964	severe	11	//	3/60	3/60	//	//	//	//	//	//	//
BF638	1972	severe	11	//	//	//	//	//	//	//	//	//	//
EECB12	2010	severe	7	7	//	//	Mild	Mild	No	No	Group 1	8	
EEI18	1954	severe	8	69	0.5/12	0.5/60	Extensive	Extensive	No	Yes	//	//	//
EES77	2009	severe	7	13	6/48	6/48	Mild	Mild	No	No	Group 1	12	
MH1365	//	severe	//	//	//	//	//	//	//	//	//	//	//
MH1406	//	severe	13	//	//	//	//	//	//	//	//	//	//
MH1462	1963	severe	12	//	//	//	Extensive	Extensive	//	//	//	//	//

While caution should be taken when basing conclusions on clinical data of small numbers of individuals, as is often the case with rare diseases, we think these data can still give some insights on the severity of the variant. When phenotypic data is grouped by severity of the second allele, it becomes clear that the effect of c.161G>A is quite profound. When combined with a mild variant, the median BCVA is 6/48 (n=10 eyes) at a median age of 48 (range 27-65), while it is down to 6/60 (n=10 eyes) at a median age of 36 (range 8-38) when c.161G>A is combined with a moderate variant. Information on visual acuity was only available for 2 homozygous individuals, but median visual acuity would be 3/60, which is similar to the median BCVA in patients with a severe allele *in trans* with c.161G>A (3/66, n=10 eyes), though age at last assessment was only available for 3 individuals. Drusenoid deposits were identified in 6 out of 15 patients for whom this information was available and retinal fibrosis was found in 2 individuals. ERG data cannot be reliably compared, as this data is only available for one or two probands per group. As Stargardt is a progressive degenerative disorder, not just age, but time that has passed since the onset of symptoms should be taken into account when comparing phenotypes. For example, when comparing S77 and I18, who have similar ages of onset, retinal atrophy is much more extensive in the older person, implying continued degeneration over time. Further division of individuals grouped by severity of variants into subgroups based on time since onset of symptoms complicates the assessment of their phenotypes, as n-numbers become low. More comprehensive conclusions can be made when more clinical data becomes available on this cohort.

5.3.3. Non-ABCA4 pseudo-Stargardt phenotypes

As mentioned before, Stargardt disease type 1 is caused by mutations in the *ABCA4* gene (Allikmets *et al.* 1997). However, mutations in various other genes can cause pseudo-Stargardt phenotypes, including Stargardt disease type 3 and type 4, which are caused by *ELOVL4* and *PROM1*, respectively. Another gene causing Stargardt-like phenotypes is peripherin-2 (*PRPH2*). In this section, an overview will be provided on these genes in the Target 5000 cohort.

5.3.3.1. Autosomal dominant retinal dystrophy caused by *PRPH2* mutations

The *PRPH2* protein is located in the rim region of photoreceptor cells. While the exact molecular function of this tetraspanning membrane protein is still unclear, several studies suggest it plays a vital role in stabilisation and initiation of outersegment (OS) disc generation. Mouse studies showed that *Prph2*^{-/-} mice couldn't form OS discs (Zulliger *et al.* 2018) whereas mice carrying the C150S or the Y141C heterozygous mutations in *Prph2* showed reduced *Prph2* function and abnormal interaction with ROM1 (Zulliger *et al.* 2018; Chakraborty *et al.* 2020). Lewis *et al.* (2022) suggest that oligomerisation of *PRPH2* is essential for enclosure of newly generated OS discs. The clinical phenotype caused by mutations in *PRPH2* is very diverse, with rod and cone photoreceptors dysfunction varying greatly, even in individuals harbouring the same mutation. Recently, Ikelle *et al.* (2023) concluded that rod and cone photoreceptor respond differently to mutant *PRPH2* protein; whereas cone deterioration appears to be mainly caused by haploinsufficiency of *PRPH2*, rod-associated retinal degeneration may be linked to dominant negative defects in this protein. *PRPH2* not only forms homomers, but it also forms oligomers with ROM1. Several cases of recessive digenic RP have been attributed to heterozygous *PRPH2* and *ROM1* variants (Dryja *et al.* 1997).

Target 5000 *PRPH2* cohort consisted of 32 people of 16 pedigrees harbouring 12 unique pathogenic variants in *PRPH2*, which are presented in Table 5.8. Twelve of these individuals were also found to carry one or more disease-causing variants in *ABCA4*. Interestingly, two unrelated probands were found to carry a pathogenic and autosomal dominant variant in *PRPH2*, but also biallelic *ABCA4* mutations (BF522 and EEO6, respectively). At the moment of writing, not clinical data was available on these individuals but a future study on their phenotypic presentations compared to those affected by only biallelic *ABCA4* or only autosomal dominant *PRPH2*-disease would be extremely interesting. In our T5000 cohort one pedigree was found to carry heterozygous variants in both the *PRPH2* and *ROM1* genes, respectively (F59 pedigree). Interestingly, while Dryja *et al.* found the *ROM1* variant to segregate with disease in one pedigree, we found that only individuals carrying the c.637T>C mutation in *PRPH2* in addition to *ROM1* c.793G>A present with a phenotype. However,

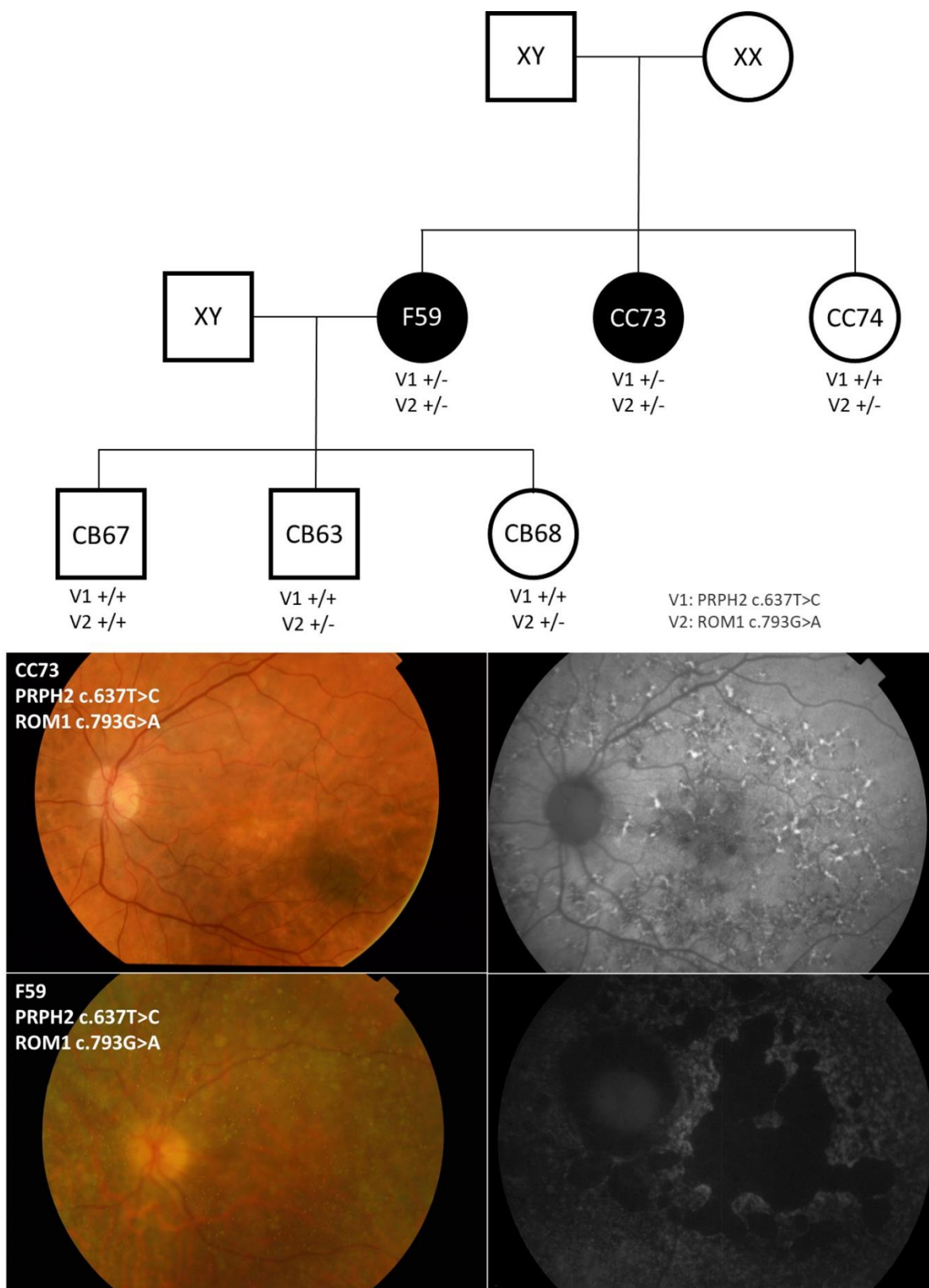


Figure 5.5. Schematic pedigree of a family affected with an RP-like phenotype. Two affected siblings are heterozygous for *PRPH2* c.637T>C and *ROM1* c.793G>A. Family members that only harbour the *ROM1* variant appear non-affected so far. □ = male, ○ = female, ● = affected. B. Fundus images of two affected individuals, showing moderate peripheral and chorioretinal atrophy.

the age of onset in both individuals (F59 and CC73) was in their 7th decade of life, so a small possibility is still present that the younger generation may develop some symptoms later in life.

Peeters *et al.* performed a systematic review of all variants in PRPH2 published up to July 2020; (2021). Interestingly, the pathogenic variant they found to be most common in PRPH2, c.424C>T (p.Arg142Trp) was not found in our cohort at all. However, the authors noted that this mutation was only seen in individuals from a Caucasian background and indicated there might be a Dutch or German founder effect, which would explain why this variant was not seen in the Irish IRD cohort. Instead, we found the c.634A>G (p.Ser212Gly) variant to be the most common, accounting for 12 out of 32 (37.5%) of alleles. Only 14 instances of this variant have been reported in LOVD and both gnomAD and ExAC estimate the global allele frequency to be 0.000008. Figure 5.12 shows fundus images of two siblings with a retinitis pigmentosa phenotypes caused by PRPH2 c.634A>G. Functional assessment through ERGs revealed that both individuals have rod and cone dystrophy, even though visual acuity is still good (CA29: 6/12 and 6/12; CI23: 6/6 and 6/6), possibly indicating foveal sparing. A more extensive study on the prevalence of this variant in Ireland and worldwide and the phenotype it causes might give valuable information on PRPH2 function.

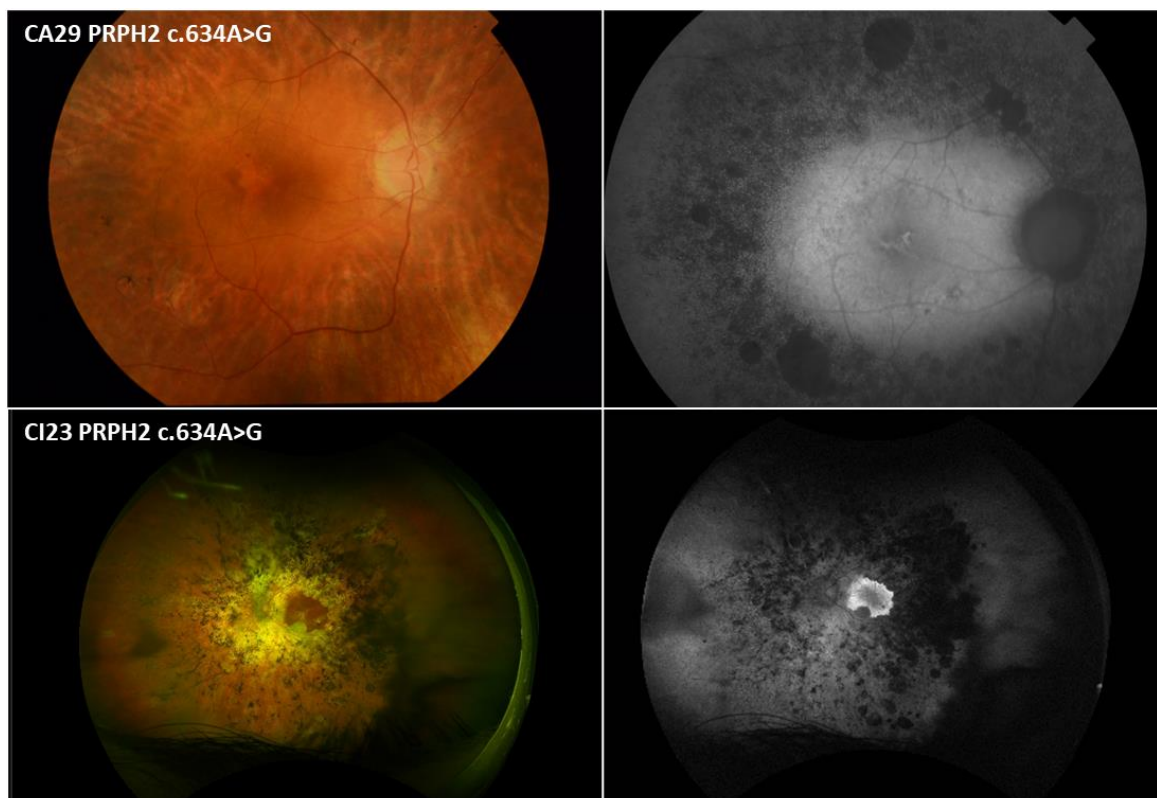


Figure 5.6. Coloured and Autofluorescence fundus images of two siblings carrying the dominant PRPH2 c.634A>G variant.

Table 5.9. A summary of probands solved with pathogenic variants in *PRPH2*. CRD = cone-rod dystrophy, RP = Retinitis Pigmentosa, BEM = Bull's Eye Maculopathy, MD = Macular Degeneration, STGD1 = Stargardt disease, WES = Whole Exome Sequencing, smMIPs = small molecule Molecular Inversion Probes, Sanger f.v. = Sanger sequencing of variant previously found in affected family member(s).

Barcode	Pedigree	Phenotype	<i>PRPH2</i> Variant (DNA)	<i>PRPH2</i> Variant (protein)	Sequencing method	Putative modifier gene	Putative modifier variant 1	Putative modifier variant 2
BF604		STGD1	c.1A>T	p.(Met1Leu)	ABCA4-smMIPs			
BF522		STGD1	c.37C>T	p.(Arg13Trp)	ABCA4-smMIPs	ABCA4	c.1715G>A; c.5312+3A>T	c.[2588G>C;5603A>T]
BF603		STGD1	c.394del	p.(Gln132Lysfs*7)	ABCA4-smMIPs	ABCA4	c.1792G>A	
BF605		STGD1	c.440dup	p.(Gly148Trpfs*29)	ABCA4-smMIPs	ABCA4	c.6148G>C	
MH1348		STGD1	c.441delT	p.(Gly148fs)	Target Capture			
EECF63		STGD1	c.457A>G	p.(Lys153Glu)	ABCA4-smMIPs	ABCA4	c.5603A>T	
MH1345		MD	c.457A>G	p.(Lys153Glu)	Target Capture	ABCA4	c.1927G>A	
EEJ26	N2	RP	c.464C>T	p.(Thr155Ile)	Sanger f.v.			
EEN2	N2	RP	c.464C>T	p.(Thr155Ile)	Target Capture			
EEP56		BEM	c.623G>A	p.(Gly208Asp)	Target Capture			
BF030		RP	c.633C>G	p.(Phe211Leu)	WES			
BF441		RP	c.633C>G	p.(Phe211Leu)	WES			
EEA5	A5	RP	c.634A>G	p.(Ser212Gly)	Farrar <i>et al.</i> 1992			
EECA29	A5	RP	c.634A>G	p.(Ser212Gly)	Sanger f.v.			
EECI23	A5	RP	c.634A>G	p.(Ser212Gly)	Sanger f.v.			
EEF22	A5	RP	c.634A>G	p.(Ser212Gly)	Sanger f.v.			
EEC1	C1	RP	c.634A>G	p.(Ser212Gly)	Target Capture	ABCA4	c.5603A>T	
EEH6	C1	RP	c.634A>G	p.(Ser212Gly)	Target Capture	ABCA4	c.5603A>T	
EEI17	C1	RP	c.634A>G	p.(Ser212Gly)	Target Capture	ABCA4	c.5603A>T	
EEU12	C1	RP	c.634A>G	p.(Ser212Gly)	Target Capture	ABCA4	c.5603A>T	
EEU3	C1	RP	c.634A>G	p.(Ser212Gly)	Target Capture	ABCA4	c.5603A>T	
EECE60	O6	BEM	c.634A>G	p.(Ser212Gly)	Sanger f.v.	ABCA4	c.466A>G	

EECF42	O6	RP	c.634A>G	p.(Ser212Gly)	Sanger f.v.	<i>ABCA4</i>	c.1792G>A	
EEO6	O6	RP	c.634A>G	p.(Ser212Gly)	Target Capture	<i>ABCA4</i>	c.466A>G	c.1792G>A
EECC73	F59	RP	c.637T>C	p.(Cys213Arg)	Sanger f.v.	<i>ROM1</i>	c.793G>A	
EEF59	F59	RP	c.637T>C	p.(Cys213Arg)	Target Capture	<i>ROM1</i>	c.793G>A	
MH1351		CRD	c.638G>A	p.(Cys213Tyr)	Target Capture			

5.3.3.2. Photoreceptor degeneration caused by PROM1

Though the exact function of PROM1 is not fully explained, it is understood that it plays a major role in regulation of photoreceptor disk morphogenesis (Yang *et al.* 2008; Permanyer *et al.* 2010). The phenotype can vary greatly; RetNet links PROM1 dysfunction to recessive RP, dominant Stargardt-like Dystrophy, BEM, dominant CRD, and dominant MD. In the Target 5000 Stargardt cohort, 4 individuals were genetically diagnosed with defects in PROM1 instead of *ABCA4*. This led to the further exploration of PROM1 in the full Target 5000 cohort; 15 individuals in 10 pedigrees are likely solved with variant in PROM1. Table 5.8 gives an overview of these individuals and their variants.

IRDs caused by defects in PROM1 can be inherited in either a dominant or a recessive fashion. In recent years, the difference between recessive and dominant PROM1-related retinal disease has been investigated extensively. It has been established that truncating mutations, such as frameshift variant c.1354dupT, were likely recessive (Liu *et al.* 2016; Cehajic-Kapetanovic *et al.* 2019). Additionally, it has been suggested that a dominant inheritance pattern, however, was associated with a milder phenotype; photoreceptor dysfunction and degeneration was mostly restricted to the macula, whereas patients with two recessive mutations presented with more severe dystrophy throughout the whole retina (Cehajic-Kapetanovic *et al.* 2019). While the phenotype of patients caused by dominant PROM1 mutations sometimes resembles *ABCA4*-associated retinopathy (Wolock *et al.*, 2019), the recessive form resembles the more severe RP phenotype (Maw *et al.*, 2000; Zhang *et al.*, 2007). A relatively common variant in PROM1, c.1117C>T, that caused the protein change p.Arg373Cys has been established to have a dominant effect on protein function (Michaelides *et al.* 2010). Indeed, 5 individuals in the Target 5000 cohort harbour this variant and it appears to have followed a dominant inheritance pattern in all.

However, it appears that one cannot simply generalise that missense variants cause dominant disease and frameshift or nonsense variants cause recessive disease; Zaneveld *et al.* 2015 discovered a recessive pathogenic PROM1 allele in a Chinese proband. The entire Target 5000 cohort counts 11 alleles of the c.1354dupT variant out of 2444 alleles screened at that position. All of these alleles were found in affected individuals. One of these individuals was homozygous and only two individuals harboured another variant in PROM1. The allele frequency in our cohort was 0.0045, which is significantly higher than that of the general population (0.00023 in non-Finnish Europeans according to gnomAD), though this is to be expected in a disease panel. In one pedigree of 4 affected individuals (Figure 5.13), this frameshift variant segregates with the disease, while a missense variant of unknown significance does not exacerbate the phenotype. Furthermore, in one pedigree implicated by the c.1354dupT frameshift variant the affected proband (O18) also harboured an intronic, pathogenic variant in *ABCA4* (c.4253+43G>A), while their brother, who

remains functionally unaffected but exhibits some macular changes, is only heterozygous for c.1354dupT. Similarly, Lee et al have described a phenomenon in which the effect of a dominant PROM1 variant was exacerbated by additional variants in *ABCA4* (Lee *et al.* 2019). LOVD counts 56 reported alleles, of which at least 8 appear to be the only potential disease causing variant. Available clinical data and fundus images of affected individuals harbouring the PROM1 c.1354dup variant are presented in Table 5.10 and

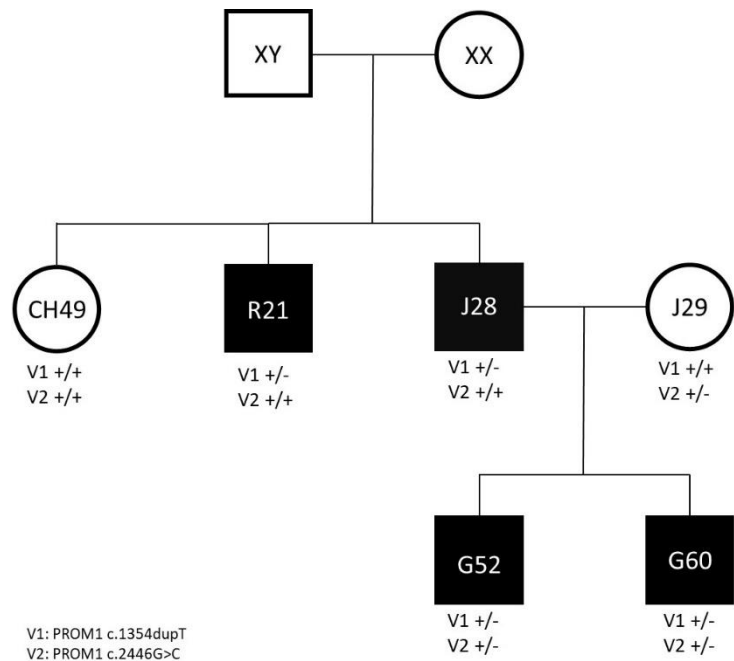


Figure 5.7 Schematic overview of a pedigree with an RP phenotype and two PROM1 mutations. □ = male, ○ = female, ● = affected

Figure 5.14. An overview of all individuals with phenotypes due to pathogenic PROM1 variants is presented in Table 5.11

Table 5.10. Clinical information indicating severity of phenotype in probands with PROM1 c.1354dup variant. YOB = Year of Birth, BCVA = Best Corrected Visual Acuity, RE = right eye, LE = left eye, ERG = Electroretinogram, (H) = homozygous, PL = perception of light, // = not available. Extent of peripheral retinal atrophy and chorioretinal atrophy was categorised into mild (atrophy confined to retinal arches), moderate (atrophy extends to mid-periphery), or extensive (atrophy extends to far periphery). ERG results were also categorised into groups; group 1 indicates reduced macular function, group 2 indicates cone dystrophy, and group 3 indicates cone-rod dystrophy

ID	Genotype	YOB	Age at Onset	BCVA RE	BCVA LE	Extent peripheral retinal atrophy	Extent chorioretinal atrophy	ERG	Age at ERG
G52	c.1354dup c.2446G>C	1975	30	6/9	6/6	Mild	Mild	//	//
G60	c.1354dup c.2446G>C	1977	39	6/60	6/24	Mild	Mild	Group 3	39
J28	c.1354dup	1948	65	6/6	6/6	//	//	Group 3	65
R21	c.1354dup	1953	64	//	//	Extensive	Extensive	Group 3	64
O18	c.1354dup	1975	//	6/24	6/24	Mild	Mild	Group 1	37
S36	c.1354dup (H)	1954	5	PL	PL	Extensive	Extensive	No response	62

Figure 5.8. Colour fundus photo's and fundus autofluorescence photos of all individuals with a clinical phenotype in the Target 5000 cohort harbouring the PROM1 frameshift variant c.1354dupT (p.Tyr452fs). G52, G60, J28 and R21 are part of the same pedigree, G52 and G60 also carry the c.2446G>C (p.Ala816Pro) missense variant. O18 harbours a single splice-altering variant with a moderate effect on mRNA splicing in ABCA4 (c.4253+43G>A) in addition to the PROM1 frameshift variant. S36 is the only individual we have identified who carries this variant homozygously.

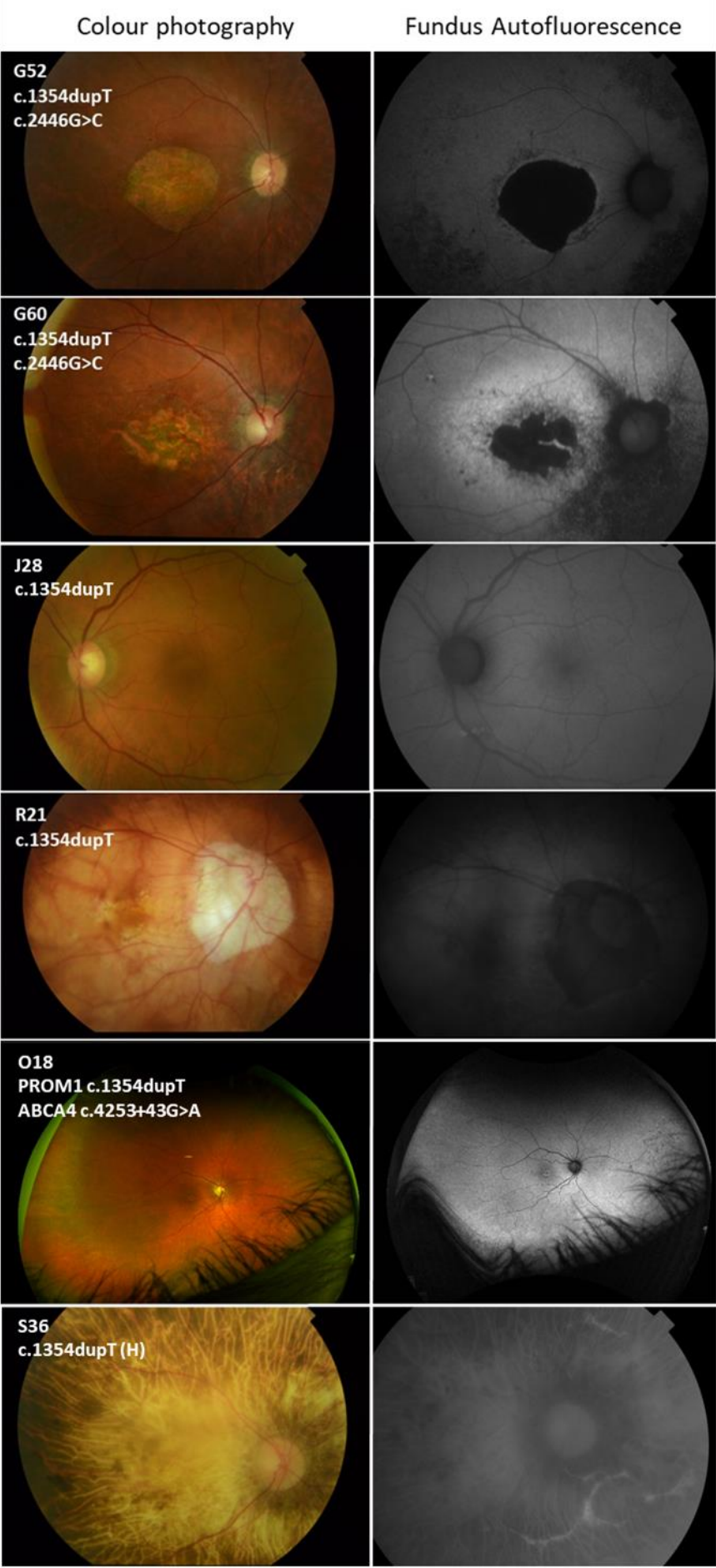


Table 3.11. Overview of individuals affected by PROM1 variants. CRD = cone-rod dystrophy, EOSRD = early-onset severe retinal degeneration, GA = gyrate atrophy , RP = retinitis pigmentosa STGD = Stargardt disease, H = homozygous, // = no significant variants found

BARCODE	PEDIGREE	CLINICAL PHENOTYPE	PROM1 VARIANT (DNA)	VARIANT (PROTEIN)	PREDICTED INHERITANCE	GENE VARIANT 2	VARIANT 2 (DNA)	VARIANT 2 (PROTEIN)
BF118	BF1	CRD	c.1117C>T	p.Arg373Cys	dominant	//		
BF136	BF1	CRD	c.1117C>T	p.Arg373Cys	dominant	//		
BF151	BF1	CRD	c.1117C>T	p.Arg373Cys	dominant	//		
EED3	D3	EOSRD	c.1632G>T (H); c.1946C>T (H)	p.Gly544Valfs*34; p.Ser649Leu	recessive, homozygous	//		
EEG52	G52	RP	c.1354dupT	p.Tyr452fs	recessive	PROM1	c.2446G>C	p.Ala816Pro
EEG60	G52	RP	c.1354dupT	p.Tyr452fs	recessive	PROM1	c.2446G>C	p.Ala816Pro
EEJ28	G52	RP/ glaucoma	c.1354dupT	p.Tyr452fs	recessive	//		
EER21	G52	RP/ glaucoma	c.1354dupT	p.Tyr452fs	recessive	//		
EEH74	H74	GA/RP	c.1414C>T (H)	p.Arg472*	recessive, homozygous	//		
EEO18	O18	STGD	c.1354dupT	p.Tyr452fs	recessive	ABCA4	c.4253+43G>A	p.[=,Ile1377Hisfs*3]
EES36	S36	EOSRD	c.1354dupT (H)	p.Tyr452fs	recessive, homozygous	//		
EEW8	W8	STGD-like	c.1117C>T	p.Arg373Cys	dominant	ABCA4	c.6079C>T	p.Leu2027Phe
MH1487		STGD	c.1354dupT	p.Tyr452fs	recessive	//		
MH1344		CRD	c.1142-1G>A (H)	p.? (splice defect)	recessive, homozygous	//		
EEG11		STGD	c.1117C>T	p.Arg373Cys	dominant	//		

5.3.3.3. Autosomal dominant Stargardt disease caused by *ELOVL4*

Stargardt disease type 3 is an early onset macular degeneration caused by autosomal dominant loss-of-function mutations in the *ELOVL4* gene (ELONGation of Very Long chain fatty acids 4) (Zhang *et al.* 2001). While the exact function of *ELOVL4* is not fully clarified, it is understood that this protein plays a vital role in biosynthesis of specific fatty acids in retina, skin, and brain tissue (Vasireddy *et al.* 2007; Agbaga, 2016). The effect of *ELOVL4* dysfunction appears to be extremely tissue-specific; a Stargardt-like phenotype can develop due to disrupted biosynthesis of polyunsaturated fatty acid chains (VLC-PUFA), while *ELOVL4*-related skin or brain abnormalities are linked to failure to correctly synthesise saturated fatty acid chains (VLC-SFA) (reviewed by Yeboah *et al.* 2021). Interestingly, the location of mutations in this gene seem to have a direct effect on the protein function and, subsequently, the clinical phenotype. Mutations causing retinal dysfunction are found in either the promoter-region or the final exon and show a dominant inheritance pattern (Zhang *et al.* 2001; Edwards *et al.* 2001; Bernstein *et al.* 2001; Maugeri *et al.* 2004; Donato *et al.* 2018), while missense or truncating variants in the central exons are likely to cause pathologies in brain or skin (Cadieux-Dion *et al.* 2014; Ozaki *et al.* 2015).

Clinical features of STGD3 have a lot of overlap with STGD1, with both disorders leading to progressive loss of central vision that can present as early as the first or second decade of life. Fundus images of individuals affected by STGD3 show progressive atrophy that can include ‘flecks’ and lipofuscin-based autofluorescence (Maugeri *et al.* 2004; Agbaga *et al.* 2014). In the Target 5000 Stargardt Cohort, only one pedigree was genetically diagnosed with Stargardt disease type 3. Patient CC34 was solved through MD-smMIPs and CC35 through TCS sequencing. Cascade analysis confirmed the presence of a frameshift variant in *ELOVL4* (c.789_793delinsAAC; p.Asn264fs) in both CC34 and CC35 and subsequently also identified this variant in two other affected family members (CC36 and CC37, respectively). This variant is extremely rare; allele frequencies through databases as gnomAD or clinVAR are not available, and the only report in LOVD is from the Target 5000 study (Dockery *et al.* 2017). However, other deletions within 20 basepairs of this variant, such as c.790del, c.794del, or c.797_801del, make up 42/130 entries in LOVD. Figure 5.15a depicts a schematic pedigree of this family with Sanger sequencing traces pointing out the variant. Figure 15.5b presents fundus images of these family members, showing degeneration of the retina that is mostly confined to the retinal arches in all individuals. Additional information, such as visual acuity and age at presentation is depicted in Table 5.12.

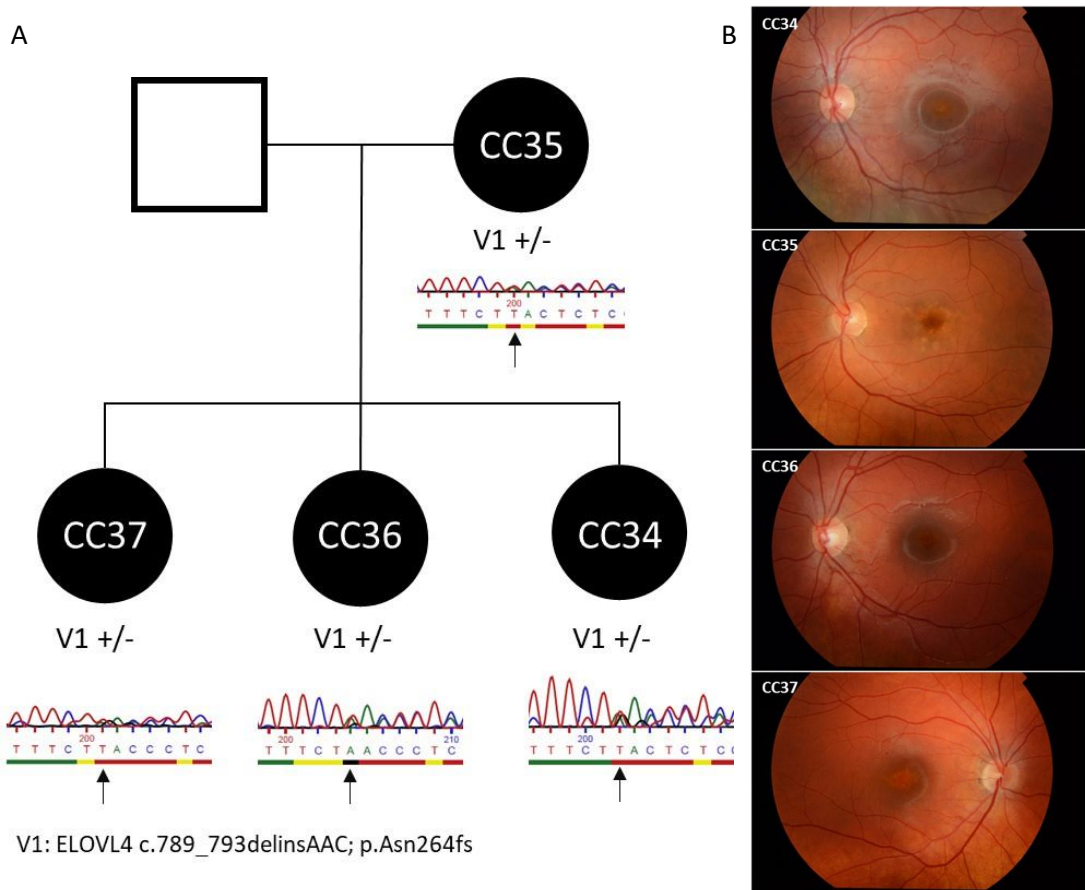


Figure 5.15. Overview of a family harbouring a dominant negative variant in *ELOVL4*. A. Schematic pedigree tree of family and segregation of *ELOVL4* c.789_793delinsAAC. B. Fundus images of affected members in this pedigree.

Table 5.12. Overview of clinical features in affected members in CC34 pedigree that carry the *ELOVL4* c.789_793delinsAAC frameshift variant. YOB = Year of Birth, Dx = Diagnosis, BCVA = Best Corrected Visual Acuity according to Snellen test, RE = right eye, LE = left eye, N/A=not available.

Patient Id	YOB	Age Of Onset	Age Last Visit	BCVA RE	BCVA LE	Extent Peripheral Retinal Atrophy	Extent Chorioretinal Atrophy
CC34	2010	8	8	N/A	N/A	Mild (confined to retinal arcades)	Mild (confined to retinal arcades)
CC35	1977	31 (Dx)	39	6/19	6/6	Mild (confined to retinal arcades)	Mild (confined to retinal arcades)
CC36	2009	7	7	6/6	6/6	Mild (confined to retinal arcades)	Mild (confined to retinal arcades)
CC37	2005	11	13	6/19	6/19	Mild (confined to retinal arcades)	Mild (confined to retinal arcades)

5.4. Discussion

In this thesis chapter an extensive overview of the genetic architecture of Stargardt disease in Ireland was provided, utilising clinical and genomic data collected and investigated as part of the Target 5000 project. A total of 69.7% of Irish individuals clinically diagnosed with Stargardt disease were solved through Target 5000. Biallelic mutations in *ABCA4* were attributed to 212 of the genetically diagnosed individuals (93%), while 16 people were affected by pathogenic mutations in other genes (7%). About 20-30% of affected individuals in the full Target 5000 cohort remain unsolved and the same is true for the Stargardt sub-cohort; in 34 people only one allele was found and in 37 people no candidate variants were identified at all, making a total of 21.7% of Stargardt patients we have not been able to solve, not including those who have not been sequenced yet. Whole genome sequencing may aid in the elucidation of these unsolved cases, for example through the discovery of new IRD-related genes, the interrogating of non-coding regions in known disease genes, or further clarification of the role modifier loci can play in penetrance of genetic defects.

This chapter also presented data on genes that are linked to pseudo-Stargardt phenotypes; *PRPH2*, *PROM1*, and *ELOVL4*. Mutations in *PRPH2* were the cause of phenotype in 32 individuals in the Stargardt cohort, 6 of whom had a Stargardt phenotype. In two individuals, biallelic mutations in *ABCA4* were also found in addition to the *PRPH2* variant. Clinical data was not available at the moment this thesis was written, but it would be valuable to investigate the difference between these phenotypes and those caused solely by *ABCA4* variants or a single *PRPH2* variant. Furthermore, it is remarkable that the majority of those with a Stargardt phenotype caused by *PRPH2* dysfunction also harboured a pathogenic *ABCA4* variant.

Sixteen clinically affected individuals were genetically diagnosed to harbour variants in *PROM1*. However, while some literature suggests only missense variants are capable of causing dominant disease (Cehajic-Kapetanovic et al. 2019), we found a single frameshift variant in 4 individuals who did not carry additional potentially causative variants in either *PROM1* or any other gene, and in 2 individuals who carried a *PROM1* variant of unknown significance *in trans*. These data indicate that the penetrance of *PROM1* variants may not be straightforward. Further assessment of *PROM1*, both the gene and protein, is required to aid in the interpretation of variants in this gene and how it affects phenotype. A possible explanation for the variable effect by *PROM1* is its tissue-specific expression; so far five different tissue-specific promoters have already been identified in this gene (Sompallae et al. 2013)

Other than for diagnostic reasons, even when patients are affected by monogenic diseases, it is important to understand the exact mutations and their effect on protein function. For example,

PROM1, which causes STGD4, amongst others, can be inherited in a recessive or dominant negative fashion. These dominant negative proteins would require a different therapeutic approach, as simply supplementing it with a functional gene won't be effective. The dominant negative protein can bind to a functional protein, that the patient has one copy of, and render it dysfunctional. These mutations therefore require a different approach, such as suppression and replacement, or base editing of the faulty allele.

Cornelis et al (2022) have categorised 1619 variants in *ABCA4* and utilised allele frequencies, statistics, and clinical data to calculate the chance an individual has at their offspring developing Stargardt disease, based on the severity of the alleles they carry. Interestingly, they also showed that 10% of the general population is predicted to carry the c.5603A>T (p.Asn1868Ile) allele, an additional estimated 5% carries a potentially pathogenic *ABCA4* allele. While *in silico* analysis is a great tool that aids in the interpretation of variant severity, functional analysis of proteins gives more detailed information. However, because a high-resolution structure of the *ABCA4* protein remains a work in progress, most functional assays of *ABCA4* are based on secondary outcomes of the protein function, such as ATPase activity or protein yield. Minigene and midigene assays are routinely utilised to predict the impact of specific variants on pre-mRNA splicing (Sangermano *et al.* 2018; 2019; Khan *et al.* 2020a; 2020b; Fadaie *et al.* 2019; 2021), but these assays do not always lead to correct results. As splice assays are generally carried out in HEK293 cells, some suggest that tissue-specific enhancers of the spliceosome may play a role. Indeed, when the *ABCA4* c.4539+2028C>T variant was first assessed in patient-derived fibroblasts only wild-type mRNA was produced, but in patient-derived photoreceptor precursor cells a pseudoexon is included (Albert *et al.* 2018). Additionally, evidence shows that the incorporation of larger genomic regions flanking a putative splice variant, is more likely to reflect *in vivo* splicing (Sangermano *et al.* 2018). Other methods include the isolation of complete RNA from patient blood to assess splice defects, but for tissue-specific genes such as *ABCA4*, which is only expressed in photoreceptors and RPE cells (Lenis *et al.* 2018), this would not be a viable option.

Interestingly, the common, hypomorphic variant *ABCA4* c.5603A>T was present at a much higher frequency in the 'one allele only' group (23.1% of alleles) than in either the solved cases (3.06% of alleles) or the healthy population. The gnomAD allele frequency for non-Finnish Europeans is 0.066, corresponding to 6.6% of alleles in the healthy population, including 140 homozygotes. Additionally, the complex allele c.[2588G>C;5603A>T], is also much more prevalent in the monoallelic subgroup (15.4% of alleles) than it is in the biallelic group (4.59%). The c.5603A>T variant was previously considered to be benign due to its high minor allele frequency in the global population with estimates ranging anywhere from 4-10%, but recent advances in sequencing the whole *ABCA4* gene raised the suggestion that when it is found in trans with moderate or severe

pathogenic variants it does, indeed, cause late onset Stargardt disease (Zernant *et al.* 2017; Runhart *et al.* 2018; Curtis *et al.* 2020; Cornelis *et al.* 2020).

Another known hypomorphic variant in *ABCA4* is c.4253+43G>A, which was found to be statistically more prevalent in STGD1 patients with only one other identified *ABCA4* variant when compared to patients with two pathogenic variants or the general population (Zernant *et al.* 2018). This variant has since been described to affect splicing and is therefore now considered to be a mild, but causative allele (Sangermano *et al.* 2019; Khan *et al.* 2019, 2020; Bauwens *et al.* 2019). Indeed, we identified this variant in 5 probands with only one other pathogenic *ABCA4* allele, in 1 proband that also carried the c.5603A>T variant homozygously, and in 2 unsolved probands. Interestingly, Zernant *et al.* (2022) found some evidence that genes implicated in pseudo-Stargardt phenotypes can also play a modifying role in *ABCA4*-determined Stargardt disease: rare variants ($AF < 0.005$) in *ROM1* were found to be enriched by a factor 3.8 in their Stargardt cohort compared to healthy controls. Additionally, a common *PRPH2* variant (c.1013A>G; p.Asp338Gly) was 20% more frequent in the general Stargardt cohort compared to controls and it was 80% more common in the late-onset Stargardt subgroup, though it appears to solely affect penetrance of disease as no effect on phenotype severity was observed. Indeed, the global allele frequency in gnomAD is 0.23, while in our Target 5000 cohort this allele was found 1925 times out of 4832 total alleles that were sequenced, corresponding to an allele frequency of 0.39, indicating it is present much more frequently in affected individuals. Another study demonstrated that monoallelic pathogenic variants in *ABCA4* can modify the phenotype in patients with PROM1 disease (Lee *et al.* 2019). Likewise, Poloschek *et al.* (2010) reported on a pedigree spanning 5 generations, where various individuals were affected by mutations in either *PRPH2* (c.514C>T), *ROM1* (c.686G>A), *ABCA4* (c.6148G>C), and combinations of these. They noted that, while *PRPH2*-*ROM1* digenic variation caused a stronger phenotype, two individuals only carrying the *ROM1* variant also presented a clinical phenotype fitting with macular degeneration. One mutation causing a variety of phenotypes has also been observed in other genes. For example some mutations in *BBS1*, such as M390R, can cause either Bardet-Biedl Syndrome, but also non-syndromic autosomal recessive RP (Estrada-Cuzcano *et al.* 2012).

Hanany and colleagues created a database containing 276,921 sequence variants that were identified in 187 autosomal recessive IRD genes and estimated that 2.7 billion people worldwide (36% of the population) are healthy carriers of at least one autosomal recessive IRD mutation (Hanany *et al.*, 2020). Personally, I think it would be extremely interesting to investigate how many carriers of monoallelic pathogenic mutations in recessive IRD genes end up developing some form of AMD. With whole genomes sequencing becoming more and more affordable every year, it could be a great opportunity to further investigate the overlap between IRDs and AMD.

With the elucidation of genetic defects in IRD patients, and indeed in AMD patients in the future, gene therapies are on the horizon too. Other than supplementation of genes, common mutations can potentially be repaired *in vivo* with CRISPR/Cas9-based techniques. Indeed, various groups have already successfully corrected *ABCA4* mutations *in vitro* (Siles *et al.* 2023; De Angeli *et al.* 2022), but the challenge lies in translating this research to the clinic. In the Irish Stargardt cohort, almost 25% of alleles are splice-altering variants (Table 5.1). Antisense oligonucleotides (AONs) are another promising strategy for treating genetic disorders, especially when aberrant splicing is involved. These mutation-specific nucleotides can ‘cover’ sequences that misguide the spliceosome and modulate splicing. AONs have been designed and shown to rescue splicing for many variants, both in *ABCA4* and other genes. Indeed, an AON-based therapy managed to rescue the splice defect caused by common mutations in *ABCA4* in midgene models as well as in human retinal organoids (Garanto *et al.* 2019; Kaltak *et al.* 2023a; 2023c). However, while AONs for Stargardt disease are still largely investigated *in vitro*, AON-based strategies for other genetic disorders such as USH2A-related Retinitis Pigmentosa, Huntington’s disease, or tumorous malignancies are already in clinical trials (ProQR, 2021; Wave Life Sciences, 2022; Biospace, October 2022; Biospace, December 2022). Indeed, the EMA- and FDA-approved therapy Spinraza is a treatment utilising AON-based splice rescue of a homolog in patients carrying a defective *SMA1* gene in spinal muscular dystrophy (Wurster and Ludolph. 2018). Khan *et al.* (2020a) showed that, when a splice site is disrupted through NCSS mutations, strengthening the other splice site of the same exon, can rescue the splice defect; this knowledge can provide additional options for AON-design.

In this thesis chapter, an extensive overview of the underlying genetic causes of Stargardt disease and Stargardt-like phenotypes was created. Overviews like these are important because they aid in the further understanding of inheritance patterns and genotype-phenotype correlations. The identification of causative genes and the understanding of pathogenic variants in these genes and their role in protein dysfunction is fundamental for the design of therapies for these disorders.

6. General Discussion

A total of 5.5 million people worldwide are estimated to be afflicted by some type of inherited retinal degeneration (Hanany *et al.* 2020). While IRDs are an extremely heterogeneous group and present a wide range of clinical phenotypes, they have in common that the retina progressively and irreversibly deteriorates. Caused by biallelic mutations in *ABCA4*, Stargardt disease type 1 (STGD1) is considered to be the most common monogenic retinal degeneration with an estimated prevalence of 1 in 10000 (Blacharski 1988). Loss-of-function mutations in *ABCA4* lead to accumulation of bisretinoid A2E, which is a major component of the toxic pigment complex lipofuscin. Aggregation of lipofuscin and A2E reduces autophagy, destabilises cellular membranes, disrupts mitochondrial function, and leads to increased levels of oxidative stress (Saadat *et al.* 2014; Sivapathasuntharam *et al.* 2019; Sparrow *et al.* 2012). These damages also lead to a reduced ATP production in retinal cells, which is extremely worrying as the retina is one of the highest energy-consuming tissues (Sivapathasuntharam *et al.* 2019).

At the moment of writing, no therapies for Stargardt disease have been approved, though the NIH clinical trial database reports 30 interventional studies (<https://clinicaltrials.gov>; assessed at 31/07/2023). Only two of these studies are classic gene therapies, and interestingly, neither of them utilise the *ABCA4* gene, highlighting again the difficulties in designing a therapy for Stargardt disease. Ocugen's OCU410ST gene therapy utilises AAV5 to deliver the humanised RORA gene, encoding for RAR Related Orphan Receptor A (Ocugen, 2023). This protein is involved in the regulation of many cellular processes, including inflammation, apoptosis, the circadian rhythm, autophagy, mitochondrial function, and oxidative stress levels (Jetten *et al.* 2001; Jetten 2009). Indeed, the neuroprotective properties of this protein have been shown to protect cell models of various neuronal degenerations against oxidative stress (Bouktouche *et al.* 2006; Al-Zaid *et al.* 2023). Though the clinical trial phase I/II study is only in the recruitment stage and is estimated to finish in the autumn of 2025, this is a very interesting project to follow, since it links in with our own approach to modulate mitochondrial function, as described in this thesis.

The other gene therapy in clinical trials is Nanoscope's MCO-010 (AAV2-MCO), which is being tested not only as a therapy for advanced Stargardt disease but also for RP. This approach delivers the Multi-Characteristic Opsin protein to bipolar cells via AAV2 in order to reprogram them and allow them to become light-sensitive (Nanoscope Therapeutics, 2022). Bipolar cells are more likely to survive in advanced disease, even when photoreceptor cells, that normally capture light, have died. The main objective of this therapy is improved light perception. Indeed, preliminary results of the phase II clinical trial utilising this therapy in RP patients have shown

improved vision-guided mobility and near-object recognition in the majority of test subjects (Nanoscope Therapeutics, 2023). This therapy, if approved, may be exceptionally beneficial for people with advanced stages of retinal disease, as most conventional therapies focus on prevention of further cell death and retinal cells are post-mitotic, so they are unable to regenerate once deceased.

Other than gene therapies, a popular mechanism of action in potential therapies for STGD1 appears to be regulation of vitamin A or vitamin A-derivates in the retina. Vitamin A is transported from the liver to peripheral tissues, like the retina, by retinol-binding protein 4 (RBP4) (Kanai *et al.* 1968; Noy *et al.* 1990). Two RBP4 antagonists are currently in phase I/II clinical trials for both Stargardt disease and AMD; Tinlarebant and STG-001, respectively (reviewed in Kim *et al.* 2021b). Additionally, reduction of vitamin A processing or dimerization to mitigate A2E accumulation are mechanisms currently tested in clinical trials, such as Emixustat; however, the rate of change in atrophy in patients who received the drug was the same as in the placebo-group (Kubota *et al.* 2022).

Interestingly, a clinical trial of SAR422459, a gene therapy vector delivering *ABCA4* via equine retrovirus, has been terminated after phase I/II trials as 6/22 participants presented with increased RPE atrophy, whereas only 1 participant exhibited a reduced number of macular flecks (Parker *et al.* 2022). However, this clinical trial gives extremely beneficial information, as participants were observed up to 15 years. Adverse effects were most commonly due to surgical procedure, but severe immunological effects were not observed (Parker *et al.* 2022).

Considering the physiology of the retina, most classic gene therapy designs will never be able to fully cure inherited retinal degenerations. The mechanism of action is usually focused on prevention of further deterioration by counteracting retinal cell death, as post-mitotic cells can't regenerate once they're dead. Alternative treatments are now focusing on intraocular, intravitreal, or subretinal transplantation of RPE cells generated from reprogrammed embryonal stem cells (ESC) or autologous bone marrow-derived stem cells. Indeed, the NIH clinical trial database lists various phase I/II trials investigating the safety and potential of this approach in Stargardt disease and AMD (NCT01920867 (SCOTS); NCT03011541 (SCOTS2); NCT01345006 (MA09-RPE); NCT01625559 (MA09-RPE); NCT03772938 (IKiKO-KB); NCT02903576; and NCT02749734). It will be interesting to find out whether these strategies, if safe and effective, also provide benefit in the long run, as genetic defects in the patient's own cells are not resolved.

Due to the limited AAV-cargo capacity and size of *ABCA4* various groups have been working on the optimisation of dual vector strategies, where two vectors are used to generate one functional therapeutic element. Mechanisms to combine the transgenes in both vectors include

overlapping sequences, trans-splicing approaches, and a more efficient hybrid strategy combining both methods (Trapani *et al.* 2014; 2015; Collela *et al.* 2014; McClements *et al.* 2018; Dyka *et al.* 2019). While all strategies produced full length *ABCA4*, especially the trans-splicing approach was prone to concatemerisation and the creation other non-specific byproducts. Furthermore, studies in animal models revealed that the efficacy of dual vector strategies is low with transgene levels between 1% and 20% of wild type RNA (Dyka *et al.* 2019; McClements *et al.* 2020). All in all, while dual vector AAV systems are a promising approach, more comprehensive studies will have to be carried out to reduce unwanted products and optimise expression levels.

While many teams worldwide work on the optimisation of viral vectors to improve transduction efficacies, reduce immunogenicity, and increase cargo capacity, the arrival of non-viral vectors may provide a solution to these drawbacks. Nanoparticles were utilised to deliver an *ABCA4* transgene to mouse retinas, which achieved expression up to 8 months post injection (Han *et al.* 2012). More recently self-assembling nanoparticles have been employed to transport plasmids carrying the *ABCA4* gene to retinal cells (Sun *et al.* 2015; 2020), but long term gene expression was minimal. However, as the techniques are still relatively young, optimisation and enhancement of these particles may improve their efficiency greatly. Indeed, modification of the plasmid and a strategy involving multiple injections have shown more promising results (Sun *et al.* 2021; 2022), indicating that a nano particle-based approach for supplementation of *ABCA4* could have therapeutic potential.

Because mitochondrial welfare is not only impacted in Stargardt disease, but also in other retinal disorders such as age-related macular degeneration (AMD), glaucoma, and Leber hereditary optical neuropathy (LHON), our team investigated the therapeutic benefit of modulating mitochondrial function in models of various retinal diseases (Chadderton *et al.* 2013; 2023; Millington-Ward *et al.* 2022; 2023). A yeast homolog of the NADH Dehydrogenase protein, consisting of only 1 subunit as opposed to 45 subunits in the human complex, has been optimised and cloned into AAV-vectors. In chapter 3 and 4 of this thesis, we investigated whether this therapeutic vector could also provide benefit to cell and mouse models of Stargardt disease. Indeed, AAV.CAG.ophNDI1 was found to improve mitochondrial function and reduce oxidative stress in various cell models of disease. Additionally, in *Abca4*^{-/-} mice eyes treated with this viral vector exhibited significantly lower cellular ROS levels and reduced lipofuscin intensity than eyes that received control injections. It is important, however, to further investigate the beneficial effects of AAV.CAG.NDI1 on *in vivo* models; more doses should be explored to find the optimal benefit and functional studies should be expanded.

While gene-independent strategies might not be the most straight-forward option in genetic disorders, it may be a valuable tool for disorders with large genes or for patients whose genetic diagnosis remains elusive. All in all, there are so many therapeutic strategies being developed at the moment that the future of gene therapy is full of hope.

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Appendix I: Joined Publications and other outputs

Some of the data in chapter 3 have previously been published in the following paper:

Millington-Ward, S., Chadderton, N., Finnegan, L. K., **Post, I. J. M.**, Carrigan, M., Gardiner, T., Peixoto, E., Maloney, D., Humphries, M. M., Stitt, A., Léveillard, T., Humphries, P., Kenna, P. F., Palfi, A., & Farrar, G. J. (2022). AAV-mediated gene therapy improving mitochondrial function provides benefit in age-related macular degeneration models. *Clinical and translational medicine*, 12(8), e952. <https://doi.org/10.1002/ctm2.952>.

Figures 3.15 and 3.16 and Figures 3.18 – 3.21 were printed previously in this paper. Assays for these figures were performed in close collaboration between the first authors and me. A copy of this paper is presented on the following pages.

Other joined publications that came out over the course of this PhD degree, but which are not featured in this thesis are listed below:

- Palfi, A., Chadderton, N., Millington-Ward, S., **Post, I.**, Humphries, P., Kenna, P. F., & Farrar, G. J. (2022). AAV-PHP.eB transduces both the inner and outer retina with high efficacy in mice. *Molecular therapy. Methods & clinical development*, 25, 236–249.
- Millington-Ward, S., Chadderton, N., Finnegan, L. K., **Post, I. J. M.**, Carrigan, M., Nixon, R., Humphries, M. M., Humphries, P., Kenna, P. F., Palfi, A., & Farrar, G. J. (2023). RPE-Directed Gene Therapy Improves Mitochondrial Function in Murine Dry AMD Models. *International journal of molecular sciences*, 24(4), 3847.
- Whelan, L., Dockery, A., Stephenson, K. A. J., Zhu, J., Kopčić, E., **Post, I. J. M.**, Khan, M., Corradi, Z., Wynne, N., O' Byrne, J. J., Duignan, E., Silvestri, G., Roosing, S., Cremers, F. P. M., Keegan, D. J., Kenna, P. F., & Farrar, G. J. (2023). Detailed analysis of an enriched deep intronic ABCA4 variant in Irish Stargardt disease patients. *Scientific reports*, 13(1), 9380.

Furthermore, the following page will list posters and presentations I have giving over the course of my PhD project.

Supplemental Table 1. Overview of talks and posters presented over the course of my PhD.

Presentation Type	Title	Conference	Date
Talk	AAV vector delivery targeting common pathways of disease in STGD1	StarT ITN Midterm Review	30/01/2020
Talk	AAV vector delivery targeting common pathways of disease in STGD1	StarT ITN meeting (virtual)	10/09/2020
Poster	Optimising AAV-mediated gene delivery to Retinal Pigment Epithelial cells	15th Pro Retina meeting (virtual)	09/04/2021
Poster	Optimisation of AAV-mediated gene-delivery in Retinal Pigment Epithelium	WVER 2021 (virtual)	10/9/2021
Poster	Optimisation of gene delivery by AAV vectors in retinal pigment epithelial cell models	ISHG 2021 (virtual)	17/09/2021
Talk	AAV vector delivery targeting common pathways of disease in STGD1	StarT ITN meeting	23/09/2021
Poster	Optimising gene delivery via AAV vectors in retinal pigment epithelial (RPE) cells	Retina 2021 (virtual)	05/11/2021
Poster	AAV-mediated NDI1 supplementation in cell models of Stargardt disease and AMD	ISHG 2022	16/09/2022
Poster	Supplementation of NDI1 via AAV in in vitro models of Stargardt Disease and Age-Related Macular Degeneration	ESGCT 2022	13/10/2022
Talk	AAV vector delivery targeting common pathways of disease in STGD1	Retina 2022 (StarT day)	03/11/2022
Poster	AAV-mediated NDI1 supplementation in in vitro models of Stargardt Disease and Age-Related Macular Degeneration	Retina 2022	04/11/2022

AAV-mediated gene therapy improving mitochondrial function provides benefit in age-related macular degeneration models

Dear Editor,

With an estimated 196 million people suffering from age-related macular degeneration (AMD) in 2020 and predicted to increase to 288 million by 2040,¹ dry AMD, representing 70%–90% of AMD cases, represents an enormous clinical need with no current therapies. We have demonstrated that NDII and an optimised version of NDII (ophNDII), a mitochondrial complex I equivalent from *Saccharomyces cerevisiae*, provide functional and histological benefit in two murine models of dry AMD as well as benefit in two cellular models of dry AMD. There are no drugs on the market for dry AMD. However, there are currently a small number of candidate gene therapies in clinical trial (clinicaltrials.gov). To our knowledge, this is the first demonstration that a gene therapy directly targeting mitochondrial dysfunction provides functional benefit in in vivo models of dry AMD, making this a novel approach to treating this devastating condition.

Dry AMD is characterised by the formation of drusen between Bruch's membrane (BM) and the basal lamina of the retinal pigment epithelium (RPE) and atrophic changes in the choriocapillaris followed by the death of photoreceptors in the macula and geographic atrophy, with a related loss of central vision. AMD is multifactorial with genetic and environmental factors known to contribute to the disease.² Although underlying mechanisms involved in AMD are not fully understood, mitochondrial dysfunction leading to increased oxidative stress in the RPE, DNA damage and impaired mitophagy are known to contribute to RPE and photoreceptor cell death.³ Both the RPE and photoreceptors have been shown to display mitochondrial complex I (of the electron transport chain) deficiency.⁴

The *Cfh*^{-/-} mouse⁵ has been widely used as a dry AMD model and aged *Cfh*^{-/-} mice have been reported

to display impaired visual function, thinning of the retinal outer nuclear layer, changes in BM and basal laminar deposits (BlamDs).^{5,6} In this study, we also observed electroretinography (ERG) deficits in aged *Cfh*^{-/-} mice (Figure 1A–D, Table S1), but no changes in BM or BlamDs were apparent. However, cone photoreceptors exhibited disorganised outer segments, and substantial mitochondrial alterations compared to cones of control mice. Cone mitochondria appeared shrunken and fragmented and the cytoplasm of inner segments swollen and electron-lucent. These changes in cone histology have not previously been reported and indicate mitochondrial dysfunction (Figure 1E–J).

We have investigated the utility of the nuclear-encoded NDII⁷ gene as a candidate therapy for dry AMD. NDII provided benefit in models of Parkinson's disease, Leber hereditary optic neuropathy and multiple sclerosis.⁸ NDII has also been shown to reduce reactive oxygen species (ROS) and oxidative stress in disease models.^{7,8} We utilised a codon-optimised version of NDII, ophNDII, which we observed to express ~3-fold higher than wild-type NDII in murine retina from recombinant adeno-associated viral (AAV) vectors following subretinal delivery (Figure S1).

A range of AAV2/8 and AAV2/5 viral doses (1.0×10^7 – 7.5×10^9 vg) were used to deliver ophNDII and NDII subretinally to *Cfh*^{-/-} mice. Significant and robust functional benefit was observed in 60 aged mice using ERG readouts, as well as reduced ROS, increased nicotinamide adenine dinucleotide (NADH) oxidation and increased cone photoreceptor numbers in treated versus control eyes (Figures 2A–O, S2, S3). Notably, with none of the doses used were negative effects observed even up to 7–9 months post-injection. In acknowledgement that no model recapitulates all aspects of dry AMD, a second

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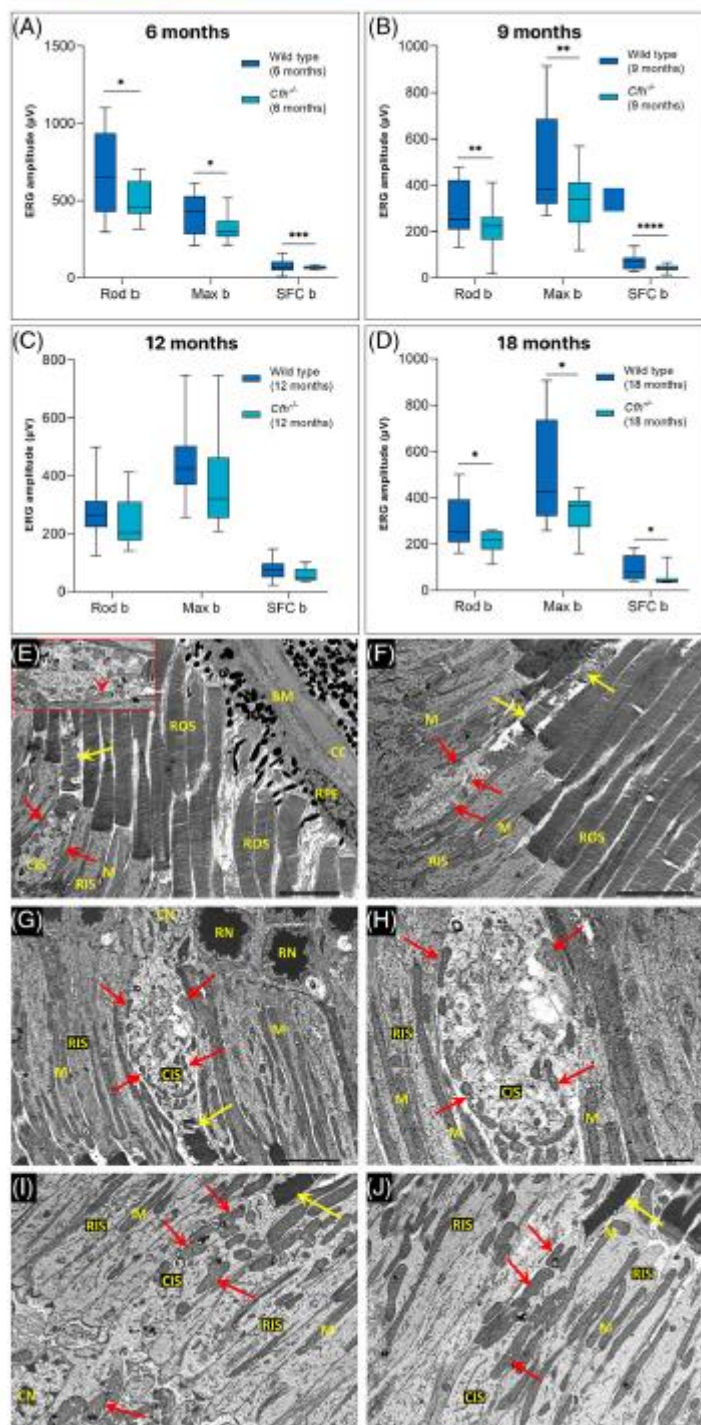


FIGURE 1 *Cfh*^{-/-} murine model. (A–D) Electretinography (ERG) analysis on C57BL/6J and *Cfh*^{-/-} at 6, 9, 12 and 18 months of age. At all ages, (A) 6 months, (B) 9 months, (C) 12 months and (D) 18 months, *Cfh*^{-/-} mice displayed significantly reduced Rod b, Max b and single flash cone (SFC) b responses compared to C57BL/6J mice. **p* < .05, ***p* < .001 and ****p* < .0001; 2-sample *t*-test. (E–J) Transmission electron microscopy (TEM) on 12 months C57BL/6J and *Cfh*^{-/-} mouse retinas. (E) The retinal pigment epithelium (RPE) in *Cfh*^{-/-} mice appeared normal with intact basal infoldings of the basolateral plasma membrane and no evidence of electron-dense deposits. Bruch's membrane (BM) was of comparable thickness to controls wild-type (WT) C57BL/6J controls (I and J) and showed no electron-dense deposits. The choriocapillaris (CC) was intact, and the endothelium was densely fenestrated at the RPE interface. The cone cells in *Cfh*^{-/-} mice (E–H) exhibited the disorganization of the outer segment (OS) membranes (yellow arrows in E–G). Also, the mitochondria (M) in the cone inner segments (CIS) of *Cfh*^{-/-} retinas were irregular in size and shape (red arrows) and generally much smaller than those in adjacent rod inner segments (RIS). Some cone mitochondria appeared to be undergoing fission (E-inset, red arrowhead). The cytoplasm of the CIS was generally more electron-lucent than that of rods (E–H) and some appeared swollen (G and H). Cone cells in WT control mice (I and J) showed normal OSs (yellow arrows) and large mitochondria, which in contrast to those in *Cfh*^{-/-} mice tended to have a greater diameter (red arrows) than the mitochondria in adjacent rods. CN, cone nucleus; RN rod nucleus

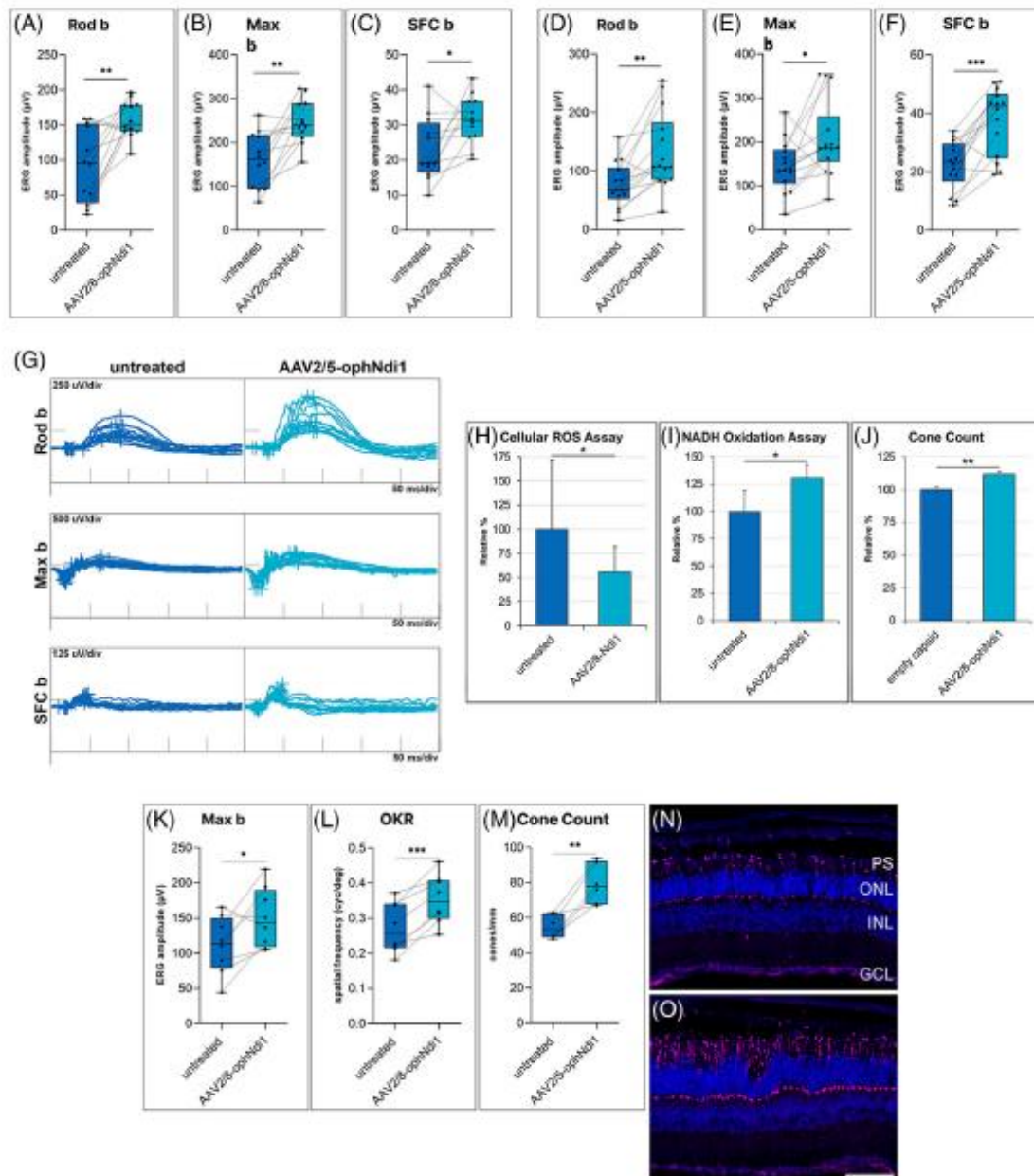


FIGURE 2 Rescue of the *Cfh*^{-/-} genetic and NaIO₃-induced murine models. (A–C) Two-month *Cfh*^{-/-} mice were subretinally injected with 1.0×10^7 -vg adeno-associated viral (AAV)2/8-ophNdi1 in one eye, whereas contralateral eyes were not uninjected ($n = 12$). Electrophysiologicals (ERGs) of treated eyes compared to untreated contralateral eyes at 9 months showed significantly greater Rod b (A, 158.1 ± 26.22 vs. $93.24 \pm 53.37 \mu V$), Max b (B, 247.7 ± 49.71 vs. $159.7 \pm 63.09 \mu V$) and single flash cone (SFC) b (C, 31.43 ± 6.968 vs. $23.03 \pm 9.006 \mu V$) responses. * $p < .05$; ** $p < .01$; paired *t*-test. (D–G) Two-month *Cfh*^{-/-} mice were subretinally injected with 5.7×10^6 -vg AAV2/5-ophNdi1 in one eye, whereas the contralateral remained uninjected ($n = 14$). ERGs (D) of treated eyes compared to untreated

murine model, the well-established sodium iodate-induced (NaIO_3) model,⁹ was also treated subretinally with AAV2/8-ophNdiI and AAV2/5-ophNdiI. NaIO_3 , a strong oxidising agent, causes catastrophic damage to the RPE leading to subsequent photoreceptor loss and reduced photoreceptor cell function, including reduced ERG amplitudes when delivered systemically.¹⁰ Similar to our findings in the *Cfh*^{-/-} mouse, subretinally delivered AAV-ophNdiI provided robust ERG benefit, as well as improved optokinetic responses and increased cone photoreceptor cell numbers in treated versus control eyes (Figure 2K–O).

To interrogate the mechanism behind the observed functional and histological benefit in the treated murine NaIO_3 model, cellular models utilising NaIO_3 were investigated; primary porcine RPE (pRPE) cells and ARPE19 cells, a well-established cell line with some characteristics of RPE. pRPE cells were transduced with AAV2/8-ophNdiI and insulted with NaIO_3 . Immunocytochemistry for 8-OHdG (oxidative stress marker), CPN60 (mitochondrial marker) and phalloidin (selective for F-actin) showed high levels of oxidative and mitochondrial stress and the absence of actin filaments in NaIO_3 -insulted versus control cells, indicating severe stress and reduced viability. In contrast, insulted cells transduced with AAV2/8-ophNdiI appeared similar to control cells (Figures 3A–O, S4). Similar rescue from NaIO_3 insult was also observed in ARPE19 cells transduced with AAV2/8-ophNdiI (Figures 4A–O, S5). These data suggest that AAV2/8-ophNdiI treatment provides significant

protection against mitochondrial stress, oxidative damage to DNA and cell death in the cellular NaIO_3 models.

Additionally, mitochondrial stress tests were performed on pRPE cells transduced with AAV2/2-ophNdiI and insulted with NaIO_3 . NaIO_3 insult significantly reduced basal oxygen consumption rates (OCRs), maximal OCRs and ATP production in cells. However, treatment with AAV2/2-ophNdiI significantly increased each of these parameters indicating a rescue of mitochondrial function (OXPHOS, Figure 3P–R). Spare respiratory capacity, the difference between maximal OCR and basal OCR, was reduced with AAV2/2-ophNdiI treatment as basal OCR was increased by more than the maximal OCR (Figure 3Q). When pRPE cells were exposed to the complex I inhibitor rotenone, OCRs were reduced to background levels in control and NaIO_3 -insulted cells. However, the addition of rotenone to AAV2/2-ophNdiI-treated cells – NDI1 is insensitive to rotenone – had minimal effect on OCR levels, which were substantially maintained (Figure 3S). Notably, similar benefits in bioenergetic profiles were also observed in NaIO_3 -insulted ARPE19 cells transduced with AAV2/2-ophNdiI (Figure 4P,Q, Table S2).

We tested NDI1 and ophNdiI, which target mitochondrial dysfunction, known to be a key factor in dry AMD. Robust benefit was demonstrated with multiple AAV-delivered NDI1/ophNdiI vectors and doses in the *Cfh*^{-/-} and NaIO_3 -induced mouse models as well as two cell models. The study represents the first demonstration globally of functional benefit in vivo in dry AMD models pro-

contralateral eyes at 9 months showed significantly greater Rod-b (E, 76.0 ± 38.7 vs. $133.4 \pm 66.2 \mu\text{V}$), Max b (F, 143.8 ± 58.8 vs. $207.1 \pm 86.9 \mu\text{V}$) and SFC b (G, 22.3 ± 8.27 vs. $37.54 \pm 11.44 \mu\text{V}$) responses. * $p < .05$; ** $p < .01$; *** $p < .001$; paired *t*-test. (H) A CellRox (ROS) assay was performed on retinal cells from *Cfh*^{-/-} untreated mouse retinas ($n = 17$) or mouse retinas treated with AAV2/8-NdiI ($n = 10$). Mice were subretinally injected with 2.4×10^8 -vg AAV2/8-NdiI at 2 months of age. CellRox was measured in dissociated retinal cells 3 months post-injection. Levels in untreated cells were taken to be 100%. CellRox levels in untreated versus treated cells were $100\% \pm 72.5\%$ and $55.9\% \pm 26.6\%$, demonstrating a significant reduction in ROS in AAV2/8-NdiI-treated retinas of 44.1% (2-sample *t*-test). (I) NADH oxidation activities in retinas of 14-month *Cfh*^{-/-} mice subretinally injected in one eye with 7.5×10^7 -vg AAV2/8-ophNdiI and in the contralateral eye with 7.5×10^7 empty capsids were compared ($n = 4$ retinas per group); levels in untreated retinal samples were taken to be 100%. NADH oxidation activity was 31.0% higher in eyes that received AAV2/8-ophNdiI compared to control eyes (paired *t*-test). (J) Sections of retinas from 1-year old *Cfh*^{-/-} mice that received 1.0×10^7 -vg AAV2/8-ophNdiI in one eye and 1.0×10^7 empty vector in the fellow eye were cryosectioned and cones stained with Arr3 immunohistochemistry. Cone numbers in untreated retinas were considered to be 100%. (K and L) Adult 129 S2/SvHsd mice ($n = 8$) were subretinally injected with 7.5×10^7 -vg AAV2/8-ophNdiI and 1.0×10^8 -vg AAV2/2-CAG-EGFP in one eye. Fellow eyes received an equal volume of PBS containing 1.0×10^8 -vg AAV2/2-CAG-EGFP. Three months post-injection 22-mg/kg NaIO_3 in .9% NaCl_2 was administered via tail vein. (K) One-week post-insult, ERG analysis was undertaken; AAV2/8-ophNdiI-treated eyes had significantly greater Max b responses than untreated eyes, 150.8 ± 42.6 versus $111.9 \pm 41.2 \mu\text{V}$. (L) Optokinetic (OKR) analysis 4-week post-insult showed better responses in treated versus untreated eyes, $.354 \pm .0652$ cyc/deg versus $.273 \pm .0667$ cyc/deg, respectively. (M) Adult 129 S2/SvHsd mice ($n = 6$) were subretinally injected with 5.7×10^8 -vg AAV2/5-ophNdiI and 1.0×10^8 -vg AAV2/2-CAG-EGFP in one eye. Control eyes received equivalent volumes of PBS containing 1.0×10^8 -vg AAV2/2-CAG-EGFP. Three months post-injection 50-mg/kg NaIO_3 in .9% NaCl_2 was administered via tail vein. Seven-day post-insult mice were sacrificed, eyes fixed, retinas cryosectioned and stained with Arr3 (cone antibody, magenta) and dapi (nuclear stain, dark blue). Sections from untreated (N) and treated (O) eyes had cone numbers of 54.84 ± 6.794 versus 79.26 ± 11.49 cones/mm, representing a 44.5% increase with treatment. * $p < .05$; ** $p < .01$; *** $p < .001$; paired *t*-test. Scale bar (O): 100 μm

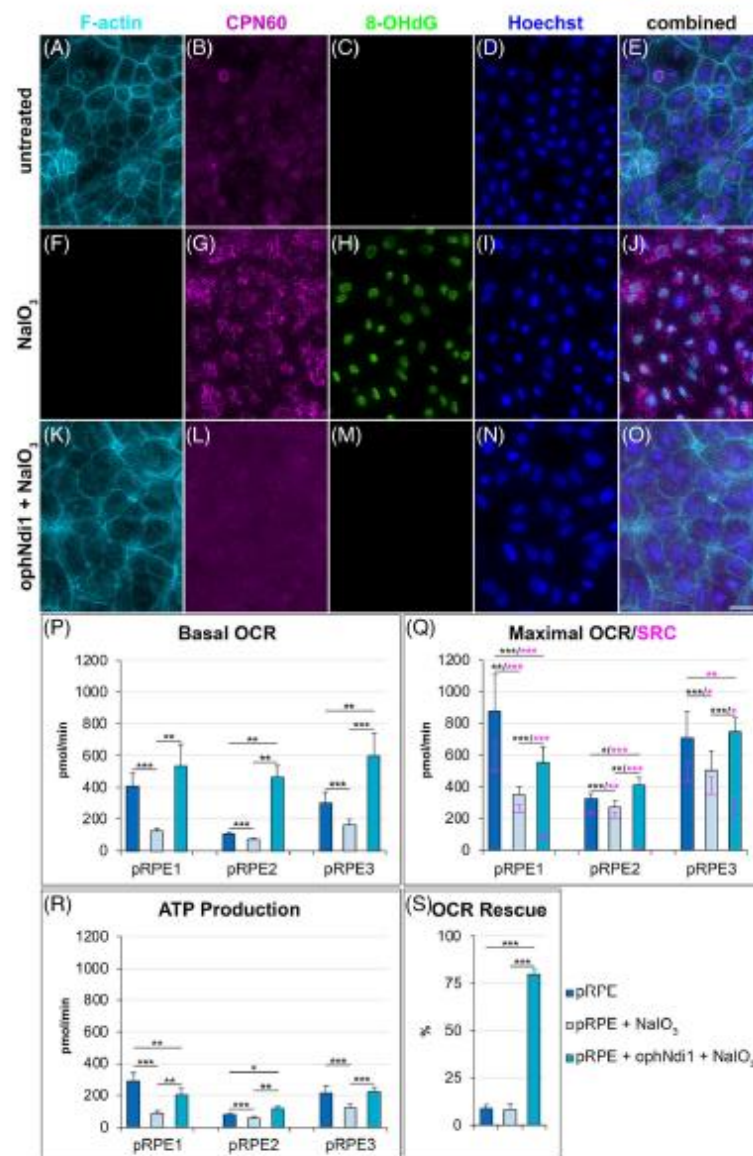


FIGURE 3 Rescues of primary porcine retinal pigment epithelium (pRPE) cells insulted with NaIO_3 . 7.5×10^4 pRPE cells (from $n = 3$ pigs) were transduced with AAV2/8-ophNdi1 5-h post-seeding ($\text{MOI} = 5.4 \times 10^5$; K–O). Twenty-eight-hour post-transduction cells were insulted with 5-mM NaIO_3 (F–O) and 24-h post-insult cells were fixed and stained with Phalloidin-4Fluor 647 (F-actin, light blue), and CPN60 (mitochondrial marker, magenta) and 8-OHdG-Alexa Fluor 488 (oxidative stress marker, green) immunocytochemistries; nuclei were counterstained with Hoechst (nuclear stain, dark blue). AAV2/8-ophNdi-treated and NaIO_3 -insulted cells (K–O) were compared to untreated (A–E) and untreated and NaIO_3 -insulted cells (F–J). Expression of ophNdi1 provides clear rescue and insulted cells treated with the virus have a similar phenotype to untreated control cells. Scale bar (O): $25 \mu\text{m}$. (P–S) 5.0×10^4 primary pRPE cells ($n = 3$ pigs; pRPE1–3) were seeded into XF96 Seahorse plates. The following day a minimum of five wells were transduced with AAV2/2-ophNdi1 ($\text{MOI} = 3.4 \times 10^5$). Twenty-eight-hour post-transduction transduced cells and untransduced control cells ($n > 15$ wells) were insulted with 6-mM NaIO_3 and 12-h post-insult cells underwent a mitochondrial stress test. (P) Basal and (Q) maximal oxygen consumption rates (OCRs), (Q) spare respiratory capacity (SRC) and (R) ATP production are indicated. (S) OCR rescue by AAV2/2-ophNdi1, post-rotenone treatment. OCR was normalised to protein. Mitochondrial stress tests on pRPE1–3 were performed on separate occasions * $p < .05$; ** $p < .01$; *** $p < .001$; paired t -test

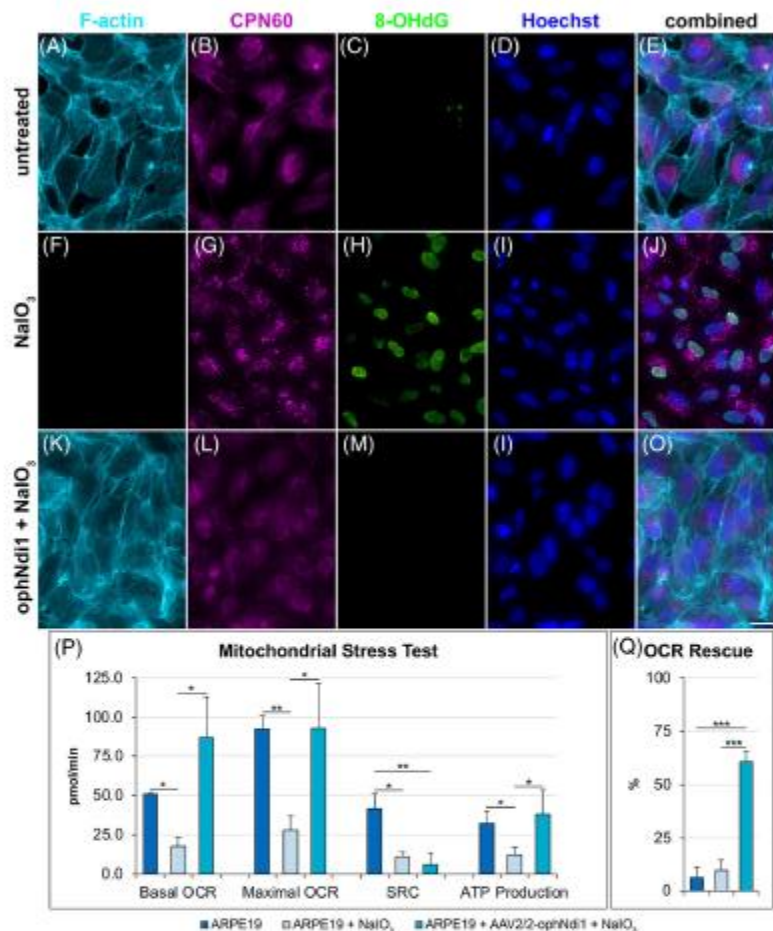


FIGURE 4 Rescue of ARPE19 cells insulted with NaIO₃. 5.0×10^4 ARPE19 cells were transduced with AAV2/8-ophNdl 5-h post-seeding; MOI = 5.4×10^5 (K–O). Twenty-eight-hour post-transduction cells were insulted with 5-mM NaIO₃ (F–O) and 24-h post-insult cells were fixed and stained with Phalloidin-iFluor 647 (F-actin, light blue), and CPN60 (mitochondrial marker, magenta) and 8-OHdG-Alexa Fluor 488 (oxidative stress marker, green) immunocytochemistries; nuclei were counterstained with Hoechst (nuclear stain, dark blue). AAV2/8-ophNdl-treated and NaIO₃-insulted cells (K–O) were compared to untreated (A–E) and untreated and NaIO₃-insulted cells (F–J). Expression of ophNdl provides clear rescue as the insulted cells treated with the virus have a similar phenotype to untreated control cells. Scale bar(O): 25 μ m. (P and Q) 5.0×10^4 ARPE19 cells were seeded into XF96 Seahorse plates ($n = 3$). The following day a minimum of five wells were transduced with AAV2/2-ophNdl (MOI = 3.4×10^5). Twenty-eight-hour post-transduction transduced cells and a minimum of 16 wells of untransduced cells were insulted with 5-mM NaIO₃ and 12 h post-insult cells underwent a mitochondrial stress test using an XF96 Seahorse. (P) Basal and maximal oxygen consumption rates (OCRs), spare respiratory capacity (SRC) and ATP production are indicated. (Q) Rescue of OCR by AAV2/2-ophNdl post-rotenone treatment is also indicated. OCRs are normalised to protein. NaIO₃ insult reduced basal OCRs, maximal OCRs, SRC and ATP production significantly compared to cells that received no insult (by 66.0%, $p < .05$; 69.8%, $p < .01$; 74.4%, $p < .001$ and 62.1%, $p < .05$, respectively). Notably, SRC (the difference in maximal and basal OCR) is further reduced by AAV2/2-ophNdl as the increase in basal OCR caused by the ophNdl expression is greater than the increase in maximal OCR. OCR was normalised to protein. * $p < .05$, ** $p < .01$, *** $p < .001$; 2-sample *t*-test

vided by a gene therapy directly targeting mitochondrial function.

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CONFLICT OF INTEREST

SMW, NC, MC, PFK and GJF are inventors on patent no. 10220102.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

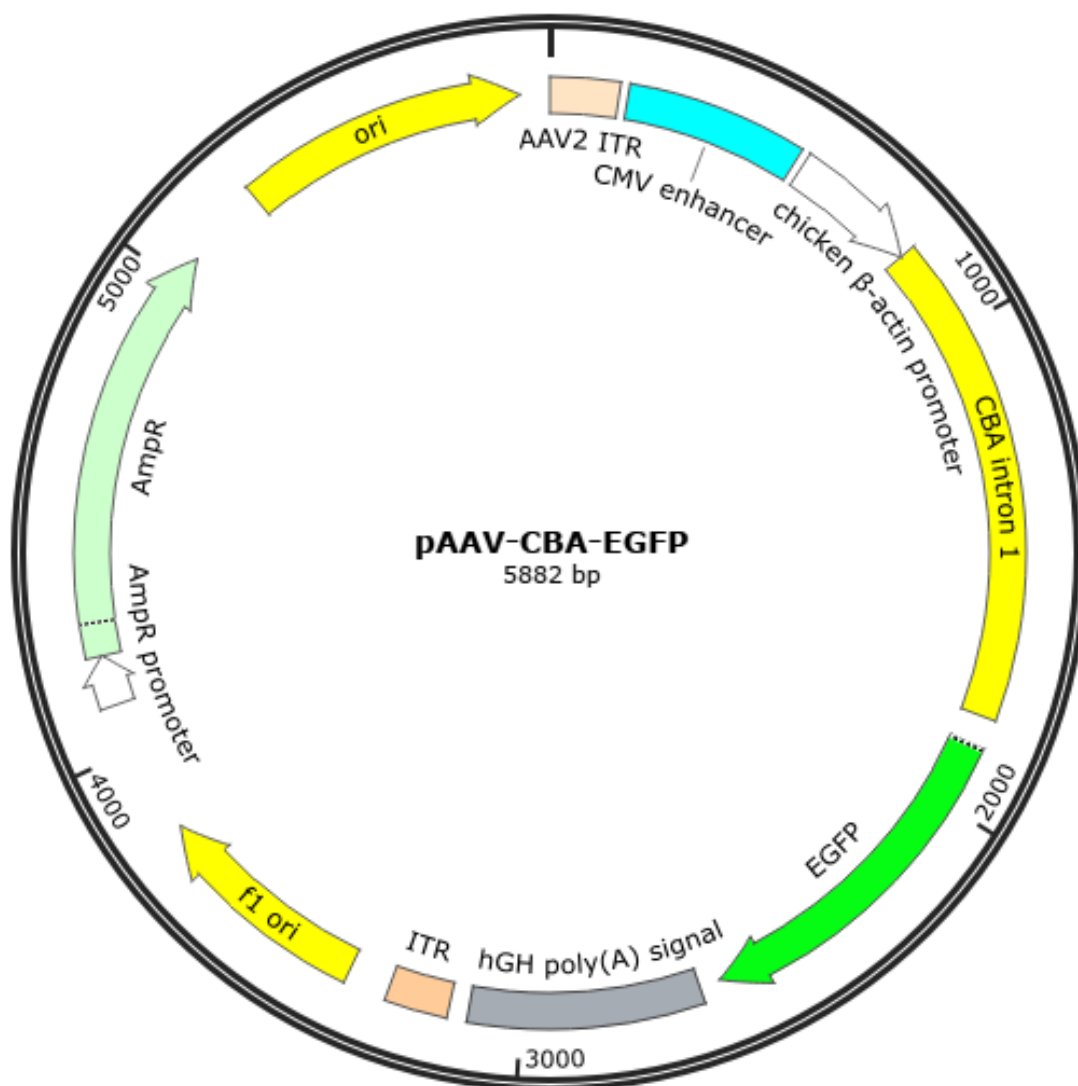
Appendix II: Construct maps and sequences

The following constructs were used and/or generated over the course of this PhD project:

- pAAV.CAG.EGFP
- pAAV.CMV.EGFP
- pCBA-RP14
- pCHOP-ophNdi
- pAAV-CBA-ophNDI1

Plasmid maps and corresponding sequences are presented below

pAAV.CAG.EGFP

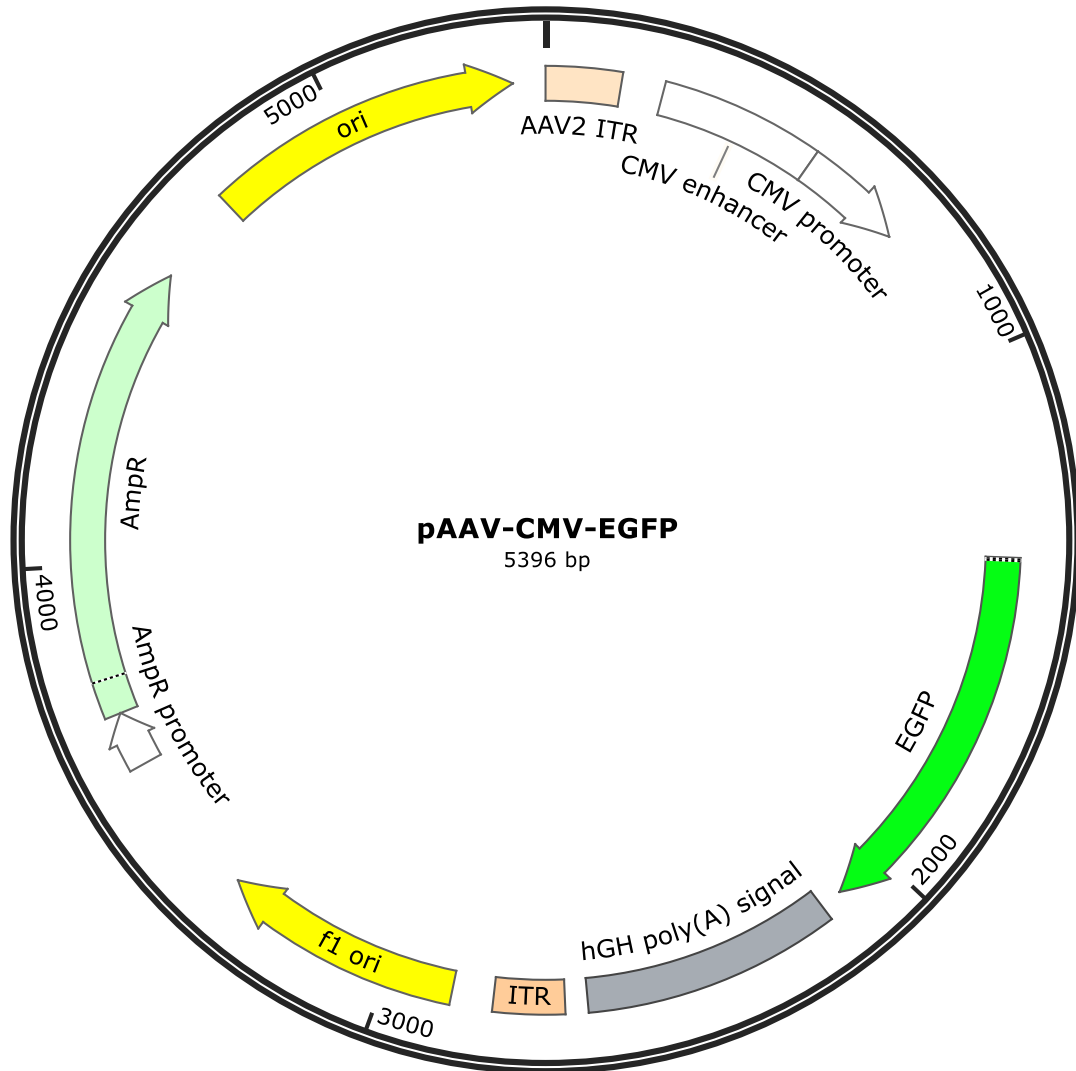


Inverted Terminal Repeats
CAG promoter
eGFP cDNA
Ampicillin resistance gene

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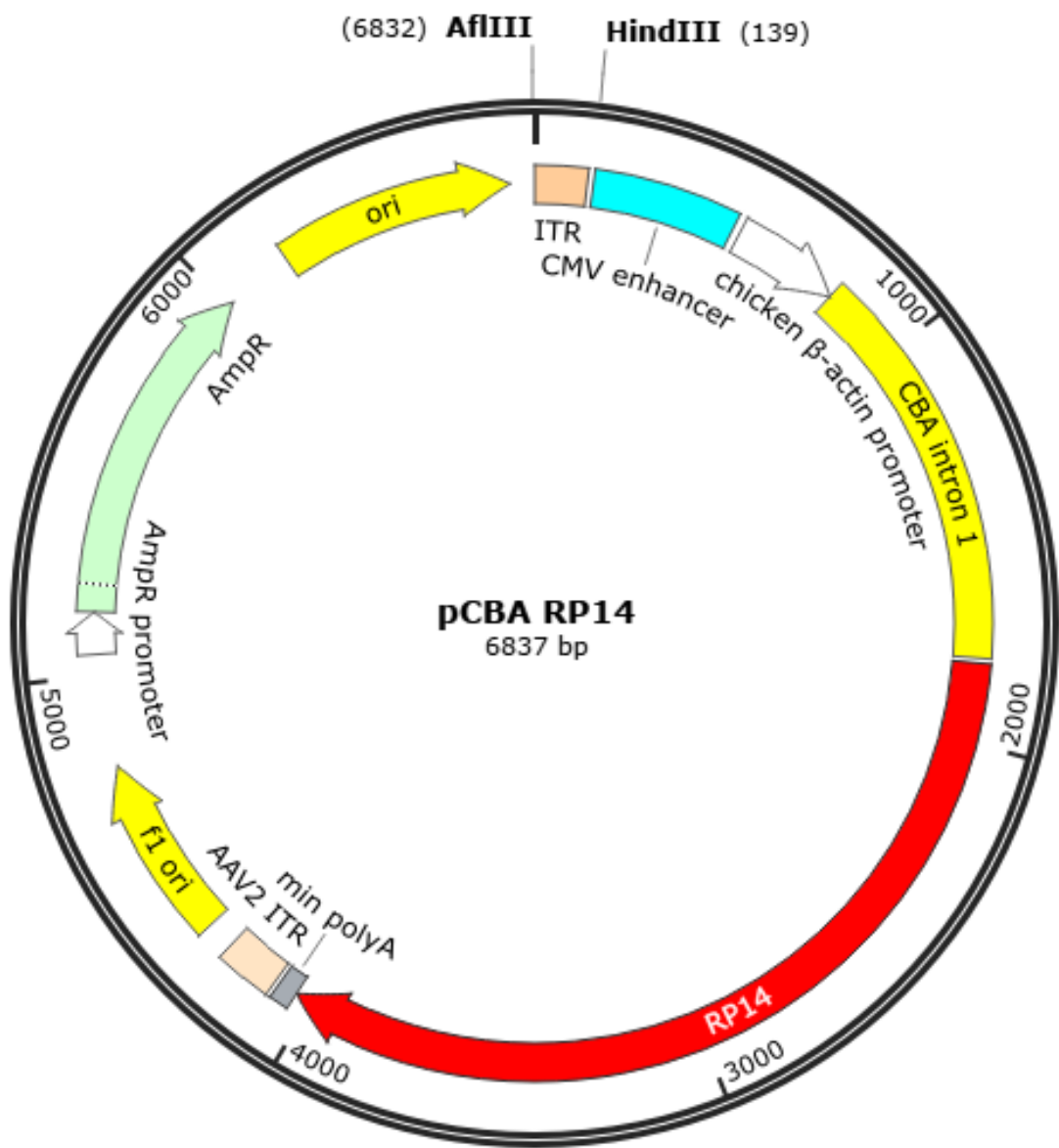
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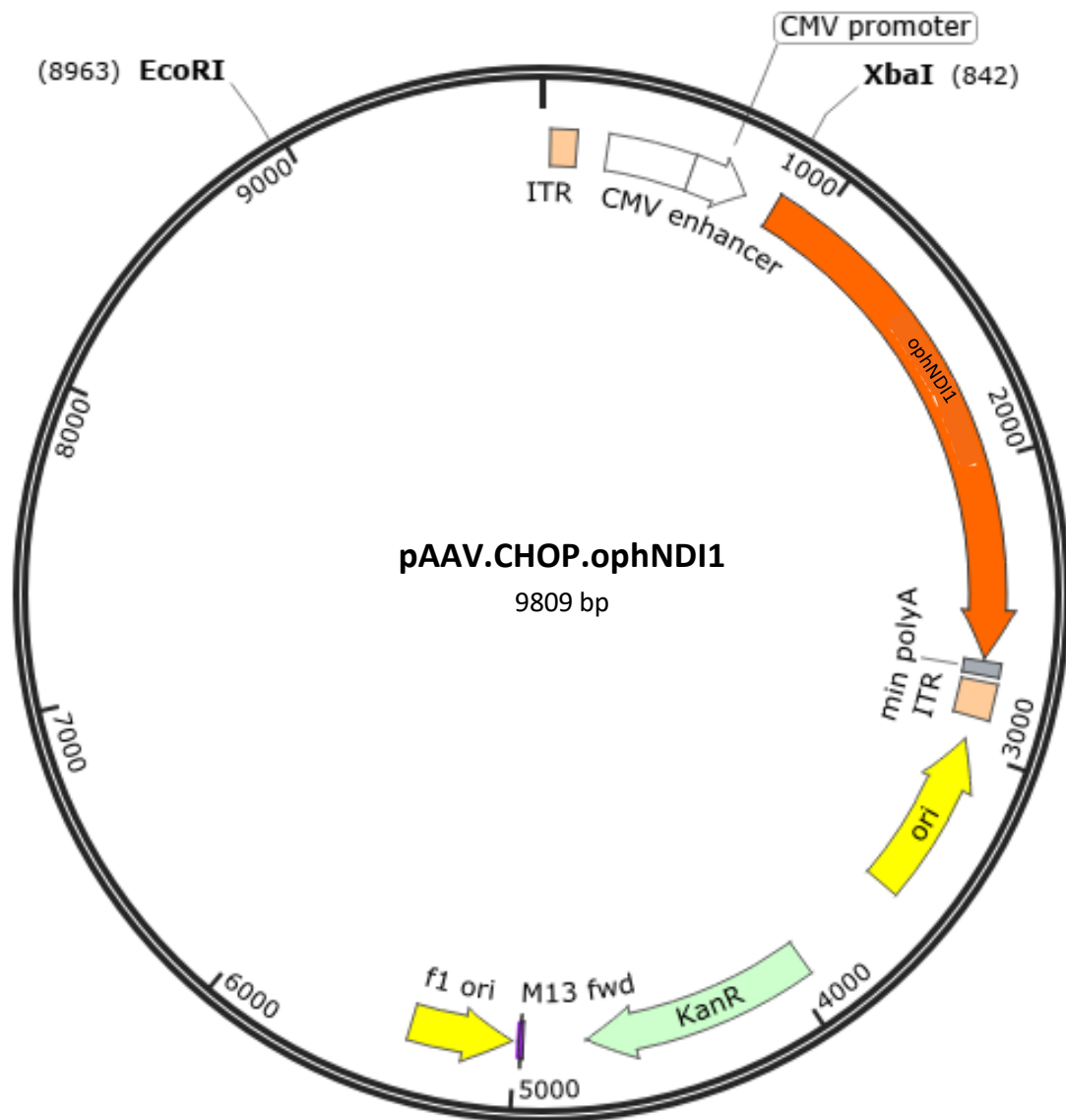
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Inverted Terminal Repeats
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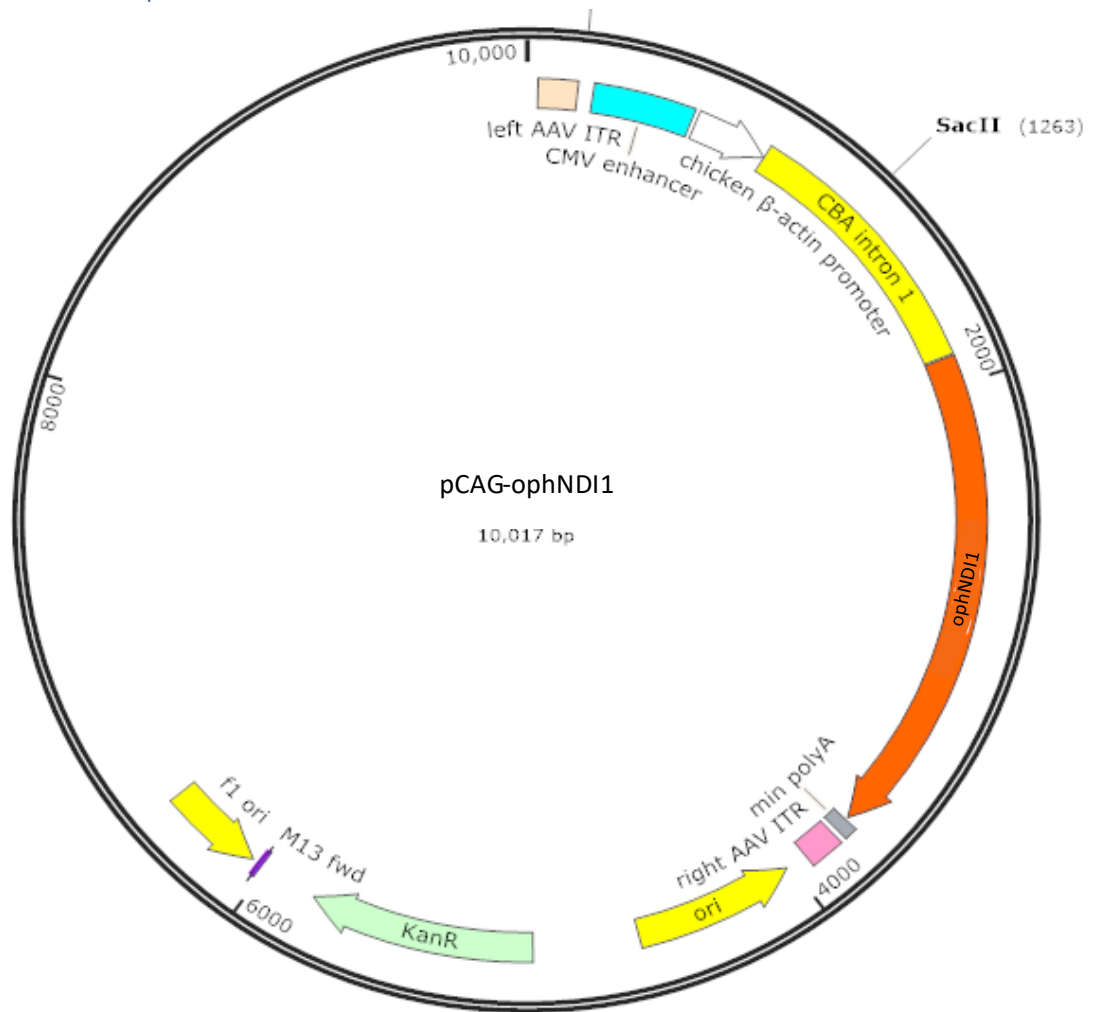
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pAAV-CAG-ophNDI1



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Left ITR

CCTGC

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CMV enhancer

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Chicken beta actin (CBA) promoter

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CBA intron 1

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GAATTC

Human NDI1 182V

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Min polyA

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Right ITR

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GCGCGCAGCTGCCTGCAGG ACATG

pUC origin of replication

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Kanamycin resistance promoter

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Kanamycin resistance coding sequence

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M13 forward

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F1 ori

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Lambda

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GCCCCGCTTCTTTGAATTTTGAATCAGGCTGTGAGCCTTATGCTCGAACTGACCATAACCAGCG
CCCGGCAGTGAAGCCCAGATATTGCTGCAACGGTCGATTGCCTGACGGATATCACCACGATCAA
TCATAGGTAAAGCGCCACGCTCCTTAATCTGCTGCAATGCCACAGCGTCCTGACTTTTTCGGAGA
GAAGTCTTTCAGGCCAAGCTGCTTGCGGTAGGCATCCCAACAGGGAAAGAAGCTGGTAGCGT
CCGGCGCCTGTTGATTTGAGTTTTGGGTTTAGCGTGACAAGTTTGCGAGGGTGATCGGAGTAAT
CAGTAAATAGCTCTCCGCCTACAATGACGTCATAACCATGATTTCTGGTTTTCTGACGTCCGTT
ATCAGTTCCCTCCGACCAGCCAGCATATCGAGGAACGCCTTACGTTGATTATTGATTTCTACC
ATCTTCTACTCCGCTTTTTTAGCAGCGAAGCGTTTGATAAGCGAACCAATCGAGTCAGTACCG
ATGTAGCCGATAAACACGCTCGTTATATAAGCGAGATTGCTACTTAGTCCGGCGAAGTCAGAGAA
GGTCACGAATGAACCAGGCGATAATGGCGCACATCGTTGCGTCGATTACTGTTTTTGTAAACGC
ACCGCCATTATATCTGCCGCGAAGGTACGCCATTGCAAACGCAAGGATTGCCCGATGCCTTGT
TCCTTTGCCGCGAGAAATGGCGGCCAACAGGTCATGTTTTTCTGGCATCTTCATGTCTTACCCCC
AATAAGGGGATTTGCTCTATTTAATTAGGAATAAGGTCGATTACTGATAGAACAAATCCAGGCT
ACTGTGTTTAGTAATCAGATTTGTTCTGTGACCGATATGCACGGGCAAAACGGCAGGAGGTTGTT
AGCGCGACCTCCTGCCACCCGCTTTCACGAAGGTCATGTGTAAAAGGCCGCGAGCGTAACATTA
CTAATGAATT

Legend

Location of Forward Primers

Location of Reverse Primers

Confirmed by sequencing

Appendix III: Primer Overviews

Many primers were used over the course of this PhD project. All primers used are presented below, grouped per chapter/project.

Supplemental Table 2. Primers used to sequence pAAV.CAG.ophNDI1

Primer name	Primer sequence	Direction	Location on pCAG - ophNDI1
CHOP_CBA_huNdi_F1	CGAAGGTCATGTGTAAAAGGC	Forward	10054-10074
CHOP_CBA_huNdi_F2	CAGCCAGAGCTCAGGACATTC	Forward	184-204
CHOP_CBA_huNdi_F3	TTTGTGTCAGCGATGGGGG	Forward	705-723
CHOP_CBA_huNdi_F4	GTGCGTGTGTGTGTGCGTG	Forward	1129-1147
CHOP_CBA_huNdi_F5	GCAGGGACTTCCTTTGTC	Forward	1609-1626
CHOP_CBA_huNdi_F6	CCATCAGCTTTCTGAAGC	Forward	2155-2172
CHOP_CBA_huNdi_F7	TGGAAAAGGCCAACCTGCTGC	Forward	2596-2616
CHOP_CBA_huNdi_F8	GCAGCAACAACATCTTCG	Forward	3082-3099
CHOP_CBA_huNdi_F9	GGTAGGCACCGTTTTTTCCTG	Forward	3545-3565
CHOP_CBA_huNdi_F10	GCTGGCGTTTTTCCATAGG	Forward	4089-4107
CHOP_CBA_huNdi_F11	AGTTGGTAGCTCTTGATCCG	Forward	4590-4609
CHOP_CBA_huNdi_F12	GAAACGTCTTGCTCGAAGC	Forward	5086-5104
CHOP_CBA_huNdi_F13	TGATGACGAGCGTAATGG	Forward	5566-5583
CHOP_CBA_huNdi_F14	TTCGCTATTACGCCAGCTGG	Forward	6017-6036
CHOP_CBA_huNdi_F15	GGAGAAAATACCGCATCAGG	Forward	6550-6569
CHOP_CBA_huNdi_F16	CTGACAGTGACAGACTGC	Forward	7059-7076
CHOP_CBA_huNdi_F17	GGTCAGACCAGATTGAAACG	Forward	7547-7566
CHOP_CBA_huNdi_F18	CTAAACCCTGTGTGCATC	Forward	8097-8114
CHOP_CBA_huNdi_F19	GGCATTTATACTCCGCTG	Forward	8570-8587
CHOP_CBA_huNdi_F20	CAGACGATGATGCAGATAACC	Forward	9055-9075
CHOP_CBA_huNdi_F21	CTACCATCTTCTACTCCG	Forward	9574-9591
CHOP_CBA_huNdi_R1	CGTCATTATTGACGTCAATGGGC	Reverse	272-250
CHOP_Cba_huNdi_R2	GGTTTCGCCACCTCTGACTTG	Reverse	4166-4145

Supplemental Table 3. Primers used for mouse genotyping

Primer Name	Primer Sequence	Direction
mouse abca4 F	AGGAGAAGCAATCAAATCAGGA	Forward
mouse abca4 wt R	GAAGATGCTCTGGATATCTCTGC	Reverse
mouse abca4 mut R	TGAGTAGGTGTCATTCTATTCTGG	Reverse
RPE65 F Leu450	ACCAGAAATTTGGAGGGAAAC	Forward
RPE65 R Leu450	CCCTTCCATTGACAGCTTCA	Reverse

Supplemental Table 4. Primers used for cascade analysis of Target 5000 Stargardt cohort

Forward Primer name	Forward Primer Sequence	Reverse Primer name	Reverse Primer Sequence
ABCA4 Exon 3 F	TAAGAGGTTAGGGGCTCAGC	ABCA4 Exon 3 R	GAGCTTGAGAGAAGAGGCCA
ABCA4 Exon 5 F	GGCATTAAAGTGATGGATTCTG	ABCA4 Exon 5 R	GACCCCAAGAACAGAACC
ABCA4 Exon 8 F	GGAAGTGGACTTTCTGGGAG	ABCA4 Exon 8 R	GTGGAAGTCAAACCAAACG
ABCA4 Exon 10 F	CAAGTGGAACTTTCTTGCC	ABCA4 Exon 10 R	CTAAGTGATAGGGGCAGAAAGAC
ABCA4 Exon 12 F	GATTTATGAGTCCAGTCTCAATCC	ABCA4 Exon 12 R	GAGTTGAGTCTTTGCAGTTGG
ABCA4 Exon 13 F	CTAAAGACATGGAAGCCTTGAGG	ABCA4 Exon 13 R	GAGAATGAGTTCCGAGTCAACC
ABCA4 Exon 15 F	CCTGTACATTTTAGCCTCACG	ABCA4 Exon 15 R	GGCATTCCTAGGATTAAAGG
ABCA4 Exon 16 F	GGTTCTGACAAAGAGCTTGAAG	ABCA4 Exon 16 R	CTTTTGGCCCTCCCCTCTAC
ABCA4 Exon 17 F	GACTCATCAGGAATCACACCG	ABCA4 Exon 17 R	GGAAGGGGTAGTCTTTCTGGG
ABCA4 Exon 21 F	CACCCTTAGAAGCTCTCCTG	ABCA4 Exon 21 R	CAGTGAGCATCTTGATTGC
ABCA4 Exon 22 F	GTCACTGATAAACCCCTTCTG	ABCA4 Exon 22 R	GCACCAAACCACTGCTGG
ABCA4 Exon 24 F	GAACAACGTGTGACCTGCAG	ABCA4 Exon 24 R	CGACTACACCCTCAAAATGCTC
ABCA4 Exon 28 F	CTCTCTCATTGGTGAAGGTCC	ABCA4 Exon 28 R	GAATGAAGATGTGTGTGCAAC
ABCA4 Exon 29 F	GGCTTCCTAGGAGGTAGTG	ABCA4 Exon 29 R	GGTGACATGCCATCAGAC
ABCA4 Exon 30 F	CCTACTCAACTGCCAGTTTG	ABCA4 Exon 30 R	GCTTGAGCAGAAACCAGC
ABCA4 Exon 31 F	GAATGGGGCCCTCAAATC	ABCA4 Exon 31 R	GTGAGAACTAGGGAGATGCAG
ABCA4 Exon 33 F	CTCATTCATGGTAGTTAAGCAAGTC	ABCA4 Exon 33 R	CGAGAGAGCTACTAGTAGGCGTG
ABCA4 Exon 35 F	TTCAGGGGTTGGCTAATGAC	ABCA4 Exon 35 R	GCGTCTCAGATGTCTCTCTCC
ABCA4 Exon i36, +1137 F	CAATGTGGTACCTGCAGTTG	ABCA4 Exon i36, +1137 R	GGTGAATATTGGAATTCCTG
ABCA4 Exon 37 F	TCAGAAGCCAGACAGATCCC	ABCA4 Exon 37 R	GGGGTAGGAGGACTAGTGACAG
ABCA4 Exon 38 F	GCACTCATCCGCATACAGC	ABCA4 Exon 38 R	CCAACAAATGGAGTATCTCTCTGG
ABCA4 Exon 39 F	CACAGTCTGATGCAGGAGC	ABCA4 Exon 39 R	GGACTCTCTGTCTGCAGG
ABCA4 Exon 40 F	CATTGTGGAGTGGGGCTC	ABCA4 Exon 40 R	CTTGCTCAGTTCCACATAAGG
ABCA4 Exon 42 F	GAATAGCTCTGCCTTATGG	ABCA4 Exon 42 R	CGAGATGTCTCAGTACTCAC
ABCA4 Exon 43 F	GATCTTTCAGGGCCTCAGAG	ABCA4 Exon 43 R	GGGGCCTGACATTTTAGAC
ABCA4 Exon 44 F	GAATGAATGAATAGCACGC	ABCA4 Exon 44 R	GGAAGAATCTGACAGGATTC
ABCA4 Exon 45 F	CCAAGACACAGAGAGAAAGAGC	ABCA4 Exon 45 R	CGTGACTTGTTTTTCTCCG
ABCA4 Exon 48 F	CCAATAAACAGAGGGCAAGAG	ABCA4 Exon 48 R	CTGGGATTTGAGGTGTGAG

Supplemental Table 5. Primers used in cascade analysis of Stargardt-like phenotypes

Forward Primer name	Forward Primer Sequence	Reverse Primer name	Reverse Primer Sequence
PRPH2 exon 1 F	CCACCACTACTGCATTCACG	PRPH2 exon 1 R	CTGGCTCTTCATCCCAAGG
PRPH2 exon 2 F	CTAGAACAGTGACTGGCAGG	PRPH2 exon 2 R	CTCCACTGAAGGCTGTTTC
PROM1 exon 11 F	GCCTCTCTACTCGTACTGAG	PROM1 exon 11 R	CAATGCAATACTTGGAAC
PROM1 exon 12 F	GGTGGTTCAGGCTTTTCTG	PROM1 exon 12 R	GGCCTCCTTGTACAATTTGC
PROM1 exon 13 R	GAAGGCAAAGGGTGTGAAGG	PROM1 exon 13 F	GATCTCTCCTCCTCGCGAC
D3 PROM1 exon 15 R	GTGAGCTGAGATCGCGCC	D3 PROM1 exon 15 F	TAGTGCATCGTAGGGTCACC
PROM1 exon 18 R	AATGTTGCCACCTGTTTAAGA GT	PROM1 exon 18 F	CTCTCCAGCAGCTTCCCTAA
PROM1 exon 23 F	GGAGCTCAGGCAGTAACATAA G	PROM1 exon 23 R	GTGAGGAAAGAAGACAACCG
PROM1 exon 24 F Iris	GTTTGGGGTGGTTAGAAGTTG G	PROM1 exon 24 R Iris	GCAACTCTTTGAAGACAGCAC C
ELOVL4 exon 6 R	CAGTGTGGCAAAATTTGGGC	ELOVL4 exon 6 F	TTGTCTTTTCTCCCCACCCC
ROM1 exon 2 F	GCTGCCTTGAATCCCCAC	ROM1 exon 3 R	CTGTTCCCTGGTGGTTCTGAC

Appendix IV: Genes included in panel-based NGS

Supplemental Table 6. Overview of genes whose exonic regions are covered in Target 5000 Target Capture Sequencing (further described in Carrigan *et al.* 2017; Dockery *et al.* 2019; Whelan *et al.* 2020).

ABCA4	CABP4	DRAM2	INVS	NPHP4	PRDM13	RTN4IP1	WDR19
ABCC6	CACNA1F	DTHD1	IQCB1	NR2E3	PROM1	SAG	WFS1
ABHD12	CACNA2D4	EFEMP1	ITM2B	NR2F1	PRPF3	SDCCAG8	DFNB31
ACBD5	CAPN5	ELOVL4	JAG1	NRL	PRPF31	SEMA4A	ZNF408
ADAM9	CC2D2A	EMC1	KCNJ13	NYX	PRPF4	SLC24A1	ZNF423
ADAMTS18	CDH23	EXOSC2	KCNV2	OAT	PRPF6	SLC25A46	ZNF513
ADGRA3	CDH3	EYS	KIAA1549	OFD1	PRPF8	SLC7A14	
ADGRV1	CDHR1	FAM161A	KIF11	OPA1	PRPH2	SNRNP200	
ADIPOR1	CEP164	FLVCR1	KIZ	OPA3	PRPS1	SPATA7	
AGBL5	CEP250	FSCN2	KLHL7	OPN1LW	RAB28	SPP2	
AHI1	CEP290	FZD4	LAMA1	OPN1MW	RAX2	TEAD1	
AIPL1	CERKL	GDF6	LCA5	OPN1SW	RB1	TIMM8A	
ALMS1	CFH	GNAT1	LRAT	OTX2	RBP3	TIMP3	
ARL2BP	CHM	GNAT2	LRIT3	PANK2	RBP4	TMEM126A	
ARL3	CIB2	GNB3	LRP5	PAX2	RCBTB1	TMEM216	
ARL6	CLN3	GNPTG	LZTFL1	PCDH15	RD3	TMEM237	
ASRGL1	CLRN1	GPR179	MAK	PCYT1A	RDH11	TOPORS	
ATF6	CLUAP1	GRK1	MAPKAPK3	PDE6A	RDH12	TREX1	
ATXN7	CNGA1	GRM6	MERTK	PDE6B	RDH5	TRIM32	
BBIP1	CNGA3	GUCA1A	MFN2	PDE6C	RGR	TRNT1	
BBS1	CNGB1	GUCA1B	MFRP	PDE6G	RGS9	TRPM1	
BBS10	CNGB3	GUCY2D	MFSD8	PDE6H	RGS9BP	TSPAN12	
BBS12	CNNM4	HARS	MIR204	PDZD7	RHO	TTC8	
BBS2	COL11A1	HGSNAT	MKKS	PEX1	RIMS1	TTL5	
BBS4	COL2A1	HK1	MKS1	PEX2	RLBP1	TTPA	
BBS5	COL9A1	HMCN1	MTTP	PEX7	ROM1	TUB	
BBS7	CRB1	HMX1	MVK	PGK1	RP1	TUBGCP4	
BBS9	CRX	IDH3B	MYO7A	PHYH	RP1L1	TUBGCP6	
BEST1	CSPP1	IFT140	NBAS	PITPNM3	RP2	TULP1	
C12orf65	CTBP2	IFT172	NDP	PLA2G5	RP9	UNC119	
C1QTNF5	CTNNA1	IFT27	NEK2	PLK4	RPE65	USH1C	
C21orf2	CYP4V2	IMPDH1	NEUROD1	PNPLA6	RPGR	USH1G	
C2orf71	DHDDS	IMPG1	NMNAT1	POC1B	RPGRIP1	USH2A	
C8orf37	DHX38	IMPG2	NPHP1	POMGNT1	RPGRIP1L	VCAN	
CA4	DMD	INPP5E	NPHP3	PRCD	RS1	WDPCP	

Supplemental Table 7. Overview of genes included in MD-smMIPs panel and associated phenotypes (as described in Hitti-Malin *et al.* 2022). Ad = autosomal dominant, ar = autosomal recessive, xl = x-linked. ACHM = Achromatopsia, AMD = Age-related Macular Degeneration, BBS = Bardet-Biedl syndrome, CD = Cone Dystrophy, CRD = cone-rod dystrophy, CSNB = congenital stationary night blindness, CYMD = cystoid macular dystrophy, iMD = inherited macular dystrophy, IRD = inherited retinal degeneration, LCA = Leber Congenital Amaurosis, MD = Macular Degeneration, NCMD = North Carolina macular dystrophy, RP = retinitis pigmentosa, SFD = Sorsby's fundus dystrophy, RS = retinoschisis, STGD = Stargardt disease, STGD1 = ABCA4-related Stargardt disease type 1, USH = Usher syndrome

Gene/Locus	Associated maculopathy	iMD or IRD phenotype 1	iMD or IRD phenotype 2	iMD or IRD phenotype 3
ABCA1	AMD			
ABCA4	iMD/RP-LCA	STGD1	RP ar	CD/CRD ar
ADAM19	AMD			
ADAM9	iMD	CD/CRD ar		
AIPL1	iMD/RP-LCA	LCA ar	CD/CRD ad	
APOE	AMD			
ARHGEF18	iMD/RP-LCA	RP ar		
ARMS2	AMD			
ATF6	iMD	ACHM ar		
BCAR1	AMD			
BEST1	iMD/RP-LCA	MD ad	RP ar	RP ad
C1QTNF5	iMD	MD ad		
C2	AMD			
C21orf2 (CFAP410)	iMD	CD/CRD ar		
C2ORF71 (PCARE)	iMD/RP-LCA	RP ar		
C3	AMD			
C8ORF37	iMD/RP-LCA	RP ar	CD/CRD ar	BBS ar
C9	AMD			
CABP4	iMD/RP-LCA	CSNB ar	LCA ar	
CACNA1F	iMD	CSNB xl	CD/CRD xl	other xl
CACNA2D4	iMD	CD/CRD ar		
CD46	AMD			
CD55	AMD			
CDH3	iMD	AR retinopathy		
CDHR1	iMD	CD/CRD ar		
CEP250	iMD	USH ar		
CEP78	iMD	USH ar	CRD ar	
CERKL	iMD/RP-LCA	RP ar	CD/CRD ar	
CETP	AMD			
CFB	AMD			
CFH	iMD/AMD			
CFHR1	AMD			
CFHR2	AMD			
CFHR3	AMD			
CFHR4	AMD			
CFHR5	AMD			

CFI	AMD			
CLN3	iMD/RP-LCA	RP ar	CRD ar	
CNGA3	iMD	ACHM ar	CD/CRD ar	
CNGB3	iMD	ACHM ar	CD/CRD ar	
CNNM4	iMD	CD/CRD ar	other ar	
COL4A5	AMD			
COL8A1	AMD			
CRB1	iMD/RP-LCA	LCA ar	RP ar	other ad
CRX	iMD/RP-LCA	LCA ad	LCA ar	RP ad
CTNNA1	iMD	MD ad		
CYMD locus	iMD	CYMD		
DRAM2	iMD	MD ar		
EFEMP1	iMD	MD ad		
ELOVL4	iMD	MD ad		
ESRRA	AMD			
EYS	iMD/RP-LCA	RP ar		
FBN2	iMD/AMD	MD ad		
GNAT2	iMD	ACHM ar		
GUCA1A	iMD	CD/CRD ad		
GUCY2D	iMD/RP-LCA	LCA ar	CD/CRD ad	
HK1	iMD/RP-LCA	RP ar	MD ar	
HMCN1	iMD	MD ad		
HTRA1	AMD			
IFT81	iMD	CRD ar		
IMPG1	iMD	MD ar	MD ad	
IMPG2	iMD/RP-LCA	RP ar		
IRX1/MCDR3 locus	iMD	NCMD ad		
KCNV2	iMD	ACHM ar	CD/CRD ar	
KIR2DL4	AMD			
LIPC	AMD			
MFSD8	iMD	MD ar		
NMNAT1	iMD/RP-LCA	LCA ar		
OPN1LW	iMD			
OPN1MW	iMD			
OPN1SW	iMD	Colour blindness ad		
OTX2	iMD/RP-LCA	LCA ad	MD ad	
PDE6C	iMD	ACHM ar	CD/CRD ar	
PDE6H	iMD	ACHM ar	CD/CRD ar	
PHF12	AMD			
PITPNM3	iMD	CD/CRD ad		
POC1B	iMD	CRD ar		
PRDM13/MCDR1 locus	iMD	MD ad		
PROM1	iMD/RP-LCA	RP ar	CD/CRD ad	MD ad
PRPH2	iMD/RP-LCA	MD ad	CD/CRD ad	RP ad
PUS7	AMD			

RAB28	iMD	CD/CRD ar		
RAX2	iMD	CD/CRD ar		
RDH12	iMD/RP-LCA	LCA ar	RP ad	
RDH5	iMD	CSNB ar	CD/CRD ar	
RIMS1	iMD	CD/CRD ad		
RLBP1	AMD/RP-LCA	RP ar	other ar	
ROM1	iMD/RP-LCA	RP ad		
RP1	iMD/RP-LCA	RP ad	RP ar	
RP1L1	iMD/RP-LCA	RP ar	MD ad	
RPGR	iMD/RP-LCA	RP xl	CD/CRD xl	MD xl
RPGRIP1	iMD/RP-LCA	LCA ar	CD/CRD ar	
RPGRorf15	iMD			
RS1	iMD	RS xl		
RXFP2	AMD			
SCN10A	AMD			
SLC16A8	AMD			
SPEF2	AMD			
TACC2	AMD			
TIMP3	AMD/RP-LCA	SFD ad		
TLCD3B	iMD	CRD ar		
TTLL5	iMD	CD/CRD ar		
TULP1	iMD/RP-LCA	LCA ar	RP ar	
UNC119	iMD	CD/CRD ad		
VPS13B	AMD			

Appendix V: Allele counts of all ABCA4 variants identified in solved probands

Supplemental Table 8. List of allele counts of all ABCA4 variants found in solved STGD1 probands.

Variant	Count	Variant	Count	Variant	Count	Variant	Count
c.161G>A	40	c.4469G>A	3	c.191C>T	1	c.466A>G	1
c.4139C>T	28	c.4957G>A	3	c.1933G>A	1	c.4793C>A	1
c.[302+68C>T; 4539+2028C>T]	24	c.5929G>A	3	c.2160+1G>C	1	c.4919G>A	1
c.4577C>T	24	c.6089G>A	3	c.223T>C	1	c.4925G>T	1
c.5603A>T	21	c.1018T>C	2	c.2311A>T	1	c.5113C>T	1
c.[2588G>C;5603A>T]	18	c.1804C>T	2	c.2382+1dup	1	c.5196+1G> A	1
c.3056C>T	18	c.2041C>T	2	c.2453G>A	1	c.5312+1G> A	1
c.3322C>T	16	c.223T>G	2	c.2483C>T	1	c.5329A>G	1
c.5882G>A	13	c.3329-1G>A	2	c.2549A>G	1	c.5348T>C	1
c.6079C>T	13	c.4225A>G	2	c.2564G>A	1	c.5381C>A	1
c.[5461-10T>C; 5603A>T]	12	c.4254-5T>A	2	c.2588G>C	1	c.550-551 TC>CG	1
c.[4222T>C;4918C>T]	12	c.4326C>A	2	c.2617T>C	1	c.5594dupA	1
c.3323G>A	8	c.4861A>T	2	c.2701A>G	1	c.5644A>G	1
c.4539+2028C>T	7	c.5312+3A>T	2	c.286A>C	1	c.5773A>G	1
c.[2827C>T;5603A>T]	6	c.5351T>C	2	c.2894A>G	1	c.587C>T	1
c.4253+43G>A	6	c.5908C>T	2	c.2895A>G	1	c.5898 +5G>A	1
c.4363T>C	6	c.5921A>G	2	c.302+68C>T	1	c.618C>G	1
c.1317G>A	5	c.6286G>A	2	c.3137T>G	1	c.6221G>A	1
c.5917delG	5	c.6472A>G	2	c.3212C>A	1	c.6416G>C	1
c.6098T>G	5	c.[185C>T; 5603A>T]	1	c.3305A>T	1	c.6448T>C	1
c.[1622T>C;3113C>T]	4	c.[2852T>C; 5603A>T]	1	c.3352C>G	1	c.655A>T	1
c.3113C>T	4	c.[3481C>A; 5603A>T]	1	c.3364G>A	1	c.6658C>T	1
c.4685T>C	4	c.[3758C>T; 5882G>A]	1	c.3386G>T	1	c.66G>T	1
c.5196+1137G>A	4	c.[466A>G; 4771G>A]	1	c.3754G>T	1	c.6743T>C	1
c.5714+5G>A	4	c.[5113C>T; 5882G>A]	1	c.4229A>G	1	c.731T>C	1
c.6449G>A	4	c.1037A>C	1	c.4234C>T	1	c.735T>G	1
c.[1715G>A;2588G>C; 5603A>T]	3	c.1222C>T	1	c.4283C>T	1	c.752delT	1
c.[4222T>C; 4918C>T]	3	c.1532G>A	1	c.4320del	1	c.768G>T	1
c.[4469G>A;5603A>T]	3	c.1715G>A	1	c.4468T>C	1	c.868C>T	1
c.1253T>C	3	c.1805G>A	1	c.4537dup	1	c.869G>A	1
c.1519G>T	3	c.1817G>A	1	c.455G>A	1		
c.1906C>T	3	c.1865G>A	1	c.4577del	1		