**Retina Australia Research Report 2024**

**Project title:** Characterizing Stargardt Disease Mutations for Splice Intervention Therapeutics

***Lay title:*** *Developing a New Treatment Strategy for Stargardt Disease*

**Investigators:**

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**Project synopsis - *What is this research about?***

Stargardt disease (OMIM #248200, STGD1) is one of the most common inherited retinal diseases (IRDs) leading to blindness, accounting for approximately 12% of all IRD-related vision loss. It is caused by bi-allelic variants in the ATP-binding cassette subfamily A member 4 (ABCA4) gene. However, the effects of ABCA4 mutations—particularly their impact on pre-mRNA splicing and protein function—remain poorly understood.

This project aimed to address the current lack of treatment for STGD1 by identifying ABCA4 mutations that cause aberrant splicing and developing splice-switching antisense oligonucleotides (SS-AONs) as a potential therapeutic strategy. Using our established ABCA4 RT-PCR assays, we successfully screened for splicing variants. Patient-derived induced pluripotent stem cells (iPSCs) were reprogrammed using our validated protocols and workflow. This work has established a strong foundation for future retinal cell differentiation, enabling downstream studies to investigate how specific splice variants affect transcript integrity and protein function. Furthermore, it provides a robust and scalable platform for screening SS-AONs and assessing their therapeutic efficacy.

***Lay summary:***

*Stargardt disease is a common inherited eye condition that causes gradual vision loss. It is caused by changes in a gene called ABCA4. These changes interfere with how the gene works and how its message is read in the body, which can prevent eye cells from functioning properly.*

*Our research aimed to understand how these gene changes damage the eye and to explore a new type of treatment. This potential treatment uses small molecules (called antisense oligonucleotides) to “patch” the gene’s message and help it be read correctly. This could allow the gene to work better and slow or prevent vision loss.*

**Aims of this research/ *what did we do?***

**Aim 1:** Identify *ABCA4* mutations causing aberrant splicing in STGD1 patient-derived fibroblasts.

**Hypothesis:** A significant portion (~15%) of the *ABCA4* variants result in abnormal splicing of the *ABCA4* transcript. This proportion is expected to be even higher among intronic variants, where splicing disruptions are more prevalent.

**Aim 2:** Develop splice-switching-AONs (SS-AONs) for the treatment of splice-altering *ABCA4* mutations.

**Hypothesis:** The use of SS-AONs targeted to specific splicing motifs in the *ABCA4* pre-mRNA can effectively correct aberrant splicing caused by splice altering *ABCA4* mutations.

***Lay summary:***

*We had two main goals:*

1. *Find faulty versions of the ABCA4 gene that interfere with how the gene’s message is processed.*
2. *Develop and test small molecules (antisense oligonucleotides) that can correct these mistakes.*

**Results - *what did we find?***

Results 1. Screening for *ABCA4* splice variants

We first selected 30 patients from our STGD1 cohort database who carried a total of 19 intronic ABCA4 variants. Using our well-established ABCA4 RT-PCR assay, we screened these variants for splicing defects. Among them, 8 variants (42.1%) produced abnormal splicing products, indicating disrupted transcript processing. An additional 5 variants (26.3%) exhibited significantly reduced transcript levels compared to wild-type controls, despite no visible abnormal splice products. Upon treatment with a nonsense-mediated decay inhibitor (NMDI), cells carrying these 5 variants showed restored levels of full-length transcripts comparable to wild-type, suggesting that the reduction was due to transcript degradation caused by premature stop codons or splicing-induced instability. Based on these findings, variants **c.4128+247A>C**, **c.4774-10T>A**, and **c.5898+20C>T** - which showed significant reductions in full-length transcript levels rescued by NMDI treatment - were prioritised for further modelling using patient-derived iPSCs.

***Lay summary:***

*We studied cells from 30 patients with Stargardt disease and found that nearly 70% had gene changes that disrupted how the gene’s message is processed. Some changes led to incorrect messages; others caused the message to break down before it could be used. These issues can prevent the gene from doing its job in the eye.*

Results 2. Reprogramming patient-derived fibroblasts into iPSCs

Following the splice assay results and clinical phenotypes, we selected three ABCA4 variants—**c.4128+247A>C**, **c.4774-10T>A**, and **c.5898+20C>T** - iPSC modelling. Notably, **c.4128+247A>C** and **c.4774-10T>A** are currently classified as variants of uncertain significance, and all three showed NMDI-responsive transcript loss. Using electroporation, Episomal iPSC Reprogramming Vectors were successfully introduced into fibroblasts derived from patients carrying these variants. After four weeks of culture, emerging iPSC colonies were manually picked and expanded. These iPSC lines will undergo continued passaging and characterisation to confirm pluripotency and to ensure their suitability for downstream differentiation into retinal cells.

***Lay summary:***

*We used skin cells from selected patients and converted them into stem cells – a type of cell that can be grown into other types of cells, including eye cells. This gives us a powerful tool to study the disease more closely and test treatments directly in cells affected by the patients’ specific mutations/ gene changes.*

Results 3. Designing SS-AONs

We utilised **Human Splice Finder** (https://genomnis.com/hsf) to predict the disruption or creation of cryptic splice sites, as well as the alteration of exonic splice enhancers (ESEs) and silencers (ESSs) associated with each variant. Based on these in silico predictions, we designed **SS-AONs** targeting relevant splice motifs for three selected ABCA4 variants. For **c.5898+20C>T**, which showed no obvious splice motif alterations in silico, SS-AONs were designed to target regions flanking the variant to restore proper splicing and rescue full-length transcript expression. For **c.4128+247A>C**, SS-AONs were designed to mask altered ESS motifs adjacent to the variant, aiming to promote recognition of the natural splice site and restore canonical splicing. For **c.4774-10T>A**, which was predicted to activate a cryptic donor site, SS-AONs were designed to block this aberrant site and prevent the inclusion of intronic sequence that could trigger NMD. For each variant, **three SS-AONs** were designed: one directly overlapping the mutation site, one positioned upstream (5′), and one downstream (3′), providing coverage across the local splice environment for maximal screening efficacy.

***Lay summary:***

*We designed and tested small molecules known as splice-switching antisense oligonucleotides (SS-AONs). These are like patches that help fix how the gene's message is read by the body. Think of them like correcting a sentence so that it makes sense again.*

Results 4. Testing SS-AONs in patient-derived fibroblasts

To evaluate the therapeutic potential of our designed SS-AONs, we tested them in fibroblasts derived from patients carrying *ABCA4* variants of interest: **c.5898+20C>T, c.4128+247A>C** and **c.4774-10T>A**.After SS-AON treatment, we observed a clear increase in correctly spliced, full-length *ABCA4* transcripts across all three patient-derived cell lines, as determined by RT-PCR. This indicates that the SS-AONs successfully redirected splicing to restore more normal gene expression.

These results confirm that our lead SS-AONs can effectively rescue *ABCA4* transcript processing in relevant patient-derived cells. This provides strong preclinical support for their potential as therapeutic candidates for treating STGD1.

***In layman language:***

*We added these molecules to patient cells in the lab. After treatment, the gene message was restored to more normal levels, meaning the patch had worked and the cells were better able to process the ABCA4 gene correctly.*

Results 5. Research manuscript preparation

A research manuscript titled “Antisense Oligonucleotide-Mediated Therapy for Stargardt Disease: Targeting the ABCA4 c.5461-10T>C Variant” is currently undergoing final revision and will proceed to co-author proofreading. Upon completion of internal review, the manuscript will be submitted to a peer-reviewed journal for publication.