Marie Curie Innovative Training Network

Training researchers to Diagnose, Understand and Treat Stargardt Disease, a Frequent Inherited Blinding Disorder

(StarT project)

Eye diseases are among the most common inherited human disorders. Around one third of the known genetic defects or syndromes involve the eye. Vision research has often blazed a trail for many disciplines to follow, giving a lead in omics, genome editing, stem cell biology, animal models of disease, and the development of novel therapeutic approaches such as gene therapy.

StarT will create an interdisciplinary and intersectorial European training network focusing on different aspects of autosomal recessive Stargardt disease (STGD1), a frequent inherited blinding disorder that affects an estimated 925,000 persons worldwide, representing more than one-third of all inherited retinal disorders. StarT research aims to uncover the regulation of its disease gene ABCA4 and its missing heritability, in order to develop novel treatments.

STGD1 is due to ABCA4 mutations, however up to 35% of STGD1 cases carries one or no ABCA4 coding mutation. New unconventional classes of ABCA4 mutations were recently discovered by us, the significance of which largely remains elusive. In order to understand the mechanisms triggered by these missing ABCA4 mutations and to design new therapies for STGD1 cases, challenging research questions will be addressed by the integration of unique skills from this network.

Early-Stage Researchers will perform cutting edge research using innovative and interdisciplinary approaches: (functional) genomics and transcriptomics, bio-informatics, CRISPR/Cas9 genome editing, generation of stem cell and animal disease models and design of new treatments.

Individual project descriptions

ESR1 Partner 4 (TIGEM): Identification of the gene networks that modulate ABCA4 expression
We have recently generated a comprehensive mRNA and microRNA (miRNAs) transcriptome of the human retina and have acquired expertise in co-expression analysis. ESR1 will capitalise on these resources to gain insight into the organisation of the ABCA4 transcriptional unit and into the dissection of the gene networks that participate in the modulation of the expression and function of ABCA4 in human retina. ESR1 will further define the genomic organisation of the ABCA4 gene using our vast RNA-seq dataset to identify and validate the presence of alternative transcript variants. By integrating already available and newly-generated transcriptomics data carried out both in human and in mouse, ESR1 will reconstruct the transcriptional gene networks that modulate ABCA4 expression in photoreceptors in both physiological and pathological conditions. ESR1 will pay particular attention to the contribution of miRNAs and will combine target predictions with our data on miRNA expression in the retina to select candidate miRNAs predicted to regulate ABCA4 and validate their biological relevance using in vitro and in vivo assays. **Supervisor**: Prof. Dr. S. Banfi.

ESR2 Partner 6 (CSIC): Interaction map of cis-regulatory elements controlling ABCA4 in human retina
Although the ABCA4 gene was identified over a decade ago, its transcriptional regulation has not been studied in relevant human cells such as retina. Data about CTCF binding sites, DNase I hypersensitivity sites and histone modifications (H3K4me1, H3K27ac) generated in other cell types can be found in publicly available databases (e.g. ENCODE, Roadmap Epigenomics). Here, ESR2 aims to identify and functionally characterise cis-regulatory elements (CREs) controlling ABCA4 expression in adult human retina. ESR2 will first use the recently developed chromosome conformation technique HiChIP to determine the target genes of the CREs that are active in human retinal cells. To that end ESR2 will perform HiChIP using both the Pol II and H3K27ac antibodies that mark active promoters and enhancers, respectively. This will allow us to precisely determine which active enhancers are directly contacting the ABCA4 promoter in human retinal cells. ESR2 will then use stable enhancer transgenic assays in zebrafish to evaluate the spatiotemporal activity of the identified CREs. **Supervisor:** Prof. Dr. J.L. Gomez-Skarmeta.

ESR3 Partner 1 (UGent): Mapping the cis-regulatory landscape of ABCA4 in adult human retina

It is known that ABCA4 is under control of CRX, a key transcription factor (TF) for retinal development and maintenance: a preliminary search for TF binding sites revealed six CRX-binding regions (CBRs) in the ABCA4 region. To add to the cis-regulatory landscape of ABCA4 in human retina, ESR3 will perform ATAC-seq on adult human retinal tissue. To determine that transposase-accessible regions represent active promoters or enhancers, ESR3 will perform ChIP-seq using antibodies specific to H3K27ac and H3K4me2 histone marks and the TF CRX. In addition, ESR3 will perform RNA-seq on adult retina, to enrich for enhancer RNAs (eRNAs), which are bi-directional transcripts that are associated with active enhancers. Finally, ESR1, ESR2 and ESR3 will integrate the cis-regulatory data they generate. ESR3 will assess the effects of selected CREs by ex vivo massively parallel reporter assays in mouse retinas (CRE-seq). With these studies we will generate an integrated functional map of CREs controlling expression in the retina, which are candidate non-coding regions that may be affected in human inherited retinal diseases, such as ABCA4-related IRD. **Supervisor:** Prof. Dr. E. De Baere.

ESR4 Partner 1 (UGent): Functional assessment of a cis-regulatory element of ABCA4 in a frog model

Xenopus tropicalis contains the major cell types of the human eye and unlike the rod-dominated rodent retina, *Xenopus (X.)* has equal numbers of cones and rods, which is more similar to humans. In addition, abca4 is not duplicated in *X*. and is located in a region with high synteny, facilitating the identification and characterisation of possible CREs. Previous CRISPR/Cas9 based knock out a CRE of Shh (i.e. ZRS) in *X. tropicalis* resulted in a phenocopy of the human limb phenotype. This work demonstrated that *X*. can be used as a model organism to study the non-coding genome. To generate a stable knockout of a previously characterised CRE of ABCA4, ESR4 will use CRISPR/Cas9-mediated genome editing to disrupt this element in *X*. ESR4 will screen F0 or F1 animals for a STGD1 phenotype by histology, immunofluorescence and TUNEL assays. To study the effect of variations in CREs in F0 *X*. and to add to the stable transgenesis experiments of ESR2, ESR4 will use a recently described direct plasmid-injection method generating robust, promoter-typical expression in tadpoles. A reference gene present on the plasmid controls for variations in the injections. The plasmids further contain phiC31 AttB sites to favour integration and chromosomal insulators to reduce possible chromosomal position effects. **Supervisor:** Prof. Dr. K. Vleminckx.

ESR5 Partner 2 (RUMC): Identification and splice assays of deep-intronic ABCA4 variants in mono-allelic STGD1

Approximately 25% of STGD1 cases show one or no coding ABCA4 variant. Using ABCA4 locus sequencing, we and others identified deep-intronic variants. We focused on the identification of RNA splice defects and generated a complete set of Gateway-based splice vectors, denoted midigenes, that contain wild-type (WT) ABCA4 multi-exon segments of 4.7 to 11.7 kb. Using a mutagenesis protocol, we rapidly introduced new variants into these vectors and performed in vitro splice assays in HEK293T cells. We assessed the effect of all reported 47 non-canonical ABCA4 splice variants and tested 10 deep-intronic variants identified in 40 mono-allelic Dutch STGD1 cases. Splice defects were visualised by RT-PCR using primers annealing to flanking ABCA4 exons. For selected variants, we also confirmed their effect on patient-derived photoreceptor progenitor cells (PPCs). ESR5 will develop a cost-effective sequencing method for the ABCA4 locus using single molecule Molecular Inversion Probes (smMIPs), and sequence 400 mono-allelic STGD1 cases that have been recruited by P2-RUMC. Sequence data (variants) of ESR5, ESR6 and ESR7 will be compiled. Hundred variants predicted to affect splicing will be introduced into WT midigenes. The effect of selected variants will be analysed in patient-derived PPCs and retinal pigment epithelium (RPE) cells. **Supervisor:** Prof. Dr. F. Cremers. url: https://www.ru.nl/donders/research/theme-2-perception-action-control/research-groups-theme-2/blindness-genetics/

ESR6 Partner 5 (UNIVLEEDS): Long-read sequencing of the ABCA4 locus

The establishment and optimisation of novel enrichment and sequencing strategies are necessary to detect missing heritability of ABCA4. Technologies which enable allelic phasing of identified genetic variants are of paramount importance in the absence of additional family DNA, particularly when assessing cis-acting modifier alleles. ESR6 will design and optimise a CRISPR-Cas9 mediated enrichment method for the entire ABCA4 gene and surrounding DNA for use in long-read sequencing on a PacBio Sequel. In addition, ESR6 will employ Illumina TruSeq synthetic long-read phased sequencing. These approaches will be applied to 50 mono-allelic STGD1 cases. The data generated by ESR6 will be integrated with the smMIPs, TLA and HaloPlex data generated by ESR5 and ESR7. ESR6 will interrogate this data set, along with cases and controls from the UK 100,000 Genome data, to look for disease associated haplotypes, non-coding or mild hypomorphic or modifier variants. Where appropriate, potential modifier and hypomorphic variants or haplotypes will be investigated functionally, and their potential contribution to phenotype/genotype correlations will be investigated. **Supervisor:** Dr. C. Toomes.

ESR7 Partner 1 (UGent): Identification and characterisation of structural variants and non-coding sequence variants of ABCA4

Targeted Locus Amplification (TLA) is based on the physical proximity of nucleotides within a locus of interest and requires just one primer pair complementary to a short locus-specific sequence to amplify 10.000s of basepairs of surrounding sequence information. TLA-based sequencing results in complete sequence information and enables haplotyping, which drastically reduces the filtering of candidate variants in genomic regions. ESR7 will design TLA for the entire ABCA4 gene as a detection tool both for SNVs and SVs in 50 monoallelic STGD1 cases. In addition, TLA will be used for the characterisation of 6 SVs previously found by us in monoallelic STGD1 cases. Second, ESR7 will develop an improved HaloPlex design for cost-effective resequencing of the ABCA4 locus in 300 mono-allelic European STGD1 cases. Both TLA and HaloPlex sequence data generated by ESR7 will be integrated with the smMIPs and long-read data from ESR5 and ESR6. We and P2-RUMC previously identified 10 putative cis-regulatory variants in mono-allelic STGD1 cases. Third, ESR7 will assess the effects of previously and newly identified non-coding variants by ex vivo massively parallel reporter assays (CRE-seq) optimised by ESR3. **Supervisor:** Dr. F. Coppieters.

ESR8 Partner 9 (NewCells): ABCA4 transcript analysis of retinal cells derived from mono-allelic STGD1 cases

ABCA4 locus sequencing in genetically unexplained STGD1 cases on average yields three rare sequence variants per patient. RNA splice site prediction programs can select variants that potentially affect RNA splicing. Currently, two functional assays are being used to assess the effect of putative splice variants. First, employing splice midigenes containing wild-type or mutant sequences that are transfected into HEK293T cells, splice defects are visualised using RT-PCR and gel analysis. Second, photoreceptor precursor cells (PPCs) can be derived from induced pluripotent stem cells (iPSCs) that were obtained through reprogramming of patient skin fibroblasts or blood cells. Currently, the ABCA4 transcript is analysed using PCR-amplification of tiled cDNA segments. Here, ESR8 will augment ABCA4 transcript analysis by generating PPCs and mature photoreceptor cells from ten patient-derived iPSCs of mono-allelic STGD1 cases, available from P2-RUMC. Using PacBio single molecule (long-read) sequencing technology, ESR8 will assess the entire spectrum of aberrant transcripts in PPCs of unsolved STGD1 cases. ABCA4 locus sequencing on genomic DNA will be performed in collaboration with ESR5. **Supervisor:** Prof. Dr. M. Lako.

ESR9 Partner 2 (RUMC): Antisense oligonucleotide-based splice modulation for deep-intronic mutations in ABCA4

Antisense oligonucleotides (AONs) are molecules that are able to modulate pre-mRNA splicing of target genes, either by blocking or by recruiting factors that are essential for splicing. P1-UGent, P2-RUMC and others have identified several deep-intronic ABCA4 variants that result in the insertion of pseudexons into ABCA4 mRNA (e.g. c.4539+1100A>G; c.4539+1106C>T; c.4539+2001G>A and c.4539+2028C>T). In this project, ESR9 will employ AONs to redirect ABCA4 splicing in order to produce wild-type ABCA4 transcripts, employing patient-derived fibroblasts or minigene splicing assays. Following the identification of the most potent AON molecules for each mutation iPSC-derived PPCs from patients with the corresponding mutations will be generated. Subsequently, AONs will be administered to these PPCs, to further optimise the efficacy of the AONs in a relevant cell model. ESR9 will employ transcriptomics to study potential off-target effects of AON administration. These data will shed light on the pre-clinical efficacy and safety of AON-based splice correction therapy for ABCA4-associated IRD, and allow to identify new targets for initiating clinical studies in humans. **Supervisor:** Dr. R. Collin.

ESR10 Partner 10 (ProQR): Optimisation, delivery and tolerability of antisense oligonucleotides to treat STGD1 patients with the most common splice mutation in ABCA4

ProQR is currently in Phase IIa clinical development with a splice modulating AON (QR-110) targeting the deep-intronic c.2991+1655A>G mutation in LCA type 10. In addition, they have two additional AON programs in Usher syndrome targeted all exon-13 mutations (QR-421a) and c.7595-2144A>G (QR-411a), respectively, in pre-IND/CTA enabling studies. ProQR is also in the discovery phase with regards to an intronic ABCA4 mutation (c.5461-10T>C), one of the most frequent mutations underlying STGD1 that results in the exclusion of exon 39, or exon 39 and exon 40 together, from the mRNA, leading to a frameshift. ESR10 will continue this program and optimise the sequence and chemistry of the final molecule to generate a clinical development candidate. This lead candidate will be further optimised by determining the extent of target engagement using digital-droplet PCR, immune-profiling against human donor T-lymphocyte panels, delivery to photoreceptors tolerability and pharmacokinetic parameters following intravitreal injection of mouse and rabbit models. **Supervisor:** Prof. Dr. P. Adamson.

ESR11 Partner 4 (TIGEM): ABCA4 knockout pig as model for gene therapy in STGD1

Abca4-/- knockout mice are currently used as animal models of STGD1, but they recapitulate only some of the features of the disease, which might be due to the structure of the mouse retina, which largely differs from that of humans. The absence of an appropriate animal model severely limits both the understanding of STGD1 mechanisms as well as the testing of novel potential therapeutic strategies. Among non-primate mammals, the porcine eye shares many similarities with the human retina including anatomy, size, and a high cone/rod ratio. In addition, unlike in non-human primates or other large species, pig transgenesis is particularly advanced. ESR11 will generate a pig model of STGD1, by exploring either nuclear transfer from fibroblasts which have been genetically modified using TALEN technology in embryonic stem cells, or photoreceptor somatic gene transfer of CRISPR/Cas9 with adeno-associated viral vectors. The generation of in vivo models of STGD1 will provide unique tools for both studying the mechanism of STGD1 rod and cone cell death as well as testing new therapies including gene therapy approaches recently developed by us. **Supervisor:** Prof. Dr. A. Auricchio.

ESR12 Partner 3 (EKUT): CRISPR/Cas9 gene editing for excision and rescue of deep-intronic mutations in ABCA4

Deep-intronic mutations represent a considerable fraction of disease alleles in ABCA4-IRD. Since ABCA4 is difficult to target by AAV-based gene supplementation therapy, alternative therapeutic approaches are needed. Here, we propose CRISPR/Cas9 gene editing to target cryptic splice sites and make use of the default non-homologous end joining (NHEJ)-based repair pathway to delete or excise illegitimate exons or the critical splice donor/acceptor sites to eventually rescue normal splicing of transcripts and re-establish normal gene function. The advantages of such an approach are the loosened need for precision in gene editing, the once-forever intervention strategy and the circumvention of all dose-related/overdosing issues in standard approaches. First proof-of-concept of this strategy has been obtained by P3-EKUT in an in vitro assay of an OPA1 deep-intronic mutation. ESR12 will use this strategy to target two clusters of deep-intronic mutations in intron 30 (V4 cluster) and 36 (V1 cluster) of ABCA4 by a duplex CRISPR/Cas9 nuclease or quadruplex sgRNA/Cas9 nickase inducing double-strand breaks up- and downstream of the deep-intronic mutation and resulting in small deletions due to intrinsic NHEJ repair. Efficacy of the approach and its validation will be tested in minigene assays in HEK293 cells and in retinoblastoma Y79 cell clones in which common deep-intronic mutations will be introduced by CRISPR/Cas9 homology-directed repair. **Supervisor:** Dr. S. Kohl.

ESR13 Partner 7 (TCD): AAV vector delivery targeting common pathways of disease in STGD1

There is growing evidence that many different genetic forms of IRD share common disease mechanisms. Indeed, similar disease processes between ABCA4-associated STGD1 and age related macular degeneration (AMD) have been proposed. Loss of the ABCA4 transporter involves, among other disease mechanisms, a build-up of di-retinoid-pyridinium-ethanolamine (A2E), a vitamin A dimer that becomes trapped in the retinal pigment epithelium (RPE). A2E is a major component of lipofuscin, a hallmark of human STGD1, the Abca4-/- mouse model and AMD. In turn it has been clearly demonstrated that the ATP production capacity of mitochondria in RPE cells is greatly diminished in the presence of A2E. Here, a novel therapeutic strategy for STGD1 is proposed: methods to sustain the mitochondrial function and ATP production capacity of RPE cells in the Abca4-/- mouse model of STGD1 and in cell models of disease generated as part of the planned research program. ESR13 will generate AAV vectors expressing components to augment mitochondrial function and modulate oxygen consumption rates and ATP production. Methods to assess mitochondrial function will be employed and potential beneficial effects of delivery of AAV vectors targeting such common pathways of disease will be evaluated in cell and animal models of STGD1. **Supervisor:** Prof. Dr. J. Farrar.

ESR14 Partner 8 (UCL): Pharmacological protein folding manipulation of ABCA4 mutations

The majority of ABCA4 mutations found in STGD1 are missense mutations. Many of those that have been studied in detail (for example R602W, L541P) lead to protein misfolding, endoplasmic reticulum (ER) retention and protein degradation, leading to loss of function. Missense mutations in the related ABC transporter CFTR are also prone to misfolding and respond well to pharmacological manipulation to improve folding and restore function. P8-UCL has a great deal of experience of manipulating mutant rhodopsin folding, degradation and aggregation both in cells and in vivo. Therefore, we believe that ABCA4 will also be amenable to pharmacological protein folding manipulation. ESR14 will test pharmacological approaches that are effective for CFTR and mutant rhodopsin for their ability to rescue mutant ABCA4 folding, traffic and function. ESR14 will use a combination of heterologous expression of ABCA4 mutants in cell culture and human iPSC derived 3D retinal organoids that express endogenous mutated ABCA4 for these studies to provide direct proof of concept for pharmacological manipulation of ABCA4. **Supervisor:** Prof. Dr. M. Cheetham.