

Progress reports on research supported by ORIA Institute grants 2010

ORIA/RANZCO Grant

Genetic influences in retinopathy of prematurity: improved screening for eye disease in premature babies

Prof KA Williams and Prof DJ Coster

Overview and aim

Retinopathy of prematurity is a blinding condition of some premature infants who require neonatal intensive care and supplemental oxygen therapy. Severe retinal disease affects approximately 5% of these neonates and despite best available care, 10–15% will suffer significant visual impairment. There is increasing evidence that genetic factors, as yet unknown, determine susceptibility to human retinopathy of prematurity. In this work, we are investigating the molecular basis for our discovery that different strains of inbred rat exhibit differential susceptibility to oxygen-induced retinopathy, a robust animal model of human disease. Our *specific aim* is to quantify mRNAs, proteins and microRNAs expressed in the retinae of three inbred rat strains showing extremes of phenotypic susceptibility, during neonatal hyperoxic exposure. The *hypothesis* is that some will be differentially expressed in different strains in response to cyclic hyperoxia, compared with normoxia.

Background

Retinopathy of prematurity is a major cause of childhood blindness. However, not every premature infant develops retinopathy of prematurity, and a genetic propensity is highly likely to influence susceptibility to disease. Our long-term goal is to identify heritable traits that influence the development and progression of human ROP, so that infants who are at risk may be identified and appropriately treated, whereas infants who are not at risk may be spared unnecessary interventions. Our approach is predicated on the likelihood that identification of susceptibility traits in the animal model will provide strong clues to similar factors operating in humans. Furthermore, identification of those networks of genes that are differentially expressed at different time-points during development of ROP will permit targeted intervention at the most appropriate stage of human retinal development. In summary, we hope to illuminate the pathogenesis of this devastating condition, enable identification of infants at high risk of developing blinding retinopathy, streamline screening prior to disease progression, and identify new therapeutic targets for early intervention.

Progress to date

mRNA arrays. We have performed a screen of retinal mRNAs from rat strains that are either resistant or sensitive to oxygen-induced retinopathy, using Affymetrix arrays covering over 27,000 genes. As anticipated, false discovery rate analysis returned relatively few significant differences after adjusting for multiple comparisons. Genes that differed were ranked by fold change in expression, and submitted to the online database “DAVID”, to identify enriched gene groups. More genes regulated by HIF-1 α , or that were part of the oxygen-sensing pathway, were present in the susceptible than the resistant strain. Independent confirmation (real-time qRT-PCR or Western blot) showed that *Egln3* (encodes prolyl hydroxylase-3), *Nasp* (histone chaperone), *Slc16a3* (pyruvate and lactate export), hexokinase 2 (glycolysis) and *Bnip3* (Bcl-2 family, apoptosis), all of which are oxygen-regulated, were differentially expressed.

Proteomics. Using two-dimensional fluorescence difference in gel electrophoresis (2DIGE) and mass spectrometry, we examined the retinal proteomes of different rat strains during hyperoxic exposure, and identified 40 proteins (pI range 3-7) with altered levels of expression following hyperoxic exposure. The

experiment is being replicated for proteins with a pI in the range 7-11. Potentially relevant candidates are being independently confirmed (real-time qRT-PCR or Western blot) and pathway analysis will follow. One confirmed candidate is a component of the multi-subunit signalosome that aids in targeting proteins such as HIF-1 α for destruction through the 26S proteasome pathway.

MicroRNAs. MicroRNAs are short non-coding sequences of RNA, 21-22 nucleotides in length, that regulate gene expression at a post-transcriptional level. Binding of microRNAs to their target mRNAs results in mRNA degradation or inhibition of protein synthesis. A single microRNA can potentially regulate hundreds of genes, including those involved in disease pathogenesis. MicroRNAs show polymorphisms associated with disease susceptibility in humans as well as in binding sites in the 3' untranslated region of specific target mRNAs. The majority of cloned microRNAs are highly conserved across species and have been identified in many tissues including the eye. MicroRNAs play a role in development, angiogenesis, and responses to hypoxia and hyperoxia. We have now examined microRNA expression in the induction phase of oxygen-induced retinopathy in sensitive and resistant rat strains using Exiqon arrays that represent 1,500 microRNAs. MicroRNAs were identified as candidates if they targeted oxygen-related genes, were regulated by both oxygen and strain, or had significant adjusted p values ($p < 0.05$) after correction for multiple comparisons.

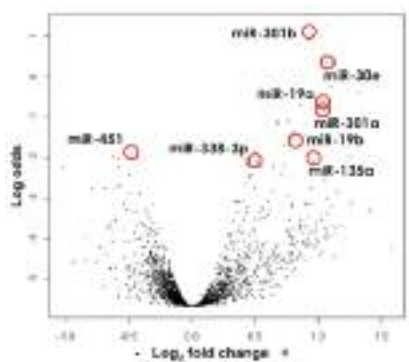


Figure. Representative volcano plot: Bayesian log odds of retinal miRNA expression versus log₂ fold change at day P5. Candidate miRs that exhibit differential expression between Fischer 344 and Sprague-Dawley rats by both strain and hyperoxic exposure are circled.

We have identified 12 microRNAs that are differentially expressed in inbred Fischer 344 and Sprague-Dawley rats exposed to hyperoxia or room air, lending credence to our hypothesis that some of the phenotypic differences in susceptibility to OIR amongst inbred rat strains involve post-transcriptional change. Several of the microRNAs are predicted to target elements of the oxygen sensing pathway, and confirmation of these interesting targets is progressing. We acknowledge the essential contributions of Ms Melinda Tea, Mr Rhys Fogarty and Dr Alex Colella to this work.

Bronchopulmonary dysplasia. The use of sustained supplemental oxygen in premature infants is associated with bronchopulmonary dysplasia, as well as ROP. To establish if hyperoxic exposure sufficient to induce florid retinopathy would induce strain-specific lung changes, lung function was assessed. No differences in arterial blood gases or protein concentration of bronchoalveolar lavage fluid were observed amongst different strains. Pulmonary vascularity increased in all oxygen-exposed animals compared with controls, but there was no difference between strains. The minor morphological

differences found in the lung did not affect pulmonary function, suggesting that mechanisms inducing eye disease and bronchopulmonary dysplasia are fundamentally different.

Publications arising from ORIA support for this project

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- Klebe S, van Wijngaarden P, Melville T, Lipsett J, De Smet H, Coster DJ, Williams KA. Exposure to cyclic oxygen sufficient for development of oxygen-induced retinopathy does not induce bronchopulmonary dysplasia in rats. *Exp Lung Res* 2010; 36: 175-82.

Publications arising from ORIA support for projects in the recent past

- Tea M, Fogarty R, Brereton HM, Michael MZ, Van der Hoek MB, Tsykin A, Coster DJ, Williams KA. Gene expression microarray analysis of early oxygen-induced retinopathy in the rat. *J Ocul Biol Dis Infor* 2009; 2: 190-201.

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ORIA Young Investigator/RANZCO Eye Foundation Grant

The effect of hyperglycaemia on experimental glaucoma

Prof R Casson and Prof P Blumbergs

Aims

Glaucoma refers to a family of optic neuropathies with multi-factorial aetiology. Lowering the intraocular pressure is currently the only evidence-based treatment approach for glaucomatous optic neuropathy. However, this does not stop progression in all patients and new treatment strategies, referred to as neuroprotective, are needed. The pathogenesis of glaucoma remains unclear, but there is good evidence that the optic nerve head (ONH) is involved early in the pathogenesis of the disease. Inadequate blood supply to the ONH may play a role, at least in some types of glaucoma. Given that vasculopathy is a hallmark of diabetes, one would expect that diabetes might exacerbate glaucoma; however, in large epidemiological studies no clear association was found. The Ocular Hypertension Treatment Study (OHTS) even suggested that diabetes protected against the conversion of ocular hypertension to glaucoma. In this project we investigated the effect of short-term hyperglycaemia on retinal ganglion cell (RGC) death and optic nerve damage in an experimental rat model of chronic ocular hypertension, which consisted of laser photocoagulation of the trabecular meshwork.

Work completed

We started the project by establishing, characterising and validating a commonly used rat model of experimental glaucoma. An ideal model should produce focal injury to RGC axons at the ONH and result in death of groups of RGC in sectors in the retina. Argon laser photocoagulation models have been used in mice and rats for a number of years. In general, the results from these methods have been consistent and the models are considered to be reliable and efficient. We were able to successfully reproduce the model described by Levkovitch-Verbin (IOVS 2002) and obtained similar intraocular pressure (IOP) profiles. We thoroughly described and characterised this model in our laboratory.

Next, we invested significant time and effort in finding and standardising outcome measurements for the quantification of optic nerve damage. Manual axon counting on transmission electron microscopy is considered the gold standard to measure structural axonal damage. However, this method is extremely time consuming and not practical to assess larger numbers of samples. We therefore adopted a protocol for semi-automated counting using light microscopy photographs of semi-thin resin cross sections. In addition, we have made use of immunohistochemical stains to set up quantification tools to analyse longitudinal paraffin processed sections of optic nerve tissue.

The final aim of this project was to investigate the influence of hyperglycaemia on experimental glaucomatous optic neuropathy. This was achieved by analysing and comparing tissue samples of two groups of experimental animals, comprising over 50 rats in total

Results

1. The optic nerve head is the site of axonal transport failure

In contrast to primates, rodents only possess a rudimentary lamina cribrosa, which extends via the neck to the transition zone of the optic nerve (ON), while mice lack any connective tissue whatsoever. As such, it is

important to ascertain whether the ONH is an important site of axonal transport failure in rodents. We achieved this aim by immunolabelling for endogenous proteins, including amyloid precursor protein (APP), synaptophysin and brain-derived neurotrophic factor (BDNF), that are routinely synthesised by RGCs and transported in an orthograde fashion. Previous reports by other groups generally used tracers injected into the vitreous cavity. An advantage of our approach is that uptake and incorporation of labelled tracer into the RGC body is circumvented. Moreover, the molecules analysed are of high, medium and low molecular weight, respectively, and have quite distinct physiological roles. Our results showed accumulation of all three proteins within axons throughout the ONH, but not distal to this location in the myelinated ON or optic tract. We found axonal cytoskeletal abnormalities, such as neurofilament beading and swellings, in the ONH at 24h after induction of raised IOP with a spatial pattern that overlapped with APP accumulation. This suggests that axonal transport disruption is mechanical, and not simply functional, in a subset of axons at very early time points. Nevertheless, in other axons, it is likely that active axonal transport dysfunction significantly preceded physical damage.

In summary, our results support the findings of others that IOP elevations of the magnitude recorded elicit an early insult at the lamina of the ONH with Wallerian-like degeneration of axons distal to the site of injury.

2. Signs of restricted axonal regeneration can be found in this experimental rat model of glaucoma

Within the central nervous system (CNS), endogenous regenerative attempts are always unsuccessful. In the visual system, for example, RGC axons display only transient, local sprouting, proximal to the lesion site after ON crush. However, unlike the catastrophic injury caused by traumatic axonopathies such as ON crush, RGCs are lost gradually during experimental glaucoma. We hypothesised that the inhibitory environment for regeneration may be less pronounced and that regeneration strategies more effective. To date, no data was available on the endogenous regenerative response of RGCs in models of glaucoma. And in fact, we observed numerous Gap43-positive axons at 7d and notably 14d after injury in the nerve fibre layer at the prelaminar ONH and continuing into the ON. On the whole, the Gap43 response was restricted to the neck and transition region of the ONH, but scattered Gap43 immunopositive punctae could be observed beyond the zone of myelination.

3. Microglial activation in the visual pathway correlates with axonal injury

We have performed an extensive characterization of the microglial response in our rat model of experimental ocular hypertension. Microglia play a central role in a number of chronic neurodegenerative conditions of the CNS, including among others Alzheimer's disease, Parkinson's disease and multiple sclerosis. In the CNS, markers of microglia have been successfully used to measure the severity of brain damage. Microglial activation occurred along the entire optic pathway and correlated closely with axonal damage. Microglia unregulated the expression of immunologic cell surface markers, including complement receptor type 3, major histocompatibility complex (MHC) class I, and MHC class II, but the expression of MHC class II was limited to cells within the white matter. Despite the increased expression of molecules associated with antigen presentation, only minor T lymphocyte infiltration was observed. Overall results advocate microglial activation as a useful adjunct quantitative tool for assessment of the status of ON damage.

4. Short-term hyperglycaemia is neuroprotective in experimental glaucoma

Experimental glaucomatous optic neuropathy was induced in a group of normoglycaemic (n = 26) and a group of hyperglycaemic (n = 26) Sprague-Dawley rats. After two weeks of elevated intraocular pressure, rats were killed by transcardial perfusion. Axonal loss was graded semi-quantitatively on transverse sections of the ON. Longitudinal sections were immunohistochemically stained for glial markers. The degree of axonal loss was significantly lower in the hyperglycaemic group of rats compared to the normoglycaemic group. Axonal damage and RGC loss was reduced by about 50%. Possible explanations for this effect are the attenuation of malperfusion induced energy failure by increasing the substrate for glycolysis or a shift in metabolism resulting in reduction of reactive oxygen species induced apoptosis. Further studies are warranted to elucidate the mechanism of neuroprotection.

References

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ORIA/R & L Lowe Grant

A transgenic model for selective ablation of Müller cells

Prof M C Gillies

Spanning the breadth of the retina from the inner to the outer limiting membranes, the Müller cell is a specialised glial cell that serves numerous functions essential to retinal homeostasis. Through the extensive arborisation of their processes, Müller cells constitute an anatomic and functional link between retinal neurons and blood vessels. They play a central role in retinal glucose metabolism and the formation and maintenance of the blood–retinal barrier (BRB), which makes Müller cells both a target and a potential key player in retinal vascular diseases such as diabetic retinopathy (DR). To date, vascular abnormalities have been the primary target for treatment of DR. Less is known about the specific role of Müller glial dysfunction in retinal vascular diseases. A causal link between Müller cell dysfunction and BRB breakdown has not been established. We hypothesise that Müller cell dysfunction is a major contributor to the breakdown of the BRB that is a hallmark of retinal vascular diseases. This study aimed to generate a transgenic model for selective and cell specific disruption of Muller cells in the retina, thus allowing us to study the interactions of Müller glial dysfunction with retinal neuronal damage and vascular abnormalities.

1. Establishment of an inducible model for Müller cell-specific gene targeting

We have generated a DNA construct containing a Müller cell-specific promoter using a Cre/Lox-P gene expression system (Fig. 1). The cell-specific promoter contains a 3-kb fragment of regulatory region of CRALBP gene (Rlbp1) which has been shown to drive robust Müller cell-specific gene expression in the retina¹.

We have produced two transgenic lines (Rlbp1-CreRE mice) and both were crossed with Rosa-LacZ



Fig.1: DNA construct used for generation of transgenic mice specifically targeting Müller cells.

reporter mice for LacZ expression after tamoxifen (TMX) induction. LacZ expression in Rlbp1-lacZ mice was specifically located to Müller cells and the levels of LacZ expression were likely controlled by doses of TMX induction (Fig. 2).

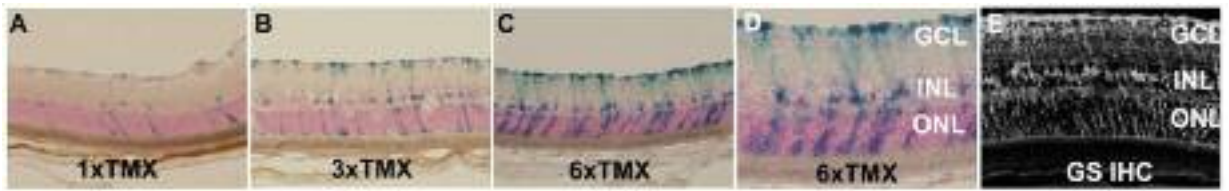


Fig. 2: LacZ expression is confined to cells spanning the full thickness of the retina (A-D), consistent with the typical morphology of Müller cells (E) at four days (4d) after TMX induction.

2. Selective disruption of Müller cells in *Rbp1-DTA* mice leads to PR injury

We have further crossed *Rbp1-CreER* mice with *Rosa-DTA176* mice^{2,3} to study the effects of selective ablation of Müller cells on retinal neurons and vasculature. DTA is a cytotoxic protein that inhibits cellular protein synthesis by inactivating elongation factor 2⁴. The toxin binds to a specific receptor on the cell surface then is internalised by receptor-mediated endocytosis, resulting in translocation of the enzymatically active A fragment to the cytosol where it inactivates elongation factor 2⁴. *Rosa-DTA176* transgenic mice carry an attenuated form of DTA (*DTA176*)³. Because mouse cells have no receptor for diphtheria toxin [4], leak or Cre-independent expression of *DTA176* is unlikely to result in bystander cell lethality even upon possible release of active *DTA176* molecules from dead Müller cells^{2,3}. However, following Cre recombination after TMX induction, more than enough *DTA176* is produced within Müller cells for desired cell killing, therefore, specific Müller cell disruption is ensured^{2,3}.

Our preliminary results show that TMX induction led to selective ablation of Müller cells (Fig. 3F) alternating with areas of glial activation (Fig. 3G-H, green) and PR injury (asterisks in Fig. 3H-I, arrows in Fig. 3J) in *Rbp1-DTA176* TG mice but not in WT and *Rbp1-LacZ* mice. Electron microscopy (EM) showed disrupted PRs with broken OLM (Fig. 3K, arrows). Deep retinal vascular capillaries were dilated but not beyond the OPL 10d after TMX induction (Fig. 3I, arrow). Analysis of dynamic cell apoptosis in the retina indicates that PR apoptosis was likely initiated by cell death in the INL 1d after TMX induction, which was consistent with the location of Müller cell bodies as revealed by TUNEL staining and immunohistochemistry (IHC) for GS (Fig. 3M-N, arrows). PR apoptosis in the ONL was predominantly observed between 3d and 7d but hardly observed at 7.5 months (7.5m) after TMX induction (O-Q).

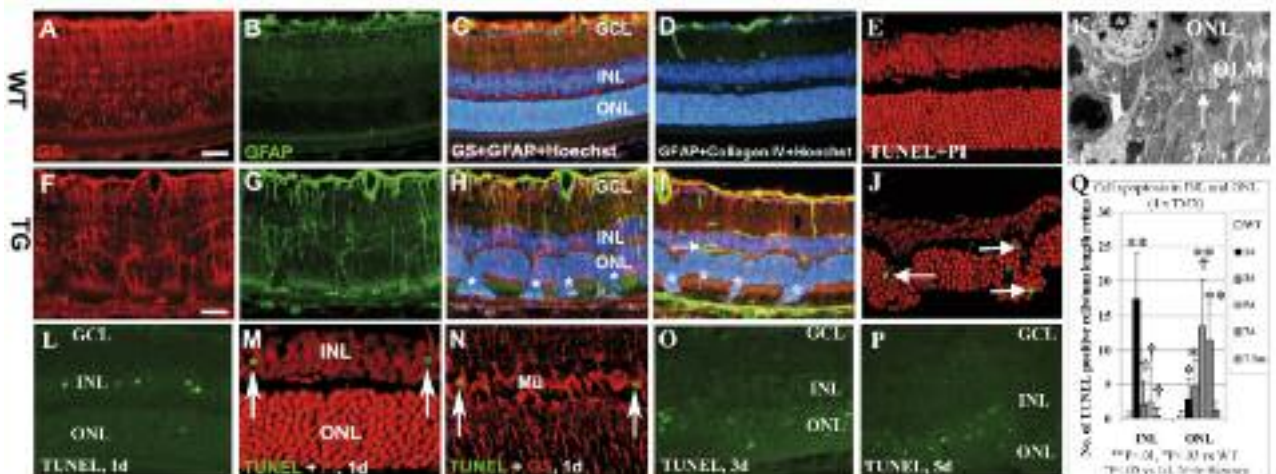


Fig. 3. Changes in Müller cells and PRs after 5 x TMX induction. A-K, 10d after TMX induction.

Electroretinography (ERG) showed decreases in a- and b-waveforms in TG mice (Fig. 4A-D). Using a twin flash paradigm and digital subtraction, we were able to isolate cone derived waveform from the rod response. Both rod and cone PRs were affected in this transgenic model (Fig. 4E).

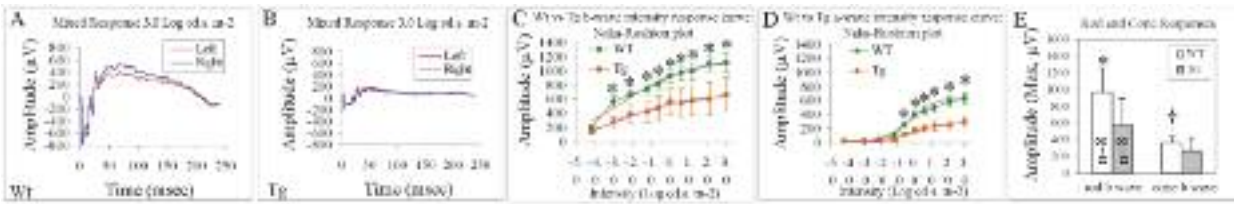


Fig. 4. ERG changes 2m after 5 x TMX. *P<0.01, †P<0.05; TG vs, WT, n=8/group, respectively.

3. Selective Müller glial disruption leads to BRB breakdown and retinal vascular abnormalities

We have developed key techniques to detect retinal vascular changes after Müller glial disruption (Fig.5). Fundus fluorescein angiography (FFA) shows that Rlbp1-DTA176 mice develop vascular leak as early as 10d after TMX induction which seems to be dose-dependent and progresses with time (Fig. 5B-E), indicating that primary disruption of Müller cells can lead to retinal vascular abnormalities. Vascular telangiectasis (Fig. 5G, arrows), altered expression of tight junction protein claudin-5 (Fig. 5K) and the development of deep retinal neovascularisation (Fig. 5I-J, and M-N, arrows) were observed in TG but not WT mice. Retinal vascular changes were closely associated with PR injury and the activation of retinal microglia (resident macrophages) as revealed by flatmount staining isolectin B4 (LB4) conjugated with Alexa Flour 594 in combination with FITC-peanut agglutinin (FITC-PNA) for PRs (Fig. 5L-N) and F4/80 for microglia (Fig. 5O, arrows).

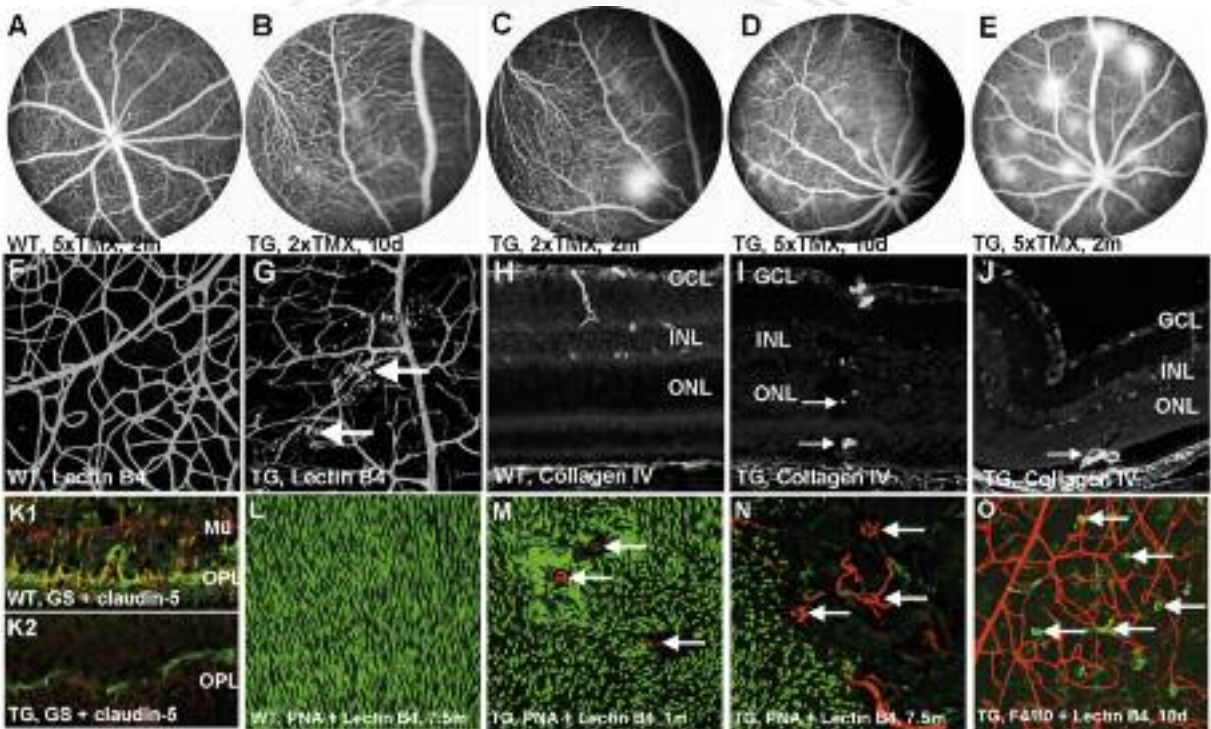


Fig. 5. Retinal vascular changes after TMX induction. F-K, 2m after 5 doses of TMX induction.

In summary, we have successfully produced an inducible transgenic model which develops dynamic changes in retinal neuronal damage, BRB breakdown and deep retinal neovascularisation. This model will be a useful tool for testing strategies on neuroprotection and anti-angiogenesis therapies. It will also be useful for studying the interactions of Müller glial dysfunction with retinal neuronal injury and vascular abnormalities.

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Publications and presentations

1. Preliminary data obtained from this study have been used for a NHMRC project grant application in 2011 (2012-2014).
2. Shen WY, Fruttiger M, Chung SH, Zhu L, Barnett N, Nguyen A, Coorey N, Murray Killingsworth M and Gillies MC. Selective ablation of Müller cells leads to photoreceptor injury, blood-retinal barrier breakdown and deep retinal neovascularization in a transgenic model. The Association for Research in Vision and Ophthalmology, Fort Lauderdale, USA, 2011 (Poster).
3. Shen WY and Gillies MC. Characterisation of a transgenic model of selective ablation of Müller cells in the retina. Oral presentation in 2011 APAO meeting, Sydney.

ORIA New Investigator Grant

Study of treatment for Retinal Vein Occlusion with Bevacizumab

Dr J S Gilhotra and Dr M Dhanapala

Retinal vein occlusion represents an important cause of vision impairment from retinal vascular diseases after diabetic retinopathy. Ischemic or hypoxic conditions of the inner retina have been associated with capillary leak, macular oedema, and neovascularisation. Neovascularisation, which can lead to secondary glaucoma and vitreous haemorrhage, is a major complication associated with central retinal vein occlusion. The standard of care so far was based on laser photocoagulation of the retina: Macular treatment for oedema from branch retinal vein occlusion, and panretinal laser photocoagulation for central retinal vein occlusion with evidence of neovascularisations. The corresponding landmark studies, the Branch Retinal Vein Occlusion Study (BVOS) and the Central Retinal Vein Occlusion Study (CVOS) were published almost three and two decades ago, respectively, and have guided evidence-based treatment since. However, these treatment patterns are now being challenged by the advent of anti-Vascular Endothelial Growth Factors (anti-VEGFs), antibodies and their derivatives which are applied intravitreally and neutralize all isoforms of VEGF-A and their biologically active degradation products. Anti-VEGF therapy with ranibizumab, a humanized, affinity-matured, anti-VEGF antibody fragment, has become the standard of care for neovascular age-related macular degeneration. Intravitreal off-label use of bevacizumab, a monoclonal antibody initially approved for systemic use in oncology, has become popular in the treatment of diabetic macular oedema. VEGF appears to be an important factor in the pathogenesis of the sequelae of retinal vein occlusive disease, which makes it an interesting target for pharmacotherapeutic intervention in this area of retinology.

We set up two single centre, randomised controlled clinical trials to compare the use of intravitreal bevacizumab versus the standard of care based on argon laser photocoagulation.

The first trial enrolls patients with BRVOs of at least three months duration with visual acuities worse or equal than 6/12. Subjects get randomised to three monthly intravitreal anti-VEGF treatments followed by monthly ‘as needed’ treatment, or standard macular laser photocoagulation following the BVOS guidelines. The length of the study is 12 months. The treatment criteria during the PRN phase are largely based on monthly optical coherence tomography (OCT). The main outcomes are visual acuity, central retinal thickness, as well as disease-related complications and treatment-associated adverse events.

Aiming for a total of 30 participants, 13 subjects have so far been randomised, of whom 6 have already completed the trial and 7 are currently being treated according to the study protocol. Table 1 shows the baseline characteristics of the enrolled participants. Table 2 summarises the interim results for the 6 subjects who have completed the trial. Both treatments performed similarly and the current data do not reveal statistically significant differences between the treatment modalities. On average, 6.7 intravitreal injections of Avastin were required. Patients in the laser arm had a mean of 3.3 laser treatments. No major safety issues have so far been identified and visual and morphological outcomes of the two approaches are comparable. However, statistical analysis is not possible at this point in time because of the low number of participants who have completed the trial to date.

Table 1: Baseline characteristics of study population for the BRVO part of the study.

		Laser treatment (n=6)	Avastin (n=7)
Age [years]	Mean	70.8	66.1
	Median	72	69
	Range	56-80	48-82
Gender	Female	3	6
	Male	3	1
Best Corrected Visual Acuity [letters ETDRS]	Mean	36.8	52.4
	Median	37	56
	Range	3-68	33-63
Central Retinal Thickness [µm]	Mean	457	492
	Median	340	530
	Range	186-908	207-756
Thickest Retinal Quadrant [µm]	Mean	564	551
	Median	443	547
	Range	338-1024	339-877

The second trial looks at patients with central retinal vein occlusion of any age with visual acuities worse or equal than 6/12. Participants get randomised to monthly intravitreal bevacizumab injections or observation following the CVOS guidelines, with panretinal laser photocoagulation if clinical evidence for new vessels becomes apparent. Likewise, the length of the study is 12 months. The outcomes are visual acuity, central retinal thickness on OCT, neovascular complications and drug related adverse effects.

Aiming for a total of 22 participants, six patients have so far been randomised. Two subjects have completed the study protocol whilst another person is still in the trial. Of note, three patients have dropped out of the trial owing to investigators clinical judgement and lack of patient compliance.

Recruitment of patients has been taking longer than initially estimated. This has been for several reasons: (1) Few referrals from practitioners outside the public system; (2) Strict study design with narrow visual acuity inclusion criteria; and (3) Patients’ preferences for one or the other treatment and refusal to random allocation of treatment. Nevertheless, we are aiming to complete the enrolment process within the coming 12 months. It will then take at least another year for all participants to complete the study protocol. The final results of the trials are to be expected in about three years.

Table 2: Interim results for the BRVO part of the study.

		Laser treatment (n=3)	Avastin (n=3)
Final Best Corrected	Mean	49.0	64.7
Visual Acuity	Median	61	72
[letters ETDRS]	Range	8-78	47-75
Letters gained	Mean	13.3	13.3
[letters ETDRS]	Median	10	14
	Range	5-25	9-17
Final Central Retinal	Mean	259	287
Thickness [μm]	Median	209	332
	Range	143-425	190-338
Change in Central	Mean	215	84
Retinal Thickness	Median	120	17
[μm]	Range	43-483	-39-275
Final height thickest	Mean	286	306
sub-quadrant [μm]	Median	265	301
	Range	248-344	289-327
Change of	Mean	289	80
sub-quadrant	Median	99	61
thickness [μm]	Range	87-680	-71-251

Very recently, the 12 month results for the BRAVO trial (Ranibizumab for the Treatment of Macular Edema following BRANCH Retinal Vein Occlusion) have been published¹. Patients in the treatment arm received monthly injections for the first six months, followed by six months of observation with treatment ‘as-needed’ on a monthly basis. On average, patients received about 8.5 injections of ranibizumab. The average letter gain in the BRAVO trial was 16.4 for the 0.3mg and 18.3 for the 0.5mg group, respectively. Importantly, mean improvement in the sham group was 12.1 letters. In summary, the letter gain from bevacizumab in the current interim evaluation is inferior to the BRAVO results. This may be due to differences in efficacy between agents, lower treatment frequency and a less stringent injection regimen in our study, or other, not yet identified factors. On the other hand, to longer mean interval between treatments could be a manifestation of the longer half life of bevacizumab compared to ranibizumab. Either way, it will be instructive to compare the final result from the current series to the larger multi centre studies and to determine whether our treatment protocol results in statistical superiority to the natural history at all.

The very limited number of patients who have completed the trial to date does not allow us to draw the final conclusions just yet. However, importantly, we intend to continue till the completion of the trial.

Publication

1. Brown DM, Campochiaro PA, Bhisitkul RB, Ho AC, Gray S, Saroj N, Adamis AP, Rubio RG, Murahashi WY. Sustained Benefits from Ranibizumab for Macular Edema Following Branch Retinal Vein Occlusion: 12-Month Outcomes of a Phase III Study. *Ophthalmology*. 2011 Jun 17. [Epub ahead of print] PubMed PMID: 21684606.

ORIA Grant

Do patients with Vogt-Koyanagi-Harada disease (VKHD) develop specific immune responses against uveal melanocytes?

Prof P McCluskey, Dr M Madigan, Dr R M Conway and Prof N Rau

Vogt-Koyanagi-Harada Disease (VKHD) affects the eye, skin, hearing and the central nervous system. However, the most damaging aspect of VKHD is severe ocular inflammation (uveitis) associated with profound potentially permanent loss of vision. There is good evidence that melanin-containing cells (melanocytes) are targets for VKHD, but little evidence that the ocular pigment cells are specifically targeted in this disease. We are investigating humoral and cell-mediated immune responses directed towards uveal melanocytes and melanocyte proteins in patients with VKHD, using sera, and peripheral blood immune cells, from patients with active and inactive (treated) VKHD patients.

We are exploring the hypothesis that human choroidal melanocytes are a target for humoral immune-mediated damage in patients with VKHD by studying the following questions:

1. Does sera from VKHD patients specifically kill normal cultured human choroidal melanocytes?
2. Does sera from VKHD patients show antibodies against normal human choroidal melanocytes, specifically, towards the melanosome-specific antigens (tyrosinase, tyrosinase-related peptide 1, MART-1 and gp100)?

Serum was collected from patients with VKHD, sympathetic ophthalmia and healthy age-matched controls. Human donor eyes were obtained from the Lions NSW Eye Bank. We established that human choroidal melanocytes can be successfully isolated and grown in culture. We characterised the expression of the melanosome-specific proteins, tyrosinase, TYRP1, MART-1 and gp100, which are all expressed by cultured human choroidal melanocytes and human choroidal melanocytes in-situ. Tyrosinase, MART-1 and gp100 proteins were also identified in the protein lysates of cultured melanocytes.

Cultured choroidal melanocytes and frozen sections of normal human choroid were used in the following experiments:

1. Cultured cells were incubated in subject sera, examined for changes in morphology and cell growth (MTT assays).
2. Indirect immunofluorescence and Western Blotting were used to examine for the expression of melanosome-specific proteins (tyrosinase, tyrosinase-related peptide, MART-1 and gp100).
3. Indirect immunofluorescence and Western Blotting were used to examine for the presence of antibodies in the various patient groups' sera.

Results

Antibody binding against cultured human choroidal melanocytes was non-specific for control and patient sera. We did not find evidence of complement-dependent cytotoxicity against choroidal melanocytes in VKHD or control sera. Antibodies against melanocytes could not be demonstrated using Western blotting or in choroid sections. We did find a novel 55kDa protein in sera from one VKHD patient that may be a potential target in VKHD. We are continuing to explore this novel protein.

Conclusion

The humoral immune system may play a role in mediating choroidal melanocyte damage involved in the pathogenesis of VKHD, but this is not due to the presence of antibodies against tyrosinase, TYRP1, MART-1 and gp100.

Increased numbers of VKHD and control patients will be studied to confirm these findings, and to investigate the role of cell-mediated immunity in the next phase of the project.

ORIA Grant

Development of antimicrobial sutureless technology for eye surgery

Dr S Watson, A/Prof L J R Foster and Dr M Sarris

We have successfully adapted our laser-activated surgical adhesive, 'SurgiLux', to incorporate a series of common antibiotics at various concentrations including: (a) amphotericin B, (b) chlorhexidine, (c) ofloxacin, (d) tobramycin and (e) vancomycin. In all cases the physiochemical and material properties have been investigated.

Salts of these antibiotics appear to act as nucleating agents promoting the material strength of the chitosan-based thin films. The SurgiLux-antibiotic composites showed negligible change in their tissue adhesion strengths which remained around 16 ± 2 kPa. SurgiLux films containing these antimicrobial agents adsorbed significantly higher volumes of water than the SurgiLux alone which increased by 40%. In contrast, amphotericin B and chlorhexidine adsorbed the largest volume of water, showing a 140–160% weight increase. Water content is likely to influence tissue adhesion *in vivo* as well as degradation of the bioadhesive.

While chitosan and SurgiLux films showed no inhibition against the common microbial species *Escherichia coli*, *Staphylococcus aureus* and *S. epidermidis*. In contrast, a composite of chitosan adhesive and the antibiotic vancomycin showed therapeutically significant release profiles greater than the Minimum Bactericidal Concentrations (MBCs) for the Staphylococci over a 28 day period. These composite films had greater crystallinity; up to 28 ± 3 compared to 8.9 ± 2 % for its unblended counterpart. Despite a significant increase in material strength from 31.4 ± 4 to 77.5 ± 5 MPa, flexibility was still maintained with an elongation to break around 5 ± 2 % and fold endurance of approximately 30 ± 3 folds. Laser irradiation had no apparent effect on the release or activity of the antibiotic which survived transient temperatures at the film-tissue interface during infra-red irradiation of around 54 °C. There was no significant change in burst pressure using *ex-vivo* bovine eyes.

Animal trials of this modified adhesive have been delayed due to a faulty laser fibre which has now been replaced. We anticipate a preliminary test using 2 rats to begin at the end of this month with the full trial following at the beginning of August.

Acknowledgement

Acknowledgement to this ORIA funding was made in the following journal publication:

1. Foster, LJR; Thomson, K; Marcal, H; Butt, J; Watson, S. & Wakefield, D. 2010 'A Chitosan-vancomycin Composite Biomaterial as a Laser Activated Surgical Adhesive with Regional Antimicrobial Activity'. *Biomacromolecules* 11(12):3563-3570.

NB: Animal ethics for the grant was obtained on the 26th May 2010, however funds were not made available until the 22nd November 2011. Consequently this constitutes a progress report rather than the final report.

ORIA Grant

Identifying genes for keratoconus from a genome wide association study

Dr K P Burdon and Dr R A Mills

This project aimed to validate and replicate findings from a genome-wide association study (GWAS) conducted on pooled DNA from keratoconus patients and unaffected controls. This was successfully achieved with the subsequent identification of the *HGF* gene in keratoconus. This gene is an excellent candidate. It is involved in cellular differentiation and migration and has also been associated with refractive error.

The top ranked SNPs from the GWAS were genotyped in individual DNA samples from all 100 cases and 216 controls that were included in the GWAS DNA pools. SNPs were considered to be validated if they

reached a nominal p-value of 9.0×10^{-3} or better. Although not of genome-wide significance, this validation threshold was chosen to allow a manageable number of candidate genes for further analysis. Of the 52 SNPs typed in individual samples, 19 reached this validation threshold, representing 13 independent loci. Each validated SNP was then assessed in the Rep1 cohort consisting of an additional 96 cases and 72 controls recruited since the GWAS was conducted. The only SNP showing any level of replication in this small cohort was rs1014091, located upstream of the *HGF* gene.

Genetic variation in the *HGF* gene was then investigated further as a candidate gene. Additional tag SNPs in the region of the gene (rs12536657, rs2286194, rs3735520 and rs17501108) were genotyped in all cases and controls (pooled and Rep1 samples) along with the SNPs from the array (rs7799610 and rs1014091). Tag SNP rs17501108 is in complete linkage disequilibrium ($r^2=1.0$) with rs1014091 typed on the array. Both these SNPs were associated with keratoconus (Table 1) and survive Bonferonni correction for the 6 SNPs analysed in this candidate gene study ($p=0.0004$ for rs1014091 and $p=0.0002$ for rs17501108). In addition, rs3735520 was also associated ($p=0.002$).

Table 1: Association results for tag SNPs in the *HGF* gene in the pooled plus Rep1 samples combined. The minor allele and its frequencies in cases and controls are shown. The Odds Ratio (OR) with 95% confidence intervals (95%CI) is expressed with respect to the risk allele. MAF = minor allele frequency. Significant p-values are highlighted bold.

SNP	Alleles Maj/Min	MAF controls (n=287)	MAF cases (n=196)	P-value	OR (95% CI)
rs7799610	G/A	0.078	0.072	0.74	1.08 (0.66-1.78)
rs12536657	G/A	0.227	0.195	0.24	1.21 (0.88-1.68)
rs2286194	T/A	0.167	0.204	0.15	1.28 (0.92-1.78)
rs3735520	C/T	0.413	0.513	0.002	1.50 (1.15-1.94)
rs17501108	G/T	0.144	0.067	0.0002	2.33 (1.17-3.69)
rs1014091	G/A	0.147	0.072	0.0004	2.22 (1.41-3.48)

To further investigate this result, rs3735520 and rs17501108 were typed in a second larger replication cohort (Rep2) consisting of 215 cases. These cases were compared for these two SNPs to publicly available data from 112 unrelated Caucasian individuals typed as part of the International HapMap Project for these two SNPs. Positive association of SNP rs17501108 was replicated in this independent cohort of Caucasian individuals (Table 2). To gain further confidence in these results, we sought collaboration with a large group from the US undertaking similar studies. Their work also showed association of SNPs near HGF with keratoconus in their first cohort US1 (Table 2). No significant association ($p<0.05$) was observed in the US2 replication cohort, however the odds ratios are in the same direction with that observed in the other cohorts.

Meta-analysis of *HGF* SNPs in all cohorts suggested that SNP rs3735520 is associated with keratoconus with genome-wide suggestive p-value of 9.9×10^{-7} . A less significant p-value of 9.9×10^{-5} was calculated for SNP rs17501108/rs1014091.

Table 2: Results of association testing of HGF SNPs in multiple cohorts. The minor allele frequency in cases and controls are shown. The Odds Ratio (OR) with 95% confidence intervals (95%CI) are also shown, expressed with respect to the risk allele defined in the pooled samples. MAF = minor allele frequency.

Cohort	rs3735520				rs17501108/rs1014091*			
	MAF controls	MAF cases	p-value	OR (95%CI)	MAF controls	MAF cases	p-value	OR (95%CI)
Rep2	0.43	0.45	0.664	1.08(0.76-1.51)	0.17	0.10	0.006	1.93(1.20-3.10)
US1	0.45	0.57	6.1×10^{-7}	1.63 (1.35-1.98)	0.11	0.07	0.018	1.56(1.07-2.24)
US2	0.46	0.47	0.655	1.05 (0.86-1.28)	0.11	0.10	0.658	1.08(0.77-1.50)

* As rs17501108 and rs1014091 are in almost complete LD, the results for rs17501108 are presented for Rep2 and rs1014091 for US1 and US2 due to different genotyping strategies in the two cohorts.

To assess the effect of SNP rs3735520 on HGF levels, serum HGF concentration meeting quality control standards was obtained from 84 control participants (serum was not available from keratoconus patients). Under a non-parametric Kruskal-Wallis test there was a significant trend for increased serum HGF concentration with each T allele of the SNP (Table 4, $p=0.036$). The minor T allele is also the risk allele for keratoconus. This association was also significant under both dominant and recessive genetic models. A similar relationship was found with the risk G allele of rs17501108 although this did not reach statistical significance (data not shown).

Table 3: Mean serum HGF concentration by genotype of rs3735520 and mean rank for nonparametric tests under genotypic, dominant and recessive models. SE=standard error of the mean

Genotype	N	Mean serum HGF ± SE	Mean rank by genotype ¹	Mean rank dominant ²	Mean rank recessive ³
CC	32	486.8±39.0	33.35	33.35)	37.66
TC	39	527.5±28.7	41.36)	44.29)	
TT	12	632.0±68.4	53.08)		53.08

1 Kruskal-Wallis test p -value = 0.036, 2 Mann-Whitney U test p -value = 0.039, 3 Mann-Whitney U test p -value = 0.032

Our collaborators in Northern Ireland have also been able to show a significant increase in HGF mRNA expression in cells from keratoconic corneas compared with cells from normal corneas.

Taken together, these data indicate a role for HGF in keratoconus. Future work of ours and our collaborators will explore the mechanism for this association. This work has been submitted for publication.

ORIA/Renensson Bequest Grant

Identification of a novel gene for Nanophthalmos in a large Australian pedigree

A/Prof J E Craig and Dr K J Laurie

This project has progressed well and all data has been collected, although due to maternity leave taken by Kate Laurie in 2010, the analysis has not been completed. Work is continuing on the data to finalise the outcomes.

Prior to the start of this grant we had identified an extended pedigree with autosomal dominant Nanophthalmos and localised the causative gene to a novel locus on chromosome 17. Subsequently, we identified a further branch of the family, resident in the UK and had recruited them into the study.

Aim 1 of the project was to expand the linkage study in an attempt to further refine the linked region. This was conducted by the typing of the additional 13 UK-based family members on the same SNP array as used for the 16 members of the Australian branch (Affymetrix 10K SNP array). Linkage was identified on chromosome 17 in the extended family, consistent with the result in the Australian branch. A LOD score of 3.9 was obtained, indicating genome-wide significance for this localisation. The inclusion of the UK branch refined the region to ~9.5Mb through a recombination observed in patient 1.32. This reduced the linked region by ~5Mb from that seen in the Australian branch alone.

Aim 2 of the project was to sequence all the linked genes in the proband to identify the causative mutation. Genomic DNA from the proband (patient 1.05) was enriched for all exons in the linked region through hybridisation to a custom Agilent SureSelect capture array. The resulting DNA still contains the full genome, but has a higher representation of the targetted region, to allow sufficient coverage of sequencing. For efficiency, the enriched sample was sequenced, multiplexed with other samples from different projects

(funded elsewhere). Thus a barcode was applied to the sample during library preparation. The sample was sequenced in a single lane of an Illumina GAII sequencer and data for the nanophthalmos patient retrieved from the mixture of patients by the use of the sequence barcode.

In total, 4,952,785 reads of 65 base pair length were obtained. Only a proportion of these map to the target region. A minimum of 10x coverage was obtained at each exon in the target region. Data is currently undergoing further cleaning and alignment to the current build of the human genome to identify both reported polymorphism and potentially novel protein coding variants. A preliminary analysis identified a total of 541 variants in the linked region and those that cause putative protein coding variants are being further investigated.

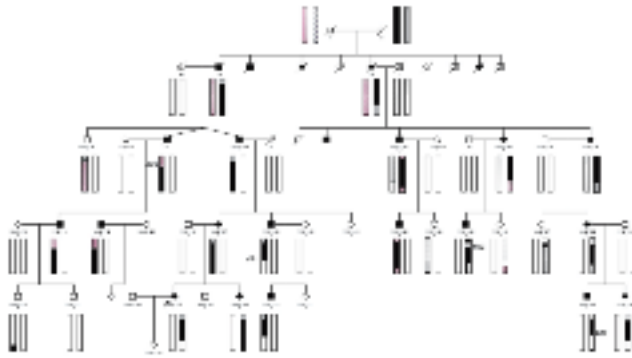


Figure 1: Haplotypes on chromosome 17 in extended nanophthalmos pedigree. The haplotype segregating with disease is coloured black. All confirmed cases share a haplotype on chromosome 17 between SNPs rs1373147 and rs1431991

ORIA New Investigator/Eye Foundation Grant

Auditory processing in glaucoma

Ms F O'Hare

Background and aims

Glaucoma pathogenesis is believed to be multifactorial. It is hypothesized that in certain individuals their optic nerves are more vulnerable to damage and the risk of developing the condition may depend upon innate differences in the global response of the central nervous system (CNS) to pathogenic factors. In light of this underlying hypothesis, preliminary research from our group identified a mild deficit in auditory temporal processing ability (a sign of auditory neuropathy) in individuals with open angle glaucoma (OAG) from a comprehensive range of auditory function tests. Thus, the purpose of the current project was to confirm this finding and tease out the nature and extent of the auditory neuropathy signs on more specific temporal processing tasks. A secondary aim of the study was to investigate similar hierarchical temporal processing function in the visual system within the same group of individuals and consider the importance of these results in terms of understanding the impact of glaucoma in regions outside the visual pathways.

Specific aims of the study were to:

1. To determine whether OAG patients with auditory temporal processing ability also have abnormalities in visual temporal processing ability.
2. To determine whether functional loss is generalized across temporal tasks or specific for subsets of temporal information in each sensory system.

Key findings

This grant allowed in depth investigation of auditory processing ability in a cross sectional sample of 25 OAG individuals compared to 25 age and gender-matched controls (median age 61 years in both groups; 28% male in both groups). Specifically, levels of temporal processing ability was examined which focused

on how well sensory neurons capture information regarding the change of sensory stimulation over time.

Aim 1: Investigation of auditory temporal processing ability in study samples identified significantly poorer speech perception under competing noise in OAG participants compared to controls. As shown in Figure 1, the average speech perception score was near 6% less in the glaucoma group compared to the control group.

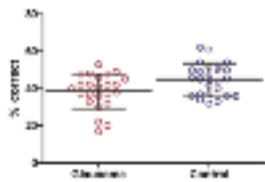


Figure 1: Individual raw scores for right ears with overlying mean (middle bar) and standard deviations (upper and lower bars) for speech perception in groups; glaucoma 38.54% ± 9.28% versus controls 44.97% ± 8.52%; p = 0.031, adjusted for age and average hearing level.

Aim 2: Coding and analysing the temporal components of both auditory and visual stimulation are considered examples of complex neural processing function. As such, it is likely that signs of ‘weak’ nerves within the CNS may manifest on tasks that selectively ‘tax’ or stress nerve function such as those that require a high degree of temporal precision. These tasks rely upon a high degree of cellular metabolism to support their complex execution. Supporting this, evidence of specific temporal processing dysfunction was identified for low frequency discrimination in both auditory and visual systems in OAG participants compared to controls. Specifically, a larger effect size or magnitude of a difference in group performance for auditory discrimination of low frequencies was identified compared to high frequencies. The ability of auditory neurons to transmit temporal information is restricted to frequencies less than 4000 hertz therefore finding that the OAG group have poorer discrimination around 500 hertz suggests selective impairment in fine temporal processing. A significant and moderate correlation was identified between speech perception scores and fine frequency discrimination in OAG participants (see Figure 2).

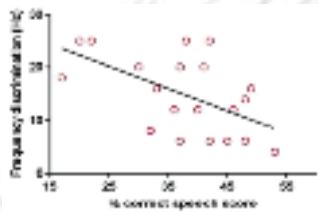


Figure 2: Correlation between fine frequency discrimination (Y axis) and speech perception score (X axis) for the glaucoma group; correlation coefficient -0.554, p = 0.007. Right ear data.

Within the visual system, specific signs of temporal processing dysfunction were identified on tasks exploring speed discrimination and global coherent motion detection. Figure 3 highlights that on average, OAG individuals have impaired speed discrimination for slow moving velocities compared with controls. Discriminatory performance for fast velocities, where spectral and contrast cues are available, was equivalent between groups.

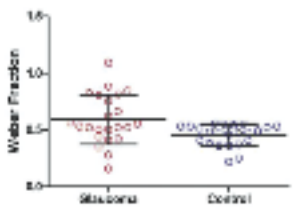


Figure 3: Individual raw scores for right ears with overlying mean (middle interval bar) and standard deviations (upper and lower bars) for speed discrimination ability for each group, reference speed 2 deg/s; glaucoma 0.58 ± 0.21 versus control 0.45 ± 0.09; p = 0.009, adjusted for age and central acuity.

Coherent global motion detection requires the integration of temporal and motion information and involves ‘higher order’ central neural processing. A mild but significant difference in global motion ability was observed between groups, accounting for a 5% drop in median scores in the glaucoma group.

The clinical implications of these findings include recommending OAG individuals who report hearing difficulty, especially within noisy environments, to undergo tests that examine central processing function in addition to standard audiogram assessment. Signs of central auditory processing dysfunction may be missed if specific tests are not performed to detect auditory neuropathy. With regard to visual findings, OAG individuals with impaired speed and motion discrimination may require interventional strategies to improve driving/pedestrian safety and navigation, an area for future research.

In summary, ORIA funding allowed us to explore and identify signs of auditory neuropathy in patients with OAG for the first time. We have been able to show novel findings of specific temporal processing impairment in both visual and auditory systems in a significant proportion of patients with OAG. Findings support a neuronal susceptibility for both and confirm the existence of changes occurring outside the visual pathways in glaucoma. We hope that with publication of the results we can prepare further applications for funding to further explore other auditory functions in glaucoma patients and investigate how having both auditory and visual impairment impacts upon communication and quality of life.

Publications

Rance G, O'Hare F, Crowston JG, Trounce I, Starr A & O'Leary S. Auditory processing deficits in individuals with primary open angle glaucoma – evidence for a systemic neuronal susceptibility outside the visual pathways (*submitted – under review*).

O'Hare F, McKendrick AMM, Rance G, Crowston JG. Is open angle glaucoma a multisensory neurodegenerative condition? Review (*in final preparation*).

O'Hare F, McKendrick AMM, Rance G, Crowston JG. Temporal processing in auditory and visual systems – evidence of wider CNS involvement in glaucoma (*in preparation*).

ORIA New Investigator/Eye Foundation Grant

Does the level of systemic inflammation in people with age-related macular degeneration influence outcomes to anti-VEGF treatment?

Dr L Lim

Background and aims

Age-related macular degeneration (AMD) is the major cause of irreversible blindness in those over the age of 60 years in both Australia and the United States. Despite new advances in the treatment of neovascular AMD with the advent of anti-VEGF (Vascular Endothelial Growth Factor) agents, approximately 10–15% of patients do not respond to this treatment and experience worse vision three months after treatment commencement.

To date, there is no way to predict whose visual acuity will improve, whose will remain stable or whose will continue to decrease with anti-VEGF treatment in the clinical setting. In addition, as injections of these treatments into the eye also have inherent risks, both ocular and systemically, limiting the number of injections would be desirable if the same visual acuity result could be achieved. As AMD has now been postulated to be the result of a chronic inflammatory process, several studies have investigated the relationship between systemic markers of inflammation and AMD. Our aim is to investigate whether inflammatory markers in the serum may be used as predictors of outcome to these new anti-VEGF treatments.

Aim of study

To investigate whether raised levels of systemic markers of inflammation [C-Reactive Protein (CRP), Interleukin 6 (IL6), Interleukin 1 (IL1), Tumour Necrosis Factor α (TNF α), soluble intercellular adhesion molecule 1 (s-ICAM1), total leukocyte count (WCC) and C3A des Arg] are associated with an increased risk of a poor response to anti-VEGF treatment in those with neovascular AMD.

Results

Over the past 12 months, baseline blood samples have been collected and stored on 110 patients with newly diagnosed neovascular AMD from the Royal Victorian Eye and Ear Hospital. Of these 110

subjects, 18 have had further samples collected at three months (just prior to their fourth ranibizumab injection).

In a pilot study on 69 subjects, baseline samples were analysed with a multiplex cytokine microarray after a 1:4 dilution in a test volume of 40 µl for cytokine and chemokine levels using the Bio-Plex human cytokine 27-plex panel (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Cytokines analysed were: CRP, IL-1b, IL-2, IL-4, IL-5, IL-6, s-ICAM1, MCP-1, MIP-1α, MIP-1β and TNF-α. The assay was read on a Bio-Plex 200 instrument and analyzed using Bio-Plex Manager V5.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

CRP was measured using a Beckman Coulter Synchron LX system Chemistry Analyser by Southern Cross Pathology Services, Monash Medical Centre, Clayton.

Participants were categorised as 'responders' (stable or improved vision) or 'non-responders' (any loss of vision) after 3 x monthly injections of ranibizumab treatment. Using this definition, 11 subjects (15.9%) were found to be non-responders, and this is in line with the findings from the MARINA and ANCHOR studies. Using a logistic regression analysis taking into account age, gender and smoking status, possible differences in baseline inflammatory biomarker levels were determined between responders and non-responders.

In this small pilot study, we obtained data on 16 inflammatory biomarkers at baseline: CRP, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12(p70), Eotaxin, Interferon-inducible protein-10 (IP-10), MCP-1, Macrophage Inflammatory Protein(MIP)-1α, MIP-1β, Chemokine ligand 5 (CCL5), and TNF-α. The effect of the level of a particular cytokine on the probability of a subject being a non-responder ranged from a slight increase (0.3% with IL-1) to a very large decrease (-300% with IL-5). An example of some of these effects are shown in Table 1.

Table 1. Results from a logistic regression analysis of baseline cytokine levels in responders vs. non-responders with neovascular AMD (CNV)

Cytokine/Biomarker	Odds Ratio	p-value	95% CI	
CRP	0.766	0.287	0.469	1.251
IL-1b	1.003	0.923	0.947	1.062
IL-4	0.917	0.621	0.650	1.294
IL-5	0.308	0.193	0.052	1.817
IL-6	0.936	0.492	0.775	1.130
MCP-1	0.981	0.827	0.823	1.168
MIP-1α	0.720	0.338	0.368	1.410

Although these results are very preliminary, with only a limited sample, they highlight our ability to detect these cytokines in our patient's serum, and show a trend that we are likely to detect a significant difference between the two treatment response groups with a larger study sample. This is strengthened by the findings that intraocular levels of cytokines such as MCP-1 are higher in those with active neovascular AMD. Recently we have also been able to detect different levels of MCP-1 in the urine of patients with AMD compared to controls, again verifying that systemic levels of these markers are measurable and do appear different in AMD compared to control subjects.

Overall, this ORIA funded pilot study supports the possibility that some of these cytokines might be able to be used as predictors of AMD treatment outcome. As a result, this exciting finding was used as the basis for an NH&MRC project grant submission in 2011. It is hoped that with further work in a larger cohort, we will be able to identify subgroups of neovascular AMD patients who will respond well, or conversely, fail to respond, to ranibizumab therapy prior to even commencing treatment.



ORIA Grant

Do the allele frequencies of AMD-related genes differ due to survival bias as the population ages?

Dr L D Robman

Genetic risks for age-related macular degeneration (AMD) have largely been determined. However, by the age when AMD manifests, in elderly, selective survival from life-threatening diseases may distort the risk factor associations with AMD. This phenomenon could not only affect the strength, but even the directions of associations, converting harmful associations into protective, and protective into harmful. We hypothesised that the genetic risk profile for AMD is different in different age groups; survival bias alters the genetic profile of the elderly; it modifies the level of genetic predispositions that could be detected at early stages of an age-related disease.

For a cost-efficient genetic risk factor analyses stratified by age, we selected the cases of early or late AMD and controls with no features of AMD, matched by age, sex and ethnicity, from the total of more than 21 thousand participants of the Melbourne Collaborative Cohort Study (MCCS), aged 50 to 80 years old. Using the ORIA-2010 funds, we finalised genotyping of DNA from the 1,600 younger participants, determining the status of 17 SNPs from various AMD-related genes. These data are collated with that from the previously genotyped 3,600 DNA samples of the elderly. The first results were presented at ARVO-2011 and further analysis is ongoing.

This funding has helped to conduct a large program of work on AMD in MCCS that has also been supported by ORIA in previous years. Together, ORIA support has contributed to the five papers published in high impact journals and also the 19 presentations at national and International levels that demonstrated the results from this study.

The first direct grant outcome:

ARVO-2011 presentation and also a submitted paper: APOE Gene-Environment Interaction In AMD Risk. MK Adams, LD Robman, JA Simpson, RH Guymer, KZ Aung, GA Makeyeva, PN Baird.

Purpose

To explore APOE e2, e3 and e4 allele frequencies and their association with AMD risk in a large age-matched case control study in Australia, and assess if associations are modified by environmental factors including age, sex and smoking.

Methods

4616 participants (2308 cases of early or late AMD and 2308 controls individually matched on sex, age and country of birth) were selected from the Melbourne Collaborative Cohort Study of 22287 participants. Fundus photographs were graded for AMD. APOE allele status was determined and genotypes were grouped into e3e3 (reference group), e2e2 e2e3 e2e4, and e4e4 e3e4. Estimates of the odds ratios (OR) were determined using conditional logistic regression.

Results

We confirmed that APOE is an AMD risk gene, with the e2 risk allele strongly associated with disease. The associations were dependent on sex, age and smoking status. In men, age modified the association (P-value for interaction 0.03) with no association seen with the e2 allele below the median age of 72 (OR 1.11 95%CI 0.75-1.64 P=0.6) but in those above age 72, those with one or more e2 alleles were twice as likely to have AMD (2.01 95%CI 1.33-3.03 P<0.001). Significant effect modification in men was observed for smoking (P=0.04) where the direction of effect of e4 was positive in non smokers and negative in former smokers. For women with one or more e2 alleles a 25% increase risk of AMD was observed, independent of age, (OR 1.25 95%CI 1.01-1.56 P=0.04) whereas the e4 allele had a borderline significant protective association (OR 0.82 95%CI 0.68-1.0 P=0.06).

Conclusions

This is the first single study to be sufficiently powered to explore gene-environment interactions in APOE and AMD. We have demonstrated significant effect modification by age and smoking on the effect of APOE, which may explain contradictory results found in previous studies that did not stratify on these variables.

Relevant presentations

Adams M, Robman L, Aung KZ, Makeyeva G, Guymer R, Baird P. Changing genetic associations with age-Apo E and age-related macular degeneration. RANZCO-2010.

Robman LD, Adams MK, Simpson JA, Aung KZ, Makeyeva GA, Giles GG, English DR, Baird PN, Guymer RH. Is AMD equally prevalent in Australians of Southern-European and Anglo-celtic origin? ARVO-2010.

Adams MK, Robman L, Simpson JA, Aung KZ, Makeyeva GA, Giles GG, English DR, Guymer RH. Abdominal obesity – not BMI – increases risk of late AMD in Men. ARVO-2010.

Publications

Adams MK, Simpson JA, Aung KZ, Makeyeva GA, Giles GG, English DR, Hopper J, Guymer RH, Baird PN, Robman LD. Abdominal Obesity and Age-related Macular Degeneration. *Am J Epidemiology* 2011.

Advanced Medical Science studies

Dr Elaine Chong, who is now a trainee successfully completed her PhD study based on this study. She will become a clinician with a strong education in Ophthalmic Epidemiology, necessary for the current leaders in Health Research.

Dr Madeleine Adams, our PhD student and practising medical doctor in the Royal Victorian Eye and Ear Hospital, is in her third year of PhD study. She is also applying for a training position.

Collaborations

The data from this study, which was funded by ORIA, have attracted attention of collaborators internationally, resulting in a NIH-2011 research grant application for a pooled dietary and genetic study on AMD that will collate our data with two other studies in the USA.

ORIA New Investigator Grant

The role of DNA Copy Number Variations in the development of myopia

Dr Maria Schache

Background

Myopia is a common condition affecting 16–26% of the population in Westernised countries such as Australia, USA and Europe. The development of myopia is a complex process influenced by both genetic and environmental factors. The primary focus of this study was the genetic causes of myopia. Prior to this work many genetic studies had been undertaken into myopia but no gene had been pinpointed. These studies focused on single genetic variations (SNPs) and used association analysis to determine a link between myopia and various genes. Even after combining this data, the genetic causes of myopia remain unclear.

It has recently become apparent that Copy Number Variations (CNVs) are also important genetic contributors to disease. CNVs are defined as a segment of DNA that is present in variable copy number in the genome. They include deletions, insertions and other complex rearrangements of segments of DNA. The contribution of CNVs to myopia is an unexplored area. Hence, this study aimed to assess the role of CNVs in the development of myopia.

Aims

Overall aim

To investigate the role of Copy Number Variations in the development of myopia.

Specific aims

1. To identify Copy Number Variations associated with myopia in the previously mapped myopia locus on chromosome 2q37.
2. To replicate putative myopia Copy Number Variations in an extended cohort.

Study group

Individuals were recruited in Melbourne between 2004 and 2009 as part of the Genes in Myopia (GEM) study. A total of 916 individuals from 290 different families have been recruited, as well as 345 monozygotic and 267 dizygotic twins. For the current study the three largest GEM Study families were utilised. These families consisted of 37 individuals with myopia (average SphE -2.76D) and 14 without myopia.

Results

A genome-wide linkage study was performed on the three GEM families described above. This resulted in the identification of a putative myopia locus on chromosome 2q37. This locus is 2.4 cM distal to the known myopia locus MYP12 and spans 1.83 cM between markers D2S2968 and D2S1391. We have previously undertaken sequence analysis on the known gene in this region as well as an association study using 151 SNPs spanning the region. Both those studies failed to identify a causative gene or gene variant in this region.

We are now undertaking a more comprehensive analysis on the chromosome 2q37 linkage region that includes both CNV analysis and DNA sequence analysis of the entire region. To achieve this, we have taken advantage of the latest DNA sequencing technology, namely Next Generation DNA Sequencing (NGS).

Data generation for the NGS analysis has been undertaken at the Australian Genome Research Facility using the Illumina GAI system. We have supplied the AGRF with DNA from ten individuals from the GEM Study families and in return the AGRF has generated raw sequence data. Due to the large volume of data, analysis is currently ongoing. Our analysis is encompassing both CNVs and DNA sequence variant analysis in the chromosome 2q37 region. Once we have identified potential CNVs and DNA sequence variants that may be associated with myopia, they will then be further analysed in the remaining GEM Study family members to confirm their role in myopia. This is an exciting study as it is the most comprehensive study into a myopia linkage region to date. We are very excited by this work and look forward to completing our analysis.

Publications and grants

Data analysis is ongoing. It is anticipated that this will be complete in the next 12–18 months, after which a number of publications will be compiled. We also plan to submit a grant proposal to the NH&MRC to expand this work and perform functional studies on the genes that are discovered to identify how they cause myopia.

ORIA/RANZCO Eye Foundation Grant

The heritability of optic disc shape

Dr P Sanfilippo and Prof D Mackey

Background and aims

Assessment of optic disc morphology is essential in the diagnosis and management of ophthalmic disorders that involve the optic nerve, including glaucoma. While much work has been conducted to examine factors affecting differences in optic disc size, there is a paucity of data considering variation in the shape of the optic disc. Additionally, studies that have measured optic disc shape have done so in terms of linear metrics

and ratios, incompletely accounting for variation in this trait (see Reprint Publication 4).

We set out to investigate whether the application of geometric morphometric (GMM) techniques in the quantification of optic disc morphology enable a more comprehensive assessment of shape variation than the traditional methods employed. We have subsequently utilized data from a large twin study and are currently analyzing the concordance of optic disc shape in identical (MZ) and non-identical (DZ) twins in order to determine the role of genetic factors (ie heritability) in this parameter.

Specific aims of this study

1. Measure optic disc shape in 1,000 twin pairs.
2. Determine the heritability of optic disc shape by comparing the correlation of optic disc shape between MZ and DZ twins.
3. Determine the genetic loci associated with optic disc shape.

Results

Prior to measuring optic disc shape in our large twin cohort it was necessary to determine an optimal method for the quantification of this morphological parameter. We thus formulated and conducted a ‘validation’ study (see Reprint Publication 3) whereby we employed discriminant function analysis to compare the efficacy of a series of the traditional shape measures in addition to several GMM techniques for correctly classifying normal and glaucomatous optic cups based on shape information alone. The construction of Receiver Operator Characteristic curves for each technique showed that the traditional shape measures performed reasonably well in discriminating optic cup shape (Area Under the Curve – AUC = 0.86), however a GMM method proved most effective in correctly classifying cups as normal or glaucomatous (AUC – 0.91). This GMM technique was then selected to measure the shape of the optic disc for all subjects in the study.

We are currently analyzing the concordance of optic cup/disc shape in our cohort. Shape information may be decomposed into a numerical quantity following a Principal Components Analysis (PCA) of the original shape data (see Reprint Publication 4). Figure 1 illustrates the graphical output of a PCA for a sample of optic cup shape data.

Preliminary analyses suggest the intra-twin pair correlation for shape is greater amongst MZ than DZ twins (Figure 2), thus implying that both aspects of disc morphology are heritable. More complex structural equation modelling will quantify this heritable component more accurately.



Figure 1: Primary modes (principal components) of shape variation for a sample of optic cups.

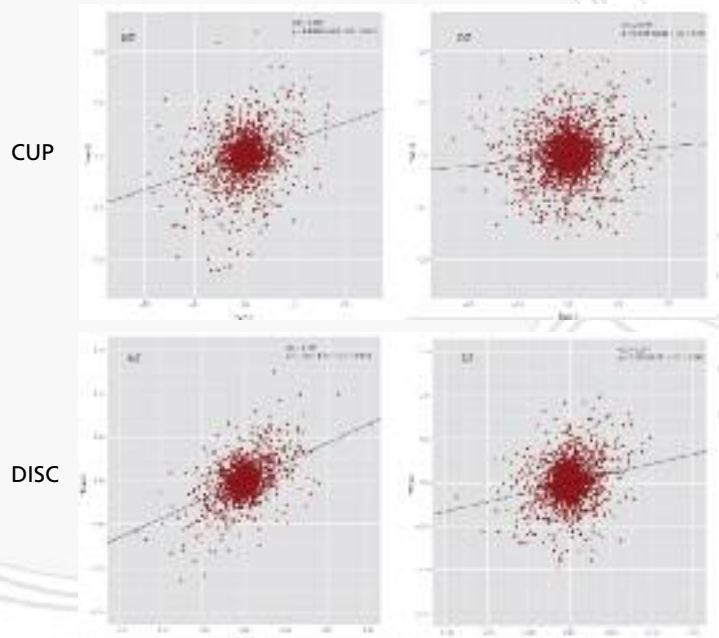


Figure 2: Within pair twin correlations for optic cup and disc shape.

In summary, being able to quantify shape variation of the optic cup and disc will provide a greater understanding of the genetic factors underlying glaucoma and also the potential for improvements in diagnostic algorithms used in computerized instrumentation (eg HRT, OCT) for the early diagnosis and monitoring of the disease.

Publications

1. Sanfilippo PG, Medland SE, Hewitt AW et al. Ophthalmic Phenotypes and the Representativeness of Twin Data for the General Population. *Invest Ophthalmol Vis Sci*. 2011 (*in press*).
2. Sanfilippo PG, Hewitt AW, Hammond CJ, Mackey DA. The heritability of ocular traits. *Surv Ophthalmol*. 2010;55:561–583.
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ORIA/Brenda A Mitchell Bequest Grant

Complement proteins and photoreceptor death in light-induced retinal degeneration

Dr K Valter-Kocsi, Dr J G Wong, Prof J M Provis and Dr M C Madigan

Age-related macular degeneration (AMD) is a multifactorial disease that is the leading cause of blindness in the Western World in those over 60 years of age. Smoking, obesity, high cholesterol and other life-style factors have been shown to be associated with increased incidence of the disease. More recently, genetic and morphological studies suggested that activation and compromised regulation of the complement pathway of the immune system play an important role in the development and progression of AMD. It remains unclear as to what initiates these immune processes in the retina. This study aimed to establish whether photoreceptor death itself could be the event that triggers activation of these retinal immune responses, and how this is regulated, once initiated.

We used a light-induced rat model of retinal degeneration to assess the relationship between photoreceptor cell death and the activation of immune responses. Our studies found that the light-induced model of retinal degeneration bears many similarities with the ‘dry’ (atrophic) AMD, the most common and insidious form of the disease.

The most severe and irreversible lesion with light damage aligned with the visual axis in an area of the rat retina that can be considered a functional equivalent of the human macula. The morphological changes were most prominent in the photoreceptor layer, RPE and Bruch’s membrane and resembled the histopathology seen in dry AMD. The primary lesion, caused by the acute bright light exposure, initiated further damage long after the stress was removed causing wide-spread cell loss in the retina. This progressive nature of the model also was common with AMD.

We observed the activation and invasion of microglia in the area of the primary lesion. These cells remove damaged cells, but may themselves cause further tissue damage. The presence of activated microglia and invasion of choroidal macrophages has been previously reported in AMD.¹

In recently published work, we analysed the differential gene expression in normal and bright light-exposed retinæ and found clusters of genes that showed significant increase in their expression levels. Among others, stress response and immune response genes families were identified.²

In the group of immune response genes, one particular gene, *Ccl2*, showed a very strong upregulation. The chemokine (C-C motif) ligand 2 (*Ccl2*) is a strong chemoattractant to monocytes (also known as the monocyte chemoattractant protein 1 (MCP-1)). In our model, the area of most severe damage showed the presence of activated microglia and invasion of monocytes, and as such, this gene became the focus of our attention.

Quantitative PCR analysis revealed a strong correlation between cell death and the upregulation of Ccl2, with increased gene expression as early as 12 hours after light exposure, just shortly after significant photoreceptor damage and loss was initiated.

Immunohistochemical studies showed that in the early stages, Müller cells located around the area of the most severe damage showed MCP-1 (ccl2) immunoreactivity, suggesting that they are responsible for the targeting of activated microglia and monocyte invasion specifically to this area. In the later stages, recruited monocytes also became immunoreactive to the protein, signifying the development of a self-perpetuating event. The presence of activated macrophages has been associated with the progression and severity of AMD and thus it is an important aspect of the disease mechanism.³

As noted above, the complement system has been implicated in AMD, and polymorphisms found on complement component and regulatory genes increase susceptibility to AMD in humans. Proteins of the complement system are also found in drusen, a characteristic feature of the disease. Further analysis of our genechip data identified 17 complement-related genes that showed differential expression following bright light-exposure. Among these were complement components of the classical (C1s, C2, C4), alternative (C3) and lectin (Ficolin B) pathways, complement receptors (CR3, CR4, C3aR1, C1qR1) as well as a number of complement regulators (CD55, CD46, SERPING1)

Spatiotemporal analysis revealed a close correlation between photoreceptor cell death and complement activation. Both complement components and regulators showed significant upregulation following large-scale photoreceptor loss and reached their peak in the post-exposure period, when apoptosis of cells showed a decrease. The persistent high expression of complement suggests they play a role in the progression of the retinal degeneration.

Immunohistochemical and in situ hybridization work demonstrated that complement was expressed in cells present in the retinal and choroidal vasculature around the area with the most severe damage and that these cells were also immunoreactive for monocyte and macrophage markers (ED-1, IBA-1).⁴

Taken together, these studies provide a better understanding of the sequence of events during the progression of retinal degeneration. These may be similar across several different types of retinal disease, the common element being photoreceptor loss. It seems that exposure to bright light causes photoreceptor damage and loss in the central area of vision, that initiates the up-regulation of a chemo-attractant molecule (Ccl2) in the Müller cells, and signals to microglia cells in the surrounding tissue. This leads to the activation and recruitment of monocytes/microglial cells from the surrounding vessels into the damaged area. Once the barrier protecting the retina is damaged, it allows the largescale invasion of monocytes/microglial cells from the choroidal vasculature, into the retina. These cells, apart from cleaning up the debris in the tissue, also produce complement proteins as well as a chemo-attractant, to recruit even more monocytes/microglial cells. This might be responsible for the close temporal relationship of the up-regulation of complement expression with photoreceptor cell death observed in this model.

Publications

1. Findings of this study were published in Rutar M, Provis J, Valter K: Brief exposure to damaging light causes focal recruitment of macrophages and long-term destabilization of photoreceptors in the albino rat retina. *Curr Eye Res* 2010.
2. Natoli R, Zhu Y, Valter K, et al.: Gene and noncoding RNA regulation underlying photoreceptor protection: microarray study of dietary antioxidant saffron and photobiomodulation in rat retina. *Mol Vis* 2010.
3. This part of the work has been recently published in Rutar M, Natoli R, Valter K, Provis J.: Early focal expression of the chemokine ccl2 by Müller cells during exposure to damageinducing bright continuous light. *IOVS* 2011.
4. These findings have been published in Rutar M, Natoli R, Kozulin P, Valter K, et al.: Analysis of complement expression in light-induced retinal degeneration: synthesis and deposition of C3 by microglia/macrophages is associated with focal photoreceptor degeneration. *IOVS* 2011.

Does genetic make up influence outcome of treatment in patients with ‘wet’ age related macular degeneration?

Dr S Wickremasinghe and Prof R Guymer

Background and aims

Age-related macular degeneration (AMD) is a potentially blinding condition. Low-grade chronic inflammation within the eye and subsequent over-expression of vascular endothelial growth factor (VEGF) are thought to play a role in its cause. Several genes that influence the inflammatory response have been shown to be associated with increased risk in the development of AMD and may influence the outcome to treatment.

This study will examine the association between polymorphisms of several genes and treatment response in individuals with neovascular AMD.

The specific aims of this study were:

1. To assess the association between variants of genes (CFH, CFHR1/3, BF/C2, C3, LOC387715/HTRA1, APOE and VEGF) that have previously been linked with the development of AMD and the outcome of treatment with anti-VEGF agents, in patients with neovascular AMD.
2. To determine whether patient characteristics such as age, gender, smoking status and delay in seeking treatment influence treatment outcome.

Aim 1

APOE gene: In 172 patients undergoing anti-VEGF treatment for neovascular AMD, after controlling for covariates, we found a significant association between the ε4 variant APOE gene and improved outcome following anti-VEGF treatment, compared to those with the ε3 or ε2 variants at 3 months (odds ratio 4.04, confidence interval 1.11, 14.70, p=0.03) and 12 months (odds ratio 2.54, p=0.20). This finding has biological plausibility with eyes of transgenic mice that express the APOE ε2 allele showing over expression of VEGF by the retinal pigment epithelium. The APOE ε2 risk allele may up-regulate angiogenic cytokines and influence CNV formation, leading to an increased amount of VEGF in the retina of these patients. The results of this study were published in *Investigative Ophthalmology and Vision Science* (2011, Epub Jan 18).

Characteristics	3 months outcome N=166		6 months outcome N= 168		12 months outcome N=149	
	OR (95% CI)	p	OR (95% CI)	P	OR (95% CI)	P
Age (years)	0.96 (0.91, 1.01)	0.18	0.94 (0.89, 0.99)	0.03	0.94 (0.88, 0.99)	0.04
Gender						
Male	1	0.20	1	0.16	1	0.44
Female	0.62 (0.30, 1.28)		0.60 (0.30, 1.22)		0.73 (0.33, 1.61)	
Baseline logMAR VA*	2.80 (1.31, 6.04)	0.001	4.18 (1.87, 9.33)	0.001	4.88 (1.89, 12.58)	0.001
APOE allele						
!2	1		1		1	
!3	2.27 (0.77, 6.69)	0.14	4.32 (1.24, 15.06)	0.02	1.93 (0.61, 6.11)	0.26
!4	4.04 (1.11, 14.70)	0.03	3.26 (0.76, 13.90)	0.11	2.54 (0.61, 10.52)	0.20

CFH, C3, LOC387715 and HTRA1 genes: We collected data in two separate cohorts, one in Melbourne (198 eyes of 192 patients) and a second cohort in Iowa, USA (148 eyes of 138 patients). In these two populations, we found no significant association between variants of the genes studied and response to treatment, after controlling for covariates such as age, gender, baseline vision and number of injections given.

Aim 2

In the process of collecting patients with neovascular AMD, we also assessed other baseline covariates that could influence outcome of vision at 12 months. We assessed factors such as baseline vision, age and gender of patients and the number of treatments given over the 12-month period. We found that baseline vision ($p=0.002$) and delay in treatment from first symptoms ($p=0.015$) were significantly associated with visual outcome. Baseline vision has previously been identified as a predictor of treatment outcome, such that eyes with poorer vision at treatment initiation are more likely to improve than eyes with good vision (ceiling effect). Similarly eyes with poor vision are less likely to deteriorate than eyes with good vision (floor effect).

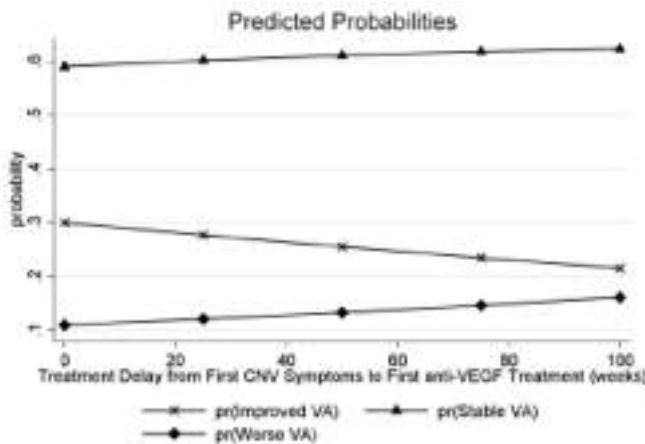


Figure 1: Graph showing the predicted probability for change in vision at six months with respect to delay from first symptoms to treatment.

pr; probability, VA; visual acuity, CNV; choroidal neovascularization, anti-VEGF; anti vascular endothelial growth factor, Improved; \geq two lines of improvement in acuity compared to baseline, Stable; within two lines of baseline, Worse; \leq two lines of baseline visual acuity.

With respect to delay in presentation, longer treatment delay from first symptoms of CNV was a highly significant predictor ($p=0.015$) of adverse outcome after adjusting for age, gender and baseline VA, with an OR of 2.62 (95% CI, 1.20, 5.68). Compared to treatment within 7 weeks of symptoms, patients treated after 21 weeks had increased the likelihood of poor response 2.6 fold despite treatment. Interestingly, a similar proportion of patients lost vision, regardless of time presentation (<7 weeks and >21 weeks). This suggests a time influence on the ability to improve vision (possibly relating to minimization of damage to the outer retina/retinal pigment epithelium), whilst there may be other factors influencing poor outcome such as genetic make-up. This work is currently under review (*American Journal of Ophthalmology*).



Ms Jacinta Spurrett, CEO of the Eye Foundation, the ORIA's fundraising arm.



A/Prof Jamie Craig presenting the Ida Mann lecture at RANZCO Adelaide 2010. A/Prof Craig currently holds an ORIA Grant. Dame Ida Mann left a significant bequest to the ORIA. The ORIA distributes an ORIA/Ida Mann Grant in acknowledgement of her legacy.