

Trinity College Dublin

Fundus Fluorescein Angiography (FFA) in Human Subjects Displays Circadian Variation

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Abstract

The relationship between retinal inner blood-retina barrier (iBRB) permeability, the circadian clock and their possible role in retinal pathology is unknown. We performed quantitative fundus fluorescein angiography (FFA) in the morning and evening in healthy human subjects aged 18 – 30 years old to assess for any changes in retinal vascular integrity. 15 volunteers were recruited, and informed consent was obtained from all participants (Appendices i & ii). Fundus colour photography, optical coherence tomography (OCT) and FFA were performed at a defined time in the morning and again in the evening, with a minimum of 48 hours between each investigation, to allow for washout of fluorescein before retesting. The chronotype of each person was determined using the Munich Chronotype Questionnaire (MCTQ). Volunteers were excluded if they had any pre-defined medical or ophthalmic history, as outlined in our ethical approval documentation.

Sodium fluorescein (500 mcg), followed by a 5mL flush of 0.9% sodium chloride, was injected via a peripheral cannulation site from which blood was also initially drawn for later analysis. FFA images of the posterior pole were captured every 15 s from completion of the infusion to 10 min. Fundal images were later independently reviewed by a consultant ophthalmologist and *ImageJ* analysis was used for quantification of OCT and FFA images. The macula was divided into 3 regions for analysis, based on the Early Treatment Diabetic Retinopathy Study grid, comprising the central fovea, inner macula and outer macula. Fluorescein signal was evident and more prolonged in the evening compared to the morning in the same subject and this was significantly increased in all macular regions analysed ($n = 15$ subjects, $***P < 0.001$). There were no significant OCT in any measured parameters, including central foveal thickness, between the time points. Cortisol levels were in line with expected normal physiology. Increased cortisol levels were measured in the morning. This supports that analysis of the MCTQ achieved the correct times to test each volunteer with regards to their circadian cycle.

We have shown that there is a significant increase and more prolonged fluorescein signal in the evening compared to the morning in healthy volunteers. This indicates a systemically injected tracer molecule in human subjects undergoes a potential size-selective passive diffusion from the inner retinal vasculature to the retinal parenchyma

with diffusion towards the outer retina and retinal pigment epithelium (RPE). An inner retina derived supply of systemic components to the photoreceptor outer segments and RPE has not been described previously and may represent a critically important physiological process central to the development of a range of retinopathies, including age-related macular degeneration (AMD). Previous non-human work from our lab indicates that a tight junction molecule, Claudin-5, whose expression varies with the circadian rhythm, plays a key role in the cycling of the iBRB permeability. The results of this current study are discussed in tandem with previous work, and for added clinical relevance, also in the setting of the pathophysiology of AMD.

Abbreviations

AMD	Age-related macular degeneration
BrM	Bruch's membrane
CC	Choriocapillaris
CNV	Choroidal neovascularisation
ELM	External limiting membrane
FFA	Fundus fluorescein angiography
GA	Geographic atrophy
GDPR	General Data Protection Regulation
HRD	Hyperreflective dots
iBRB	Inner blood retinal barrier
MCTQ	Munich Chronotype Questionnaire
MSFsc	Mid sleep on free days, SC denotes social correction applied
NHP	Non-human primate
OCT	Optical coherence tomography
RPE	Retinal pigment epithelium
VA	Visual acuity

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General Introduction

The importance of sleep in the maintenance of good health is widely accepted. Indeed, the acknowledged essential effects range from memory formation and solidification to physical recovery and performance. Vast amounts of research have been completed analysing the stages of sleep and the effect of forgoing different sleep stages compared to controls. The reparative effect of sleep has been a cornerstone of the training plans for professional athletes for some time, that muscles require rest to recover before a new day of physical effort is easy to understand (Fullagar et al., 2015; Gais and Born, 2004). That our organs, similarly, use this time to remove metabolites and waste material to optimise their functionality appears to be intuitive. From an evolutionary point of view, if we did not need to sleep, we would have lost the vulnerability that time spent sleeping subjects us to. Thus, we can infer that the positive effects are essential for continued optimal functionality. It is well known that the quality of sleep often becomes dysfunctional with advanced age (Crowley, 2011). A common dysfunctional feedback loop sees a shift to early morning waking, with afternoon napping then affecting the quality of that night's sleep, leading to progressively reduced total time of night-time sleep and in sleep quality. This feedback loop leaves us with reduced benefits of sleep and as such more susceptible to pathology. At a genetic level loss of circadian clock genes can contribute to deleterious visual effects during both development and aging. This loss of circadian genes has also been implicated in diabetic retinopathy and many other neurodegenerative diseases (McMahon et al., 2014).

Age-related macular degeneration (AMD) is a complex and multifactorial neurodegenerative disease characterised by retinal pigment epithelium (RPE) reduction and macular photoreceptor loss (Chakravarthy et al., 2010). Classification of advanced AMD splits it into wet, in which angiogenesis occurs, termed choroidal neovascularisation (CNV) or dry, in the absence of angiogenesis termed geographic atrophy (GA). Advanced AMD is present in an estimated 10 million people worldwide with 5% of those over 75 years of age affected, making it the foremost cause of vision loss in this group (Smith et al., 2001; Wong et al., 2020). AMD prevalence is increasing and spreading globally and with this the economic burden too will increase (Velez-Montoya et al., 2014). The unpredictable nature of its progression to GA or CNV or both, and the devastating effects on VA, reinforce the need for a greater understanding of the

pathogenesis. Understanding of the biology and pathophysiology at present remains limited, which in turn limits therapies (Fritsche et al., 2005). Attempts have been made to identify genetic variants associated with AMD, however translating the resulting disease associated common variants into functional consequences and biological insights is challenging (Fritsche et al., 2015). This is further complicated, as AMD is likely a group of pathologies with similar appearances under investigation. This is supported by varied interindividual responses to treatment.

This thesis deals with two main topics. Firstly, whether a physiological observation (circadian driven retinal vasculature permeability) noted in murine and non-human primate (NHP) studies previously by our group, is present in normal healthy human participants. Secondly, we investigate the relevance of such a finding beyond the not insignificant, feat of identifying a physiological process for the first time. These two topics are presented in tandem, as it was through the consideration of the potential effects of the maladaptation of this circadian process that the idea arose to investigate it, and it is easiest to understand when presented together. The process involved a review of the role of the inner retinal vasculature in AMD, which is presented in this thesis so as to understand and outline the importance of revealing and understanding this physiological phenomenon. In the experimental arm of this thesis, the process involved in organising a trial of healthy volunteers and the results are outlined. This included navigation of General Data Protection Regulation (GDPR) and the development and execution of an experimental protocol that sought the permission of the volunteers to have investigative procedures and phlebotomy completed at the extremes of their sleep cycles. We implemented strict protocols in order to ensure that should a significant effect be present we would find it and equally, if no significant finding were observed we could outline the confidence in that finding.

Finally, we will join what we have learned from the experimental data collected by this study and previous work from our lab with the literature review to discuss the importance of our findings which in itself leads to more exciting questions. From the procedural question, should physicians be performing tests that look at retinal permeability at times dictated by the patient's circadian cycle? Currently testing time is simply dictated by when a patient arrives to the clinic, and does not considering this variation of permeability affect the results? To the more profound, by preserving or restoring this circadian cycle

can pathologies such as AMD be prevented or even treated? Through presentation of our current results and discussion of previous studies we present a novel paradigm for the pathogenesis of AMD that we hope will work towards developing effective treatment and preventive options.

Introduction to Relevant Ocular Anatomy and Investigations

The anatomy of the retina (Fig. 1), the physiology of the retina, retinal changes seen in AMD and the relevant investigations, optical coherence tomography (OCT) and fundus fluorescein angiography (FFA) are introduced in this section.

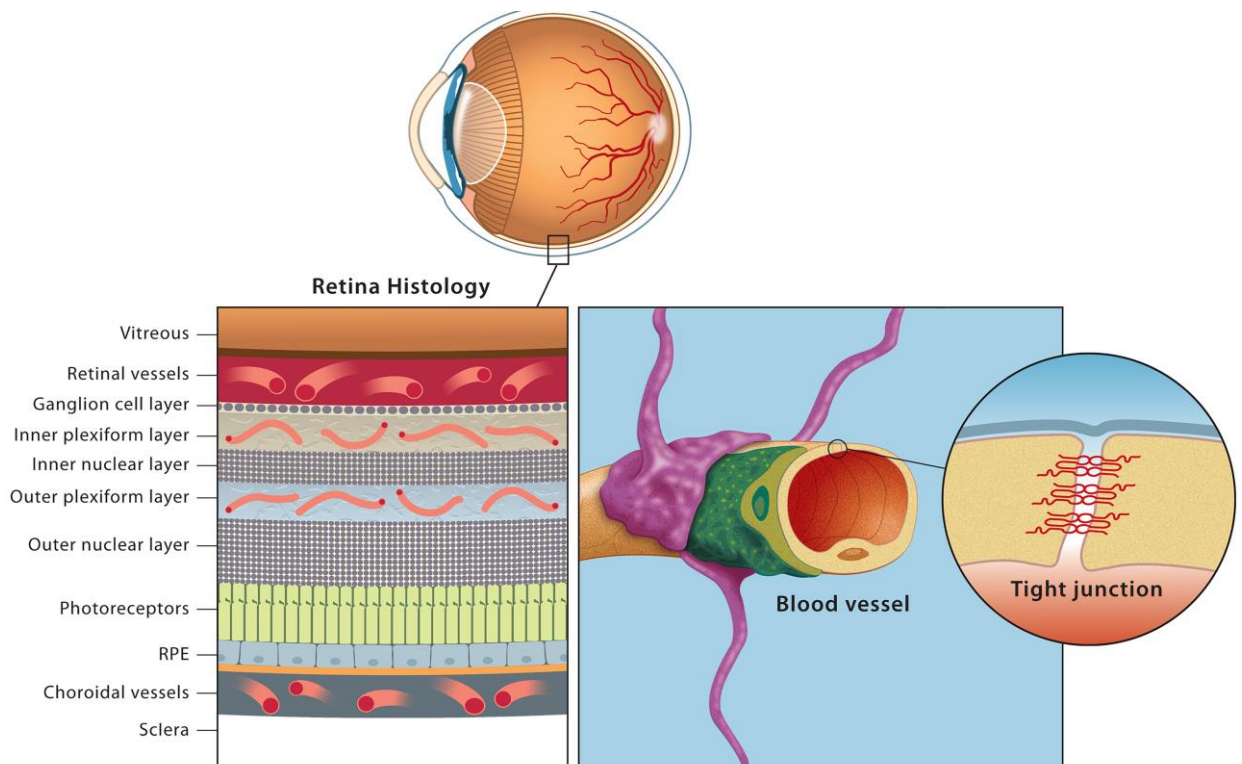


Figure 1. Anatomy of the retina and tight junctions

The macular region of the retina is responsible for our central vision and lies in the posterior pole, directly in the visual axis and is present in humans and non-human primates. This unique 6mm region develops postnatally and is relied upon for fine acuity vision. AMD preferentially affects this macular region of the retina, however the

mechanistic rationale for this is not known. Prior to the advanced stages of AMD, typically, there are earlier, clinically asymptomatic stages which are thought to be present in over 150 million individuals worldwide (Bressler, 2005; Wong et al., 2020). The accumulation of drusen, which are a collection of extracellular components located between the RPE and the choroid, marks the common major early biomicroscopic hallmark of both dry and wet AMD. In later stages of AMD and GA, some patients also display reticular pseudodrusen which are located in the subretinal space.

Each day renewal of photoreceptors requires phagocytosis of photoreceptor outer segments by RPE cells (LaVail, 1976). The fate of this phagocytosed material is multifaceted; indeed, a proportion is recycled in the formation of new photoreceptor outer segments. The remaining non-recycled material is exocytosed to the basolateral compartment of the RPE. At this point the clearance mechanism is not fully understood, with systemic clearance via the choriocapillaris considered a likely candidate (Boyle et al., 1991). The clearance of this material is highly regulated, as dysregulation could lead to a build-up of non-phagocytosed matter which would be deleterious to an individual's vision. Such a build-up seems a logical contributor in the pathophysiology of drusen in AMD and is supported by the observation in the RPE of donor eyes with AMD, of a residual body build-up in lysosomes (Kim et al., 2013). This observation begs several questions which we will now consider. The eyes provide the benefit of comparability between left and right that solitary organs lack, which is helpful here, as despite the high inter-individual variability in the location of drusen there exists appreciable symmetry as to the location of drusen in comparison of an individual's eyes (Mann et al., 2011). When paired with the observation that the retinal vasculature also displays symmetry to an equally high degree, we observe that a vascular related hypothesis is increasingly likely. The retinal blood vessels (Fig. 1) form the inner blood retinal barrier (iBRB), which plays a crucial role in the maintenance of retinal homeostasis by martialling the entrance and exit of a range of both advantageous material and other potentially damaging agents (Díaz-Coránguez et al., 2017). The highly evolved tight junctions of the epithelial cells lining the vessels (Fig. 1) block the transit of molecules above a certain, (very small) size. As well as preventing the entry of potentially harmful substances the barrier regulates macromolecule and ion exchange. Transporters acting in the transcellular pathway control metabolite efflux and the selective uptake of nutrients to the retina (Bazzoni and Dejana, 2004; Vorbrodt and Dobrogowska, 2003).

Macular translocation surgery, in which the healthy neural retina of the atrophic region of affected RPE is transferred to an area of intact RPE has been used in the treatment of CNV and GA secondary to wet AMD and dry AMD, respectively. The subgroup of patient's with subfoveal CNV showed a positive response with improved visual acuity (VA) as a result of the surgery. The follow up showed a very interesting response in that in up to 47% of cases, the translocated region went on to display RPE atrophy within the first 45 months post-surgery. This is of great importance as it highly suggests that there exists a mechanism by which the RPE cell stress and death is promoted by an inner retina derived process (Takeuchi et al., 2012; van Romunde et al., 2015).

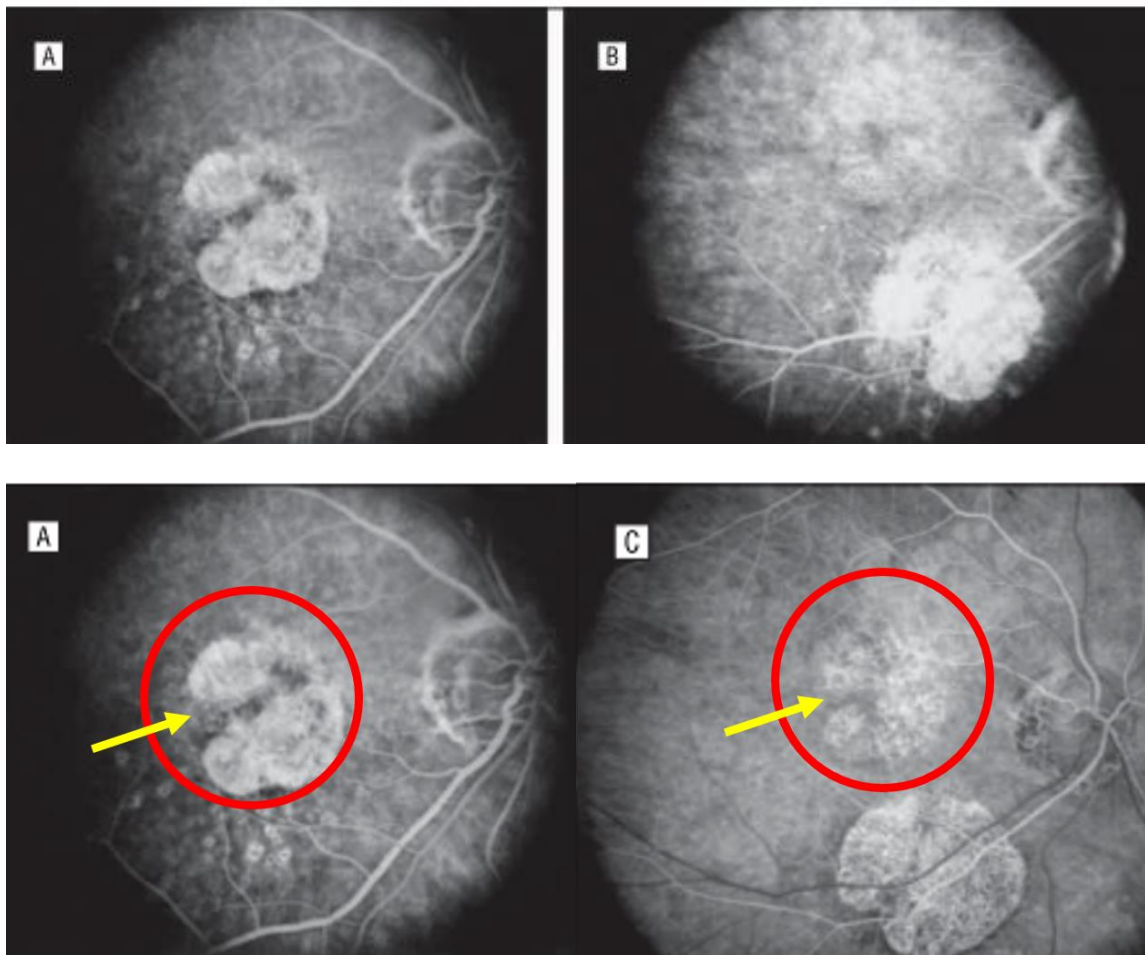


Figure 2. Macular Translocation adapted from Cahill et al. (Cahill et al., 2005). Above left: pre-operative image, above right: 4-months postoperatively and below: comparison between the preoperative and 13month post-operative image of a representative case with the area of recurrence highlighted

Figure 2 shows the retina of an eye with GA that underwent macular translocation surgery as part of a study by Cahill et al., the images show the preoperative then the four- and thirteen-month post-operative retinal fundus photographs (Cahill et al., 2005). At the four-month follow-up there was an improvement in VA and in the upper images in Figure 2 the area of damage is now seen to be shifted inferiorly which a considerably improved appearance of the macular region. However, in the paired images below in this Figure the red circle highlights the original area of damage and the area of recurrence. The yellow arrow selects out a characteristic notched area which demonstrates the high degree of similarity in the shape of the area of recurrence compared to the original area. This suggests that the inner retina is causative as the primary effector leading to damaged RPE and post translocation given sufficient time the same pathological damage in a near identical pattern is occurring.

A loss of iBRB integrity has been implicated in several ocular conditions involving vascular leakage such as diabetic retinopathy and Coat's disease (a rare juvenile retinal condition in which an acute leakage into the sub retinal space of lipid laden macrophages and blood derived cholesterol crystals) (Ghorbanian et al., 2012). Thus, Coat's disease supports the paradigm of deposition of blood derived material to the RPE emanating from a dysregulation of the inner retinal vasculature. Diabetic retinopathy differs in the sense that greatly enhanced vascular leakage is as a result of chronic breakdown of the iBRB occurring independent of RPE atrophy.

OCT

OCT is a non-invasive imaging investigation in which light waves are used to capture high resolution cross-sectional images of a retina. Multiple cross sections can be taken and summed together to form a volume OCT. In AMD, the OCT can show the presence of fluid accumulation within or behind the retina which may signify 'wet' AMD type rather than dry (where we would see atrophy without the presence of fluid). OCT is also used to monitor the response to treatments such as intravitreal injections.

FFA

FFA is utilised to investigate the retinal and choroidal circulation. Photographs are taken after the intravenous injection of sodium fluorescein, a crystalline hydrocarbon of orange-red colour, which can diffuse through most of the body fluids. Excretion is primarily via

the renal route and occurs in twenty-four to thirty-six hours. Close to eighty percent is protein bound and as such it is the remaining twenty percent that is visualised via fluorescence in the vasculature and tissues of the posterior retina.

Fluorescence is the property by which molecular excitation by light, allowing for a jump to a higher energy state with subsequent release of a photon to return to the original state. Specific excitation and barrier filters are required to capture an image of this process. Briefly, flashes of white light pass through the excitatory (blue) filter and then enter the eye, this blue light then excites the unbound fluorescein present in the retinal vasculature and those which have leaked into the parenchyma. This stimulates the fluorescein molecules to emit yellow-green fluorescence which passes through a barrier filter (yellow-green acting to block any reflected blue light) and into a camera. By analysis we can see if there is any large volume pathological 'leaking' from the vessels greater than the general low level 'leak' into the parenchyma. Our experiment looked at this leaked fluorescein and assessed if there was a difference between morning and evening levels measured in each individual. Fluorescein, being water soluble, often causes a visual change in colour in the skin, we explain this to participants that their urine will be bright yellow (excretion by the kidneys) and reassure that this will resolve within two days.

Literature Review

This review aims to outline what is known about the role of the inner retina in AMD at present, with focus on histopathological and vascular changes that occur. In any disease process it is tempting to say that the known sites of damage in a pathology are where the pathophysiology begins. However, we know that many diseases manifest in abnormalities downstream of the primary effector. As an example, in angle closure glaucoma, abnormalities at the front of the eye can cause an increase in intraocular pressure leading to a damaged optic nerve, thus it is a neuropathy in which the primary insult occurs at a relatively distant location. Therefore, when we consider AMD, we should note that although the damage manifests in the outer retina, it could be due to changes in regulation of homeostasis in the inner retina or indeed a combination of many processes. With this rationale in mind here we are looking at what is known about the role of the inner retina, to date, in AMD.

Neovascular and Atrophic AMD

AMD, in general, is pathologically characterised by degeneration of crucial components in delivering vision. This degeneration involves the choriocapillaris (CC), Bruch's membrane (BrM), RPE and the retinal photoreceptors. As discussed in the introduction, the division of advanced AMD is generally into dry and wet subtypes based on the histological differences and clinical findings. Clinically this classification is very important as it changes the treatment pathway. The nonexudative dry AMD, usually progresses slowly with the development of drusen and focal RPE atrophy with photoreceptor dysfunction and degeneration. GA is the most severe form and it involves macular loss of RPE and choroid with a paired gradual loss of photoreceptors and central vision (Curcio et al., 1996; Klein et al., 2002). The exudative wet AMD is defined by the presence of CNV, the growth, from the choroid of new blood vessels into the underlying RPE or extending into the subretinal space.

GA typically progresses slowly, however, within GA, CNV can present as an abrupt reduction in VA resulting from oedema and bleeding from new vessels encroaching on

the neural retina and RPE. In those over the age of 75 years old, AMD associated CNV occurs in about 4% of the population and is primarily macular (Smith et al., 2001). CNV occurs in 10 to 15% of individuals with AMD, despite this, it accounts for 90% of AMD severe visual loss (Ferris III et al., 1984).

On OCT, GA has classic findings, characterised by atrophy of several layers, namely the CC, RPE, photoreceptors, external limiting membrane (ELM) and outer nuclear layer, in the presence of extracellular deposits that increase OCT signal transmission beneath BrM (Wu et al., 2014). The pathogenesis of AMD is not fully understood and as such the role of the CC is controversial, whether CC impairment and resultant hypoxia is an initiating event or is a resultant or even symbiotic event with inner retinal changes as part of the pathogenesis is not fully understood (Arya et al., 2018).

Neovascular AMD sees growth of vessels into the sub-RPE and Sub retinal regions, both of which normally are avascular. This advanced stage of AMD presents as rapid loss of vision, compared to the slower progression of GA (Shah and Del Priore, 2007). This rapid and severe loss of vision means that despite neovascular AMD presenting as the minority of AMD cases, it is responsible for the majority of AMD associated blindness (Ferris III et al., 1984). Neovascular AMD can be further divided by its appearance on FFA/OCT and the location of new vessel growth. Type 1 CNV refers to growth within the sub RPE space, type 2 CNV new vessels present in the subretinal space and type 3 NV which is the presence of intraretinal proliferation (Farecki et al., 2017).

[Clinical Progression/ Natural History](#)

Broadly, the natural history of AMD can be divided into early, intermediate, and advanced stages. This is based clinically on the severity of symptoms and on fundal and OCT findings of size and number of drusen, accompanying pigment changes and whether CNV is present (Pennington and DeAngelis, 2016). Drusen are protein containing lipid rich yellowish deposits, considered to be the hallmark of AMD. Drusen deposits are present between the RPE and BrM.

Regarding the staging of AMD, dry AMD is used to describe, early and intermediate disease as well for the non-exudative late stage, GA. At present, diagnosis and staging

involves a clinical ophthalmic biomicroscopic exam of the fundus of the retina to visualise drusen, RPE pigmentary changes/ atrophy and /or the presence of exudative changes (Cook et al., 2008). FFA allows for visualisation of blood vessels and is used to detect the presence of CNV (Cook et al., 2008). OCT is also used in both the diagnosis and assessment of response to treatments. Together this information is used to confirm diagnosis and classification.

Many AMD classification systems exist, such as the Clinical Age-Related Maculopathy Staging system, based on colour fundus photography, in which early stages are separated by drusen size and number escalating up to GA and CNV (Seddon et al., 2006). Fundal images are limited in their identification of morphological features of both dry and wet AMD. Such changes in GA include RPE alterations in regions adjacent to GA and subretinal drusenoid deposits which affect the classification of AMD. OCT based classification of GA allows for consideration of a wider spectrum of phenotypic changes while avoiding staging inaccuracies (Fleckenstein et al., 2008). In GA, OCT imaging has been shown to closely resemble the histopathological characteristics. At present this may allow for risk factor modification after early recognition (Sarks et al., 1988). However, should disease modifying treatments become available, it could in the future form part of a formal screening programme.

The exact role of environmental and genetic factors in the development and progression of AMD is poorly understood. Several models have been proposed. One such paradigm for neovascular AMD suggests that accumulated drusen affect the connection between the choroidal blood supply and the RPE, inducing a hypoxic environment. This hypoxia leads to a responsive increase in pro-angiogenic factors which promote new vessel formation (Stefánsson et al., 2011). This model, however, fails to complete the picture. Several known aspects in the pathogenesis are not explained, most importantly what causes the drusen to develop and why they are not pathogenic in some. It also omits the roles of inflammation, complement activation and oxidative stress (Blasiak et al., 2014; Gemenetzi and Lotery, 2015; Stanton and Wright, 2014). It is however possible that certain aspects of such theories are correct forming part of the explanation, and that such interference in nutrient supply and waste removal plays an important role in disease progression.

AMD Histopathology

As the name suggests, age is the most significant risk factor in the development of AMD. When environmental features such as smoking, and diet are combined with genetic risk the likelihood of developing pathological changes increases. Advanced age accounts for an associated loss in the central macula of nearly 30% of rod photoreceptors, with minimal effect on the number of cones (Curcio et al., 2000).

Increasing BrM thickness is also associated with normal aging (Bird, 1992). Pathological changes have also been noted in the BrM, such as the accumulation of lipoproteins containing apolipoproteins B and E and cholesterol (Curcio et al., 2001). It is possible that accumulation of these and other materials in the macula can act as a stimulus for inflammation and as a barrier to diffusion. BrM is formed of inner and outer collagenous layers, which as the name suggests, are rich in collagen. Between these layers lies a central region of elastin and proteins associated with elastin (Chong et al., 2005). In early AMD the inner collagenous layer has been shown to be thickened, affecting nutrient diffusion (Sevilla et al., 2016).

Human donor eyes with AMD have provided histopathological data throughout the spectrum of AMD. The macula in early AMD displays several features which may lead to modest functional and VA decline, such as abnormally distributed RPE pigment and drusen which are observed in the sub-RPE space between the inner collagenous layer of BrM and the basal lamina of RPE (Sarks et al., 1999; Spraul and Grossniklaus, 1997). Lipofuscin are formed of remnants of retinoid metabolites from shed photoreceptor outer-segment membranes. These autofluorescent granules are accumulated by the RPE, and components of these are hypothesised to play a role in AMD exacerbation (Strauss, 2005). Histopathologic findings in GA include the degeneration and death of RPE cells, photoreceptors and the CC (McLeod et al., 2009).

Drusen are complex collections of material with an interesting role in AMD, subsets of which are a major risk factor for AMD progression, i.e. large or soft drusen with indistinct borders. Drusen are formed of myriad components including lipids (accounting for greater than 40% of volume), multiple proteins such as β -amyloid, vitronectin, apolipoproteins and those involved in complement regulation, as well as iron ions and zinc (Crabb et al., 2002; Curcio et al., 2009). Basal laminar deposit, a diffuse abundance

of stereotypic thickening of the basal lamina which likely reflects RPE stress levels also can be used to assess disease severity (Rudolf et al., 2008). Drusenoid deposits are observed in many patients and they confer an AMD progression risk independent of drusen (Curcio et al., 2013; Zweifel et al., 2010).

Broadly there are two main current hypotheses regarding AMD pathogenesis. One assumes that atrophy of RPE leads to a secondary loss of choriocapillaris and photoreceptor degeneration. The second places choroidal vascular insufficiency as the primary insult leading to RPE dysfunction and photoreceptor degeneration. Both hypotheses are based on the end results of advanced AMD, attempting to work back from the end point. These theories are put to the test in macular translocation surgery in which the retina is surgically detached and the macula moved, such that the macula then lies once again over healthy RPE and choroid. This represents an invasive but logical procedure, assuming that it takes many years for either of the described pathogeneses to occur at the advanced age that it presents then one should have prolonged positive results. We now know that this is not the case as in patients with GA who underwent macular translocation in their second eye, GA recurred over months and in a similar pattern as before (Cahill et al., 2005). This suggests that the primary pathology may not lie in the RPE or choriocapillaris but in the tissues transplanted in this procedure, implying that the inner blood supply and inner retina is playing an important role in the pathogenesis.

In a human post-mortem study that compared age matched controls, patients with GA and patients with CNV, Control eyes, (as would be expected) displayed relatively uniform and homogenous submacular RPE with regards to size and morphology. Their choroidal vasculature also appeared normal morphologically with an average vascular area of about 80% (McLeod et al., 2009). In patients with GA, the area of submacular RPE atrophy and degenerative choriocapillaris change was well defined. Within this well-defined area the relationship between choriocapillaris degeneration and RPE atrophy was observed to be linear and vascular area was reduced by 50% in regions of complete RPE atrophy. In patients with CNV in wet AMD, the regions beside the CNV displayed dropout of CC, with a 50% decrease in vascular area, without the presence of RPE atrophy (McLeod et al., 2009). Several animal studies have selectively destroyed the RPE either chemically or mechanically and showed that it leads to atrophy of the choriocapillaris affecting both perfusion of choriocapillaris and blood flow in the larger vessels of the choroid (Ivert et al., 2003; Korte et al., 1984; Leonard et al., 1997). This

work proves the close relationship between the CC and the RPE and shows the different scenarios in the two advanced stages of AMD. CNV may thus represent a maladaptive response in which the RPE is salvaged but with a poor visual outcome.

Inner Retinal Dysfunction in AMD

In wet AMD, punctate retinal haemorrhages can be observed. On OCT these have been noted to be found in the inner retinal layers. This shows that retinal ganglion cell loss may be related to inner retinal vascular abnormalities, which were identified in the inner nuclear, inner plexiform and ganglion cells (Coscas et al., 2013). OCT studies have also demonstrated the presence of hyperreflective dots (HRD) in the retina of patients with exudative wet AMD. The origin of these dots has been debated, but it has been shown that they show significant resolution days after treatment with anti-VEGF. The reduction in HRD continues at 1- and 3-month assessment. Moreover, they were noted as an early detectable change in recurrence (Coscas et al., 2013).

Microglia cells are found exclusively in the inner retinal layers of the young healthy retina (Provis et al., 1996). Under conditions of advanced age and disease states, including AMD, they have been shown to migrate and infiltrate the outer retina. In the aged outer retina they display morphological and immunohistochemical features of activation (Xu et al., 2009). The presence alone of microglia having migrated from the inner retina shows the development of a dynamic relationship from inner to outer retina. Their presence in the outer retina has been shown to alter cellular activity and interaction, which in itself could play a role in driving pathogenic processes (Ma et al., 2012). HRD in the outer retina have been suggested to represent swelled and activated inflammatory cells such as microglia which spread in all retinal layers. This is supported by their rapid disappearance after anti-VEGF treatment. The ELM, which corresponds to adherent junctions between photoreceptor and Müller cells in normal physiology plays an important role in restricting the migration of macromolecules and extravasated material (Marmor, 1999). However, even in cases where the ELM was intact, HRD were noted to be present in the outer retina (Coscas et al., 2013).

Loss of inner and outer blood retinal barrier (BRB) integrity in exudative wet AMD manifests as retinal swelling and cystoid oedema commonly found above areas of

subretinal neovascularisation and wound healing (Schlingemann, 2004). Alteration of the specific properties of the neovascular unit leads to the breakdown of the barrier and leakage in pathological conditions, in which many studies have investigated the tight junctions and increased paracellular transport. BRB maintenance is assisted by pericytes and microglia as well as the endothelial cells and as such BRB breakdown may be caused by or involve pathological dysfunction or loss of these perivascular cells or indeed of the connections between these cells. Tight junctions, adherens junctions and gap junctions form and regulate the paracellular transport pathway. The damage of junctional proteins in pathological eye conditions leads to defects in endothelial permeability. The direct effect of glucocorticoids on tight junction protein expression suggest this may be the mechanism by which it can affect reductions in macular oedema. In vitro retinal epithelial cell studies have shown enhanced barrier integrity by upregulating protein expression of claudin-5 and occludin in response to hydrocortisone and dexamethasone (Antonetti et al., 2002; Felinski et al., 2008).

A recent OCT based study found associations between inner and outer retinal volume reduction and superficial vascular retinal plexus alterations and the development of GA (Toto et al., 2017). In areas of GA, OCT studies have reported the development of a wedge-shaped hyporeflective band (Monés et al., 2012), and subsidence of both the outer plexiform layer OPL and inner nuclear layer (Sayegh et al., 2011). These OCT changes were also demonstrated to be present in intermediate AMD. In this longitudinal study which followed up patients on average every three months for up to thirty months, these changes were termed ‘nascent geographic atrophy’ and were demonstrated to portend drusen-associated atrophy development (Wu et al., 2014). Interestingly the changes were unique to areas that went on to develop drusen associated atrophy. As these changes occur in the inner retina, it suggests that the inner retina may be of more importance in the pathogenesis of AMD than previously thought. The increased importance of inner retinal changes is supported by outer retinal changes such as hyperreflective foci and drusen characteristics, which have been reported as risk factors for the development of atrophy, but are present in areas which do not go on to develop atrophy (Hyman et al., 2000).

Thinning of both inner and outer retina in AMD has been found in several studies which have suggested neuroretinal layer damage as a feature of AMD (Lee and Yu, 2015; Sadigh et al., 2013). Toto et al. showed that in patients who went on to develop drusen associated atrophy there was a reduction in macular thickness when they were previously

tested and had intermediate AMD. These same patients also displayed significantly thinned inner and outer retina suggesting that early damage in AMD is present throughout the retina and not confined to the outer retina (Toto et al., 2017).

Changes and damage in both choroidal and retinal vasculature are known to occur in AMD (Remsch et al., 2000; Wang et al., 2004). Indeed, doppler imaging has shown vascular deficits in both early and late AMD (Ciulla et al., 1999). It has been shown that the superficial vascular plexus can be damaged prior to advanced AMD, starting in intermediate AMD (Toto et al., 2016). This damage to the superficial vascular plexus can be seen as a reduction in flow density on OCT to which Toto et al. demonstrated a significant correlation to inner retinal layer thinning (Toto et al., 2017). Changes in perfusion have been suggested as being secondary to reduced requirement in the normally high oxygen dependent retina. There are many differences between superficial and deep plexuses. It appears that the deep plexus is preserved in intermediate AMD. As stated this may be due to the differences in the plexuses such as the pattern of each plexus the interconnected orientation of the superficial versus the polygonal units in the deep (Toto et al., 2017). Why these changes in the inner retina are occurring is a difficult question to answer using only current theories on the pathogenesis of AMD. It shows that the primary insult may be related to the inner retina, and more specifically related to the vascular changes in the inner vascular plexus/ blood retinal barrier. It also may be the case that inner and outer changes are occurring simultaneously, due to a more systemically driven pathology.

Background / Basis for this Study

At present, lifestyle alterations such as dietary modification, supplementation and smoking cessation remain the accepted treatment options in GA (Reynolds et al., 2013). Despite the high prevalence of AMD, and many studies and investigations, the underlying molecular pathophysiology of GA continues to be disputed. The RPE is the major site of evident damage in AMD, and as a result the majority of previous studies have focused on inflammatory mechanisms and aberrant signalling in the RPE. Much less research has considered whether the RPE is affected as a secondary result of a disease process which originates in another location.

The necessity for the investigation of human ocular circadian-related vascular physiology has stemmed from laboratory-based observations from our group which has discovered that the gene which encodes claudin-5, CLDN5, is regulated by the circadian clock and BMAL1 (Hudson et al., 2019). Claudin-5 is a tight junction protein expressed in large amounts at the iBRB, where it represents one of the most enriched tight junction components (Daneman et al., 2010). Our lab has shown that claudin-5 is under the control of BMAL1, a transcriptional factor, and the circadian clock. This finding led to the conception of the idea to compare FFA at different ends of the circadian spectrum, early morning and late at night. We believe that the iBRB, rather than being a relatively static barrier with a single, rigidly defined operation throughout twenty-four hours, is instead highly dynamic, and of such importance that dysregulation of its precise mechanism leads to severe pathology, with the prime candidate here being a contribution to the development of GA

Diet has been implicated as an important contributor to the formation of drusen by investigation of the extracellular material they are formed of (Curcio, 2018; Wang et al., 2010). In a murine study our lab showed that claudin-5 expression suppression paired with a diet rich in cholesterol caused notable atrophy of RPE cells. In nonhuman primates targeted claudin-5 suppression in the macular region also saw RPE cell atrophy (Hudson et al., 2019).

More evidence in support of this disrupted barrier playing a role in the development of AMD is derived from the results of macular translocation surgery. The procedure aimed

to improve the VA of individuals with GA secondary to AMD and CNV secondary to AMD. The surgery saw the ophthalmologist translocate healthy neural retina away from the region of atrophic RPE to a region of intact, healthy RPE cells. The follow up of which showed results varied dependant on the clinical subgroup. The subgroup of patients with pre-existing CNV had a successful outcome. However, of interest to our current study the patients with GA saw a recurrence of RPE atrophy in the translocated area (Cahill et al., 2005). This result suggests that the inner retina can produce an effect of stress and death of RPE cells.

Our group believes that this ability of the inner retinal vasculature to undergo circadian entrained cycling of permeability allows an osmotic drive for both the clearance of waste material from the neural retina and replenishment of substrates to the photoreceptors. With dysregulation of the circadian clock in the aging eye, both of these activities would be disrupted, leading to a build-up of metabolites and dietary components in the RPE. Over time this would lead to overloaded RPE, with the formation of drusen, which in turn leads to atrophy of the RPE as it fails to keep up in suboptimal conditions.

Changes in retinal vascular permeability were noted in murine and non-human primate studies, and linked to the circadian rhythm, where in the NHP group it displayed increased permeability of the retinal vasculature when tested in the evening compared to the morning. Experimentally induced alterations to this cycling led to changes in the retina similar to those seen in GA secondary to dry AMD. This naturally led to the question: Is this circadian cycle of permeability present in healthy humans, and is the derangement of this cycling involved in pathology? There follows an interesting situation, as often the normal human physiology is known, and the research question relates simply to the aberration from this known normal. However, in this instance, first we needed to show the presence of a presumed normal physiological process before such aberrations could be fully considered. Thus, it is that which forms the research question for my research: “Do healthy human subjects display a circadian variation in retinal permeability?”

Our Study / Methods

Introduction

This section outlines the preparation and process involved in setting-up and acquiring the data for this study. The major steps involved in gaining the required permissions and consent were: Gaining ethical approval, participant recruitment and data protection. The physical experimental methods used in data acquisition, fundus photography, OCT, FFA as well as the other steps involved on each testing day (Table 1). Finally, we also describe how the collected data was then analysed.

Day 1	Day 2
Welcome, informed consent obtained	Welcome, informed consent obtained
N.B. ask verbally regarding allergies esp. shellfish allergy	N.B. ask verbally regarding allergies esp. shellfish allergy
LogMAR VA check	LogMAR VA check
Measure weight and height	Cannulate and take blood for analysis
Cannulate and take blood for analysis	Dilating drops (tropicamide and phenylephrine) given
Dilating drops (tropicamide and phenylephrine) given	Eye examination
Eye examination and Fundus photography of both eyes	Complete OCT, section, and volume in left eye (LE)
Complete OCT, section, and volume in the LE	FFA, every 15 seconds. Empty vessels to fill for 10 minutes in the LE
FFA, every 15 seconds. Empty vessels to fill for 10 minutes in the LE	Check IOP
Check IOP	

Table 1. Brief summary of Day 1 and 2 of participant testing

Ethical Approval

The first stage in any experiment or trial involving human participants is seeking ethical approval. Ethical approval had been granted for this project prior to my involvement, however early in the preparations we realised that, based on what had been found in the

non-human arms of this project, we would need to amend the ethical approval to allow for more information to be gathered. In the time since the initial approval had been granted, European law saw the introduction of GDPR in which data controllers and processors were defined and their obligations outlined. GDPR in simple terms means that the data subject is fully aware of what data is collected, how it will be used and stored and what will be done to ensure anonymisation.

For the study, the introduction of GDPR meant that as well as our required amendments we also needed to ensure that all elements of this legislation were adhered to. Although tedious and adding a great deal of time to approval being granted while all members of the ethical approval process became accustomed to the changes GDPR, on a day to day basis the introduction meant that participants were reassured that every effort was being made to keep their data protected and also that they were fully aware of how their data would be used. The process required a 'Data Protection Impact Assessment', which involved modular assessment of each step in the data collection and processing process with risk analysis so as that any potential areas where data might be compromised could be identified and prevented ahead of time (Appendix i).

When this process was completed all, ethical approvals for the human experiments and data collection/processing were granted by the ethical committee of the Royal Victoria Eye and Ear Hospital. Informed consent was also obtained from each participant for all experiments and investigations throughout the study.

Retinal Images

Eyes were dilated and examined prior to the study. Fundus colour photography was performed using a retinal camera (Topcon TRC 50X; Topcon America Corp., Paramus, NJ) with Canon D4 digital imaging hardware was used to acquire colour fundal photographs. Images were obtained with a 30° angle of view and were independently reviewed by a consultant ophthalmologist.

OCT

OCT was acquired using the Heidelberg Spectralis™ OCT (Heidelberg Engineering, Heidelberg, Germany) machinery performed with eye tracking and HEYEX version

1.7.1.0 image capture and analysis software. Section and volume scans of the macular region were performed prior to FFA in both morning and evening sessions. The left eye was chosen for examination and images were obtained with a 30° angle of view.

FFA

Heidelberg Spectralis™ OCT (Heidelberg Engineering, Heidelberg, Germany) machinery with HEYEX version 1.7.1.0 was used to capture the images during the FFA study. Baseline colour and red-free images of the vessels in the posterior pole were taken prior to injection of Sodium fluorescein (500 mcg), followed by a 5-mL flush of 0.9% sodium chloride via a peripheral cannulation site. FFA images of the posterior pole were captured every 15 seconds from completion of the infusion for 10 minutes to visualize the blood vessels of the posterior pole. Again, the left eye was chosen for examination and images were obtained with a 30° angle of view.

Participant Recruitment / Experimental Protocol

The goal of the human study was to identify normal processes in healthy individuals, and as such we applied strict inclusion and exclusion criteria. We sought participants in the age bracket of eighteen to thirty years old with no significant medical or ophthalmic history. To confirm this all participants completed a health survey (Appendix ii). To calculate each participant's circadian rhythm, the validated Munich Chronotype Questionnaire (MCTQ) was completed by all prior to arriving for testing (Appendix iii). Based on their MCTQ results, participants were assessed once in the evening and then early in the morning, with at least 48 hours between each test to allow for complete washout of fluorescein from their system. Special care was taken to ensure no volunteers with allergies would be put at unnecessary risk, and we asked specific questions relating to a history of allergies both in the health survey and in person each testing day. Informed consent was acquired for all aspects of the study

The protocol showing the steps of each day is shown in Table 1. The two-day process began with the evening testing session. For the participant this meant meeting at the Royal Victoria Eye and Ear Hospital to begin testing at about seven or eight PM based on their MCTQ result.

On arrival volunteers were asked to read through information (written in lay terms) on the study and ask any questions they might have before signing the consent form for enrolment in the study and for the FFA. LogMAR VA was checked, and height and weight measured. Dilating agents tropicamide and phenylephrine were used to ensure adequate view and highest possible images quality for later analysis of the posterior pole. Participants were advised not to drive to the session, and that their vision would remain blurry for four to five hours.

A peripheral cannula was inserted, and bloods drawn, with the cannula left in situ for use in the FFA study. Blood Samples were immediately brought to the lab, where they were processed for serum, plasma and PBMC's. Samples were tested for cortisol levels using a cortisol ELISA kit (R&D Systems, Catalogue Number KGE008B). An ophthalmic exam was then completed to ensure no unknown pathology was present which might affect the result. Fundal images were taken for review by an independent ophthalmic consultant, again to ensure the health of the retina of each participant.

Next the volunteer underwent an OCT, which gives detailed images of the macular region, with many of the anatomical layers visible for inspection. In this study the OCT again confirmed ocular health, but the goal was to assess for changes in total and individual layer thickness between the two testing sessions. FFA was then completed. First images of the posterior pole were acquired, ensuring that the focus of the image was sharp prior to injection of the fluorescein followed by a flush of saline. In seconds, the water soluble, fluorescein reaches and fills the vessels in the eye and images were taken every fifteen seconds for ten minutes. There is a possibility of an allergic reaction to fluorescein, and we were always prepared to deal with such an incidence with an anaphylaxis emergency treatment kit and cardiac arrest trolley kept where it could be accessed without delay. Volunteers were briefed on the early signs of such a reaction and encouraged to tell us if they felt anything at all out of the normal. Luckily, there was no such adverse event in our study. After completion of the FFA, the cannula was removed and an IOP check was completed to ensure that dilation of the pupil had not caused an increase in pressure (and to treat such an occurrence is required).

The morning session was similar to that of the evening, given we were looking for differences between the two sessions. However, some of the aspects did not need to be

repeated - such as fundal photography. The shorter session was beneficial as the participants often needed to arrive close to six AM based on their MCTQ result. As with day one, cannulation with bloods drawn and sent to the lab, was followed by dilation of the pupil, OCT and FFA investigations. Again, the IOP was checked and testing was complete.

Data Analysis

For FFA analysis, the macula was divided into 3 regions based on the Early Treatment Diabetic Retinopathy Study (ETDRS) grid, comprising 1) central fovea, 2) inner macula and 3) outer macula (Fig. 3 above image). Quantification of microvascular permeability was completed with ImageJ analysis. ImageJ software was also used in the analysis of central foveal choroidal thickness and layers of the retina 500 microns either side of the fovea were measured from OCT scans (Fig. 3 image below). All images were analysed at both AM and PM testing times for later comparison.

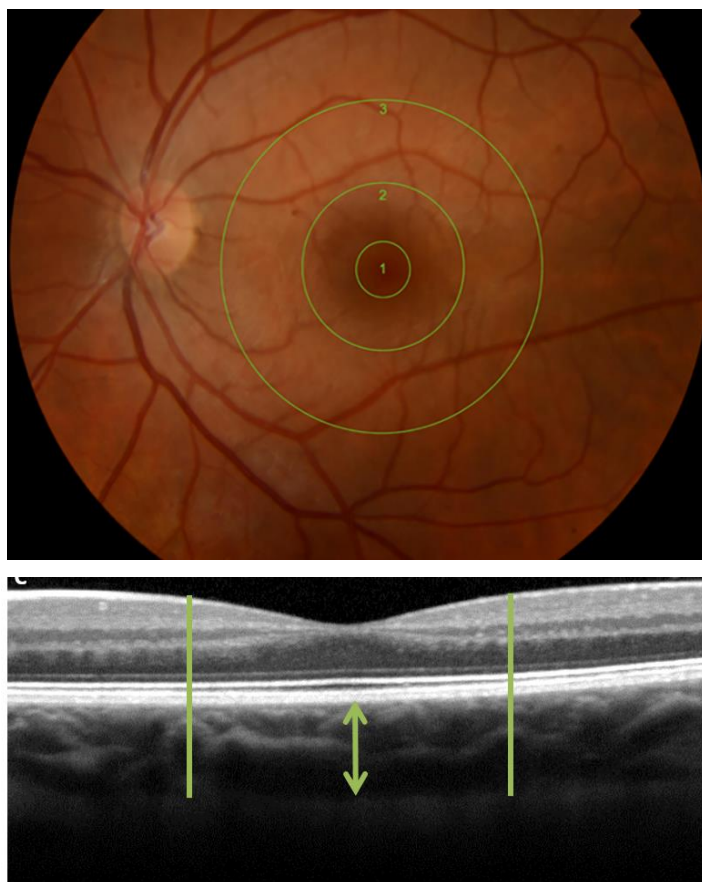


Figure 3. Above: Retina with ETDRS zones overlaid. Below: OCT showing the measurement locations of the Central Foveal Choroidal Thickness and lines drawn demonstrating 500 microns either side of fovea where thickness measurements were taken for individual layers

With regards to statistical analysis, when the two individual experimental groups were being analysed a 2-tailed Student's t test was used for statistical analysis with a P value less than or equal to 0.05 representing significance. The error was measured as standard error of the mean.

Results

Participant Demographics and MCTQ Results

Male n=11			Female n=4		
	Mean	Std Dev		Mean	Std dev
Age	24.9	3.02	Age	26.5	2.52
Height	181	6.56	Height	158	6.93
Weight	83	17.49	Weight	57.25	6.95
Race	Caucasian	11	Race	Caucasian	3 of 4
				Asian	1 of 4

Table 2. Participant basic information

The volunteer demographics (Table 2) and the results of their MCTQ (Table 3). From which we can see that patients were testing correctly in the evening prior to their correct mid sleep time and then post this time in the morning.

	MSFsc	PM time pre MSFsc	AM time post MSFsc
Average:	5.26	9.75	2.39
Std. Dev.	1.21	1.17	1.14

Table 3. Participant MCTQ analysis showing average free day, socially corrected mid sleep cycle time (MSFsc) and time pre-MSFsc for PM test and time post-MSFsc for AM test

FFA

The difference between FFA investigations at the morning and evening sessions is shown in a representative case, (Fig. 4) it is notable that the change in permeability was visible to the naked eye. Quantification analysis of the central foveal (denoted CFT in Fig. 5), inner macula, CFT/inner, outer macula and total area shows there is significant differences with the vasculature being 'leakier' in the PM compared to AM (n=15 participants) (Fig. 5). Fluorescein signal was evident and more prolonged in the evening compared to the morning in the same subject and this was significantly increased in all macular regions analysed (n = 15 subjects, ***P < 0.001).

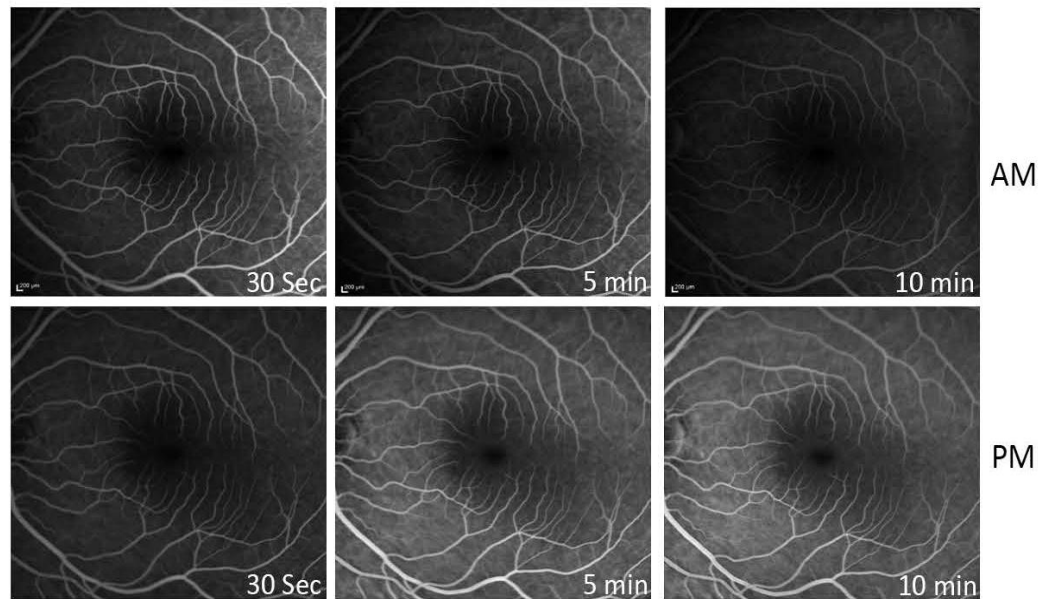


Figure 4. Representative image showing retinal permeability in the AM (top row) versus PM (bottom row) at 30 sec, 5 min and 10 min post-sodium fluorescein injection

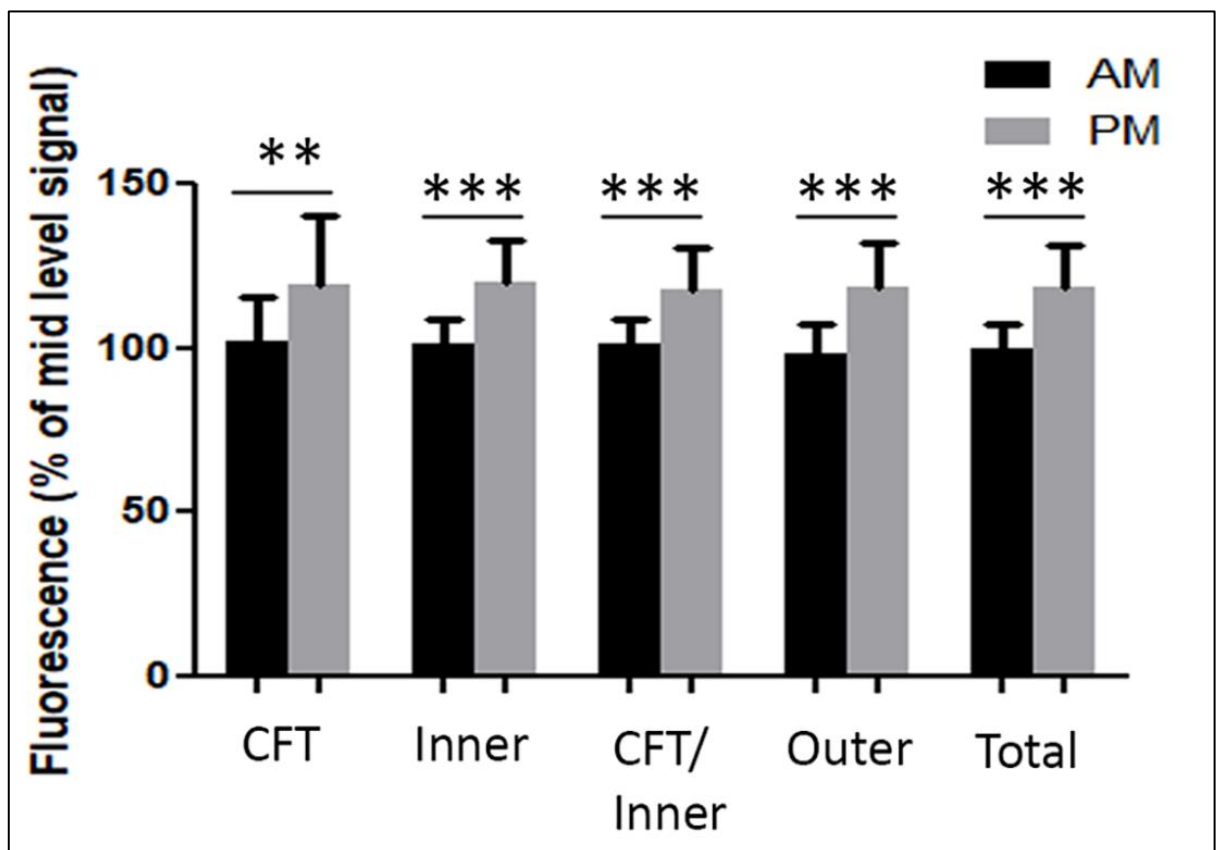


Figure 5. Quantification analysis of the central foveal (CFT), inner macula, CFT/inner, outer macula and total area shows there is significant differences with the vasculature being 'leakier' in the PM compared to AM (n=15 participants). Fluorescein signal was evident and more prolonged in the evening compared to the morning in the same subject and this was significantly increased in all macular regions analysed (n = 15 subjects, ***P < 0.001)

OCT

By analysis of the central foveal choroidal thickness, macular zone volume and individual layers of the retina at five hundred microns from the fovea (Fig 3.) there were no significant differences between morning and evening scans (Fig 6.). This measurement was affected by the maximum resolution of the OCT scans themselves, the small size of individual layers and difficulty delineating exact transition from one layer to another. It is then not possible to say that there is definitively no change in the thickness, but we can say that under the current investigative constraints no change was appreciable.

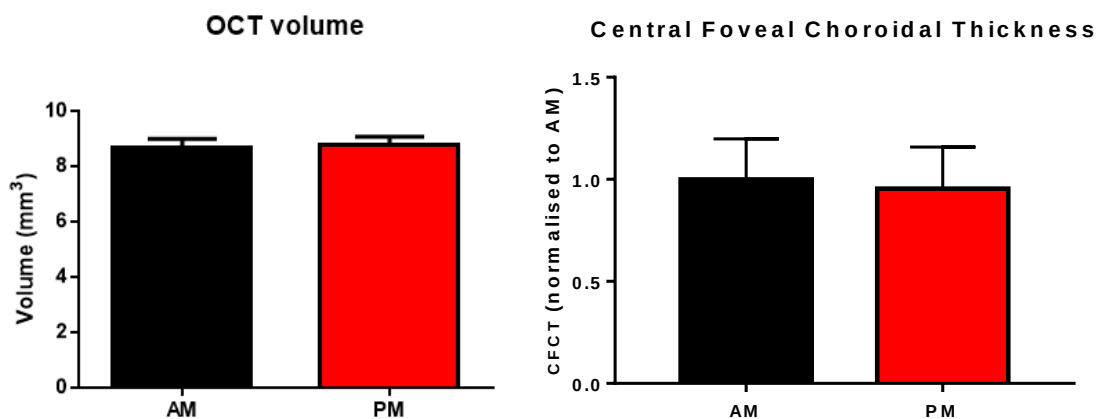


Figure 6. Volume OCT quantification (left), non-significant (n = 15). OCT, central foveal choroidal thickness (right), non-significant (n = 15 subjects). Statistical analysis by Student's t test

Blood Cortisol Levels

Blood samples were analysed using an ELISA kit (R&D Systems, Catalogue Number KGE008B) with paired samples for each individual for their morning and evening sessions. The results show a significant increase in blood cortisol in the morning sample (Fig. 7).

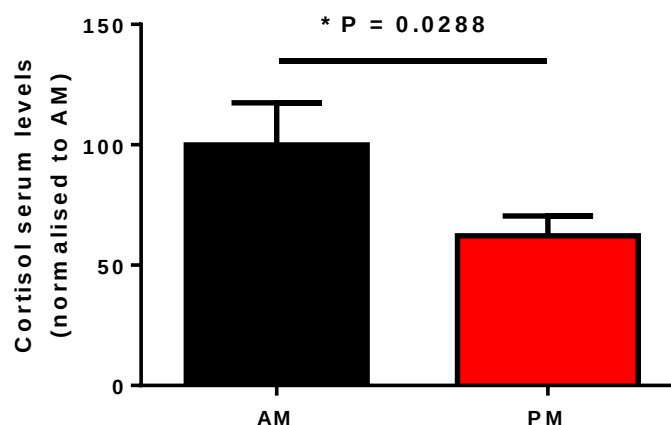


Figure 7. Cortisol ELISA, significant increase in AM compared to PM ($P < 0.05$) (n = 15)

Discussion

The primary causative factors remain unknown in AMD, be it inner or outer retina derived or a mix of both. Is it initially a cellular dysfunction or related to the vasculature? What is clear by review of the literature is that the continued assumption made by many groups is that AMD is an RPE and/or outer retinal only disease, which then secondarily affects other structures, has not yet led to a convincing explanation for the pathophysiology. This assumption does not have solid supporting evidence, and indeed, our study shows that circadian controlled clearance via the inner retinal vasculature is a strong candidate for a key mechanism driving pathology. Much of the evidence from previous studies presented does in fact support a role of the inner retina in the development of AMD. Indeed, changes in the inner retina are noted as early as those in the outer retina. However, many authors work to tie this fact into the traditional assumptions about the pathogenesis originating in the RPE, which may in fact be preventing investigation of the true origins of the pathology. Consideration of the inner retina and iBRB is thus an important step in the journey to understanding AMD.

Our study shows for the first time, to our knowledge, that the retinal vasculature of the human eye varies in permeability with respect to an individual's circadian rhythm. We believe this represents a normal physiological process. Conceptually this process is easy to hypothesise: that the eye, such a highly tuned and critical organ now and throughout our evolution, uses the time when we sleep to clear and replenish materials as needed so as to be ready for the next day. Before beginning the experiments, we believed we would find this physiological process in humans, with it being present in NHPs and mice before that. However, the strength of the signal was a shock. As shown in Figure 4 one can see the difference with the naked eye.

What, then, is the pathological relevance of this newly identified physiological process? The animal studies from our group that induced a loss or derangement of this variation in retinal vascular permeability caused a pathological process very similar to that seen in AMD. This is a significant finding and shows a strong likelihood that such a derangement in humans is leading, at least in part, to the development of AMD. An important point to consider is that even if we set aside the animal studies, given the strength of the signal that is the difference in fluorescein leak into the parenchyma between the two time points,

it is logical that it must be performing a significant physiological process. Evolution would not work to conserve such a process were it not vital. The variation in expression of genes controlled by the circadian clock allowing for, at different times, alterations in tight junctions is fascinating. This ability to produce a variation in permeability, if it were not essential for maintenance of optimal functionality of the eye, would surely have been discarded long ago.

The normal healthy human data provided in this thesis, similar to the mouse and NHP findings before it, show a dynamic iBRB. The relative opening of the iBRB in our expected sleeping hours likely pairs with the expression of claudin-5, and together would allow for removal of the material phagocytosed by the RPE each day. Aberrancy of such a process would see a build-up of such material, which we believe represents a critical role of this cycling of the iBRB in the pathophysiology of AMD.

Our group postulates that disruption of the iBRB in a size selective and chronic manner leads to the build-up of material in the RPE. The important role delivered by a variable retinal interstitial kinesis, in which retinal vasculature exhibits a cycling circadian-entrained permeability, could allow for a daily clearance of waste product material and delivery of substrate to the photoreceptors. Our group previously found that low levels of claudin-5 expression paired with a cholesterol enriched diet resulted in a rapid pathological effect on the RPE (Hudson et al., 2019). Smoking and a poor diet have long been known to be major risk factors for AMD (Clemons et al., 2005). An explanation as to why in certain individuals smoking and consuming a poor diet does not lead to AMD progression, could be that it requires risk factors in tandem with altered expression of claudin-5, with resultant dysregulated vascular permeability then leading to pathology.

The question that follows - why is this pathological process age related? There are several possible answers. Firstly, that it is a slow process that takes time for the effects of reduced delivery and clearance efficiency to cause deleterious effects. This is plausible and certainly is a factor in the presentation of patients with dry AMD who due to the effects taking place over time can get used to slight changes in vision and present later with more damage existing, compared to a process which comes on quickly as is the case in wet-AMD. This part of the explanation, however, gives little insight into or recognition of, possible causative effectors. The relationship between increasing age paired with circadian dysregulation that often occurs in advanced age offers more insight than simply

more time to occur. Indeed, the total time asleep might remain the same, with a loss of quality affecting clearance which might only occur during a single stage, such as deep sleep or REM. For now, this is simply conjecture. We do know that the proposed loss of tight regulation of this daily interstitial process of material clearance from the neural retina matches with the time in an individual's life when sleep becomes disturbed. Thus, we propose that the RPE will experience an overload of dietary components and spent metabolites which have failed to be cleared by the normal mechanisms leading to the formation of drusen.

Conclusion

Here we performed quantitative FFA in healthy human subjects aged 18 – 30 years old, in the morning and evening to assess for any changes in retinal vascular integrity. We also performed OCT and took peripheral blood samples at each time point. We have shown that the iBRB displays circadian variation in healthy subjects. By analysis of volume OCT scans there is no significant change in retinal thickness. Cortisol levels varied as expected being elevated in the morning samples which acts to confirm the correct time of testing with respect to the circadian clock.

We have shown that there is a significant increase and more prolonged fluorescein signal in the evening compared to the morning in healthy volunteers. To our knowledge this is the first time in healthy humans that a circadian driven change in permeability has been shown to exist. A systemically injected tracer molecule, fluorescein, thus in human subjects undergoes a potential size-selective passive diffusion from the inner retinal vasculature to the retinal parenchyma with diffusion towards the outer retina and RPE. Such an inner retina derived supply of systemically derived components to the photoreceptor outer segments and RPE has not been described previously and may represent a critically important physiological process central to the development of a range of retinopathies including age-related macular degeneration (AMD).

We postulate that, based on our groups previous animal studies paired with these findings in humans, this may represent a key feature in the continued optimal function of the eye and moreover, that disruption is the possible, previously unknown, driver in the pathophysiology of AMD. This is an exciting finding, as without understanding of the pathophysiology treatments will only be found with a good deal of luck. If we are correct, the possibility of restoring normal physiology with the cycling of claudin-5 and associated cycling of permeability, could prevent AMD from causing further damage and maybe even see restoration of VA.

As we do not yet know the potential changes in retinal permeability with aging, these proposed theories remain just that, theories, but exciting ones that lead to further investigation and edge us closer to understanding the pathophysiology of AMD and potentially other age related pathologies. The next steps will include repeating this

experiment on a range of different groups including those with dry AMD and age matched controls, we would expect differences in these groups where the cycling of retinal permeability is dysregulated in the affected group. It is possible that sleep analysis and FFA studies pre and post sleep may one day form the standard work-up for early AMD or even those with a family history as a screening programme in which a change in retinal permeability rather than drusen or visual loss are the presenting factors. We are very excited by this potential avenue as it has the potential to greatly reduce the global impact of this blinding condition.

In summation, this thesis outlines the finding that retinal vascular permeability varies in a manner dependant on an individual's circadian rhythm. Our lab has shown in non-human studies that this process relates to the cycling of claudin-5 which, in conjunction with our current findings implicate a component of the inner retina as the early change occurring in the pathophysiology of AMD. We propose that in humans, as is the case in the animal studies completed by our group, that claudin-5 as a key mediator in the pathology of the AMD. Claudin-5 is then a targetable effector, as by restoring the natural physiological, dynamic expression of this molecule we could restore the integrity of the iBRB. With AMD most likely a pathology relating to dysregulated clearance mechanisms, this would represent a novel therapeutic target for GA and has the potential to prevent, halt, or even reverse the pathophysiology of AMD.

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Appendices

(i) Health Questionnaire / Consent for Enrolment in Study

Volunteer Consent for Enrolment into Study

Dear Sir/Madam,

Thank you for agreeing to take part in our study. This study is related to the development of age-related macular degeneration (AMD) which is the most common cause of sight loss in those aged over 50. It causes a gradual loss of central vision, which we need for detailed work and for things like reading and driving. Edge vision (peripheral vision) is not lost.

There are two types of AMD, dry and wet. Dry AMD is treated by lifestyle changes, stopping smoking and taking specific vitamins. Dry AMD is the focus of this study.

How AMD develops is not fully understood, in this study we are investigating just this. Being part of this study will not help your own vision at this time however by understanding AMD better it will help future research into treatments helping others who may go on to develop AMD.

We believe that the changes in the retina, the 'film' in the back of the eye, are related to each person's sleep-wake cycle (circadian rhythm). The eye appears to use the time while you sleep to prepare for the next day, we want to see if changes in this normal process play a role in developing AMD.

This study involves tests which you may have had before. As we are investigating changes related to the sleep wake cycle it requires coming in on two occasions, once in the evening and once in the morning. We do this on two separate days. Volunteers will be asked to complete a questionnaire about themselves, their health and their sleep habits before the first day.

In keeping with GDPR, it is important to know that all data, we collect about you will be password protected and nothing with your name on it will be used where people not involved in the study could see.

The format of each day is as follows:

Volunteer day 1 (testing in the evening)

You will be asked to read through information on the study and sign a consent form if you have not already done so.

When you arrive, your sight will be checked, and height and weight measured.

Drops will be put in to make your pupils bigger, this will give us a better view of the back of your eye. This will cause your vision to be blurry for 4 to 5 hours and you should not drive but there is no permanent effect on vision from having any of these tests done.

A doctor will then take some blood samples and leave a cannula (a drip) in the vein for fluorescein angiography (FFA), which is explained in detail below.

Pictures of the back of the eye will be taken, these images will be reviewed by an ophthalmologist (eye specialist doctor) and if we detect something that needs further discussion we will get in touch. This is not a formal examination of your eyes and does not affect your usual eye care.

We then perform **ocular coherence tomography (OCT) which** is a test that uses special light rays to scan the retina. It can give very detailed information about the layers in the back of the eye. This is a very common, pain free test. In our clinics, this test is useful when there is doubt about whether AMD is the wet or dry form, and to monitor treatment.

Fluorescein angiography (FFA) is a photographic test of the retina, the ‘film’ in the back of the eye used in the diagnosis and to assess the effect of treatment in several eye conditions. A water-soluble dye called fluorescein is injected into a vein in your arm, where it travels through the body reaching the eyes. A special camera is used to take multiple photographs of the back of your eye as the dye passes through the blood vessels the process involves bright flashes of light and sitting still for 10 minutes.

After fluorescein angiography, your skin will turn yellow for several hours. This will fade as the dye is filtered out by the kidneys – this leads to the urine turning a dark yellow-orange colour for up to 24 hours.

Fluorescein angiography is a safe and very helpful test. The chance of adverse effects is low, but as with any medical procedure there are some risks involved. Some patients experience nausea during the angiogram, which usually passes within seconds. Some patients may vomit. Occasionally some fluorescein will leak from a fragile vein, which may cause a localised burning feeling and yellow staining of the skin. The burning usually lasts a few minutes and the staining takes a few days to disappear.

Some patients may experience allergic reactions to the fluorescein dye. The most frequent allergic reaction is a skin rash, which is often itchy and may appear within

minutes after the fluorescein injection. More severe allergic reactions are fortunately rare.

The most severe allergic reaction is called anaphylaxis, which is rare but may be life threatening. Very rarely patients may experience breathing difficulty or heart rhythm disturbances, which can be severe and even cause death. If you feel any itching, tingling in the lips or tongue, difficulty breathing or pain during or after the angiogram, let us know immediately. You may require medication to control the reaction, and your condition will be monitored until it has resolved. Delayed reactions are very uncommon, but it is important that you tell us if you experience any delayed effects that you feel may be related to your fluorescein angiogram.

After the tests are completed the doctor will then check the pressure in your eyes. This is because dilation of the pupil can sometimes cause an increase in eye pressure and by checking we ensure this has not occurred and can treat it if it has.

Volunteer day 2 (testing in the morning)

Day two is similar to day 1 however it is quicker as there are several tests which do not need to be repeated and only one eye will be dilated.

On arrival we will check your vision again and then dilate one eye and take bloods, we leave the cannula (the drip) in your vein for the fluorescein to be put in as was the case on day 1.

We then repeat the OCT and fluorescein angiogram to compare with those taken on the first day. To finish the doctor will again check the pressure in your eye and testing is complete.

What we will do with results

The blood samples will be used to test for changes between the morning and evening. We will also test for DNA changes which have been seen in AMD before. The images we collect will be analysed and compared. The goal is to combine the results from each volunteer and publish this in scientific journals. We may use your information in further studies, in which again, your data will be treated carefully, password protected and anonymised.

What you need to do

It is very important for you to fill out the questionnaire fully and to tell us about any health conditions that you have, all the medications that you are taking, and especially any allergies to medications or shellfish that you have/had in the past. It is preferred that you bring this information as a letter from your doctor. If you have had a previous fluorescein angiogram, please let us know. You do not need to fast for this test.

Fluorescein is reported to be safe in pregnancy, but we prefer to avoid this test in pregnant patients if possible. Please advise if you are pregnant or suspect you could be pregnant. If you have any further questions or concerns, please ask us.

On the day of testing it is helpful to bring sunglasses as this can help reduce blurred sight when going into the daylight after the tests.

Thank you for taking the time read this information and being involved in this study. We are happy to answer any further questions you might have. Having considered this information, we ask you to sign below and we can start organising your visits.

CONSENT FOR ENROLEMENT IN STUDY

I, _____ have read, or have had read to me, the above information concerning the procedures involved in this study. I understand this information, and any questions that I have asked have been answered to my satisfaction.

In accordance with GDPR you are consenting to our use of the information/ bio-samples you are supplying and information that will be generated from these to be used with the purpose of publishing in the scientific community in relation to this and other future studies conducted.

I consent to being enrolled in this study

Medical Practitioner _____

Date / /

(ii) FFA Consent

Fluorescein Angiography Information and Consent

What is Fluorescein Angiography?

Fluorescein angiography is a photographic test of the retina, the ‘film’ in the back of the eye. A water- soluble dye called fluorescein is injected into a vein in your arm, where it travels through the body reaching the eye. A special camera is used to take multiple photographs of the back of your eyes as the dye passes through the blood vessels, providing information about the retina and nearby tissues. Depending on the procedure employed, this may involve bright flashes of light.

Why is Fluorescein Angiography performed?

Fluorescein angiography is used to diagnose certain eye conditions, determine if treatment is possible, and plan or guide treatment. Common conditions requiring fluorescein angiography include diabetic retinopathy, macular degeneration, and retinal vascular diseases. The angiogram may need to be repeated to monitor your response to treatment or changes in your eye condition.

What are the side effects of Fluorescein Angiography?

After fluorescein angiography, your skin will turn yellow for several hours. This will fade as the dye is filtered out by the kidneys – this leads to the urine turning a dark yellow-orange colour for up to 24 hours. Your vision will be blurred and you should but there is no permanent effect on the vision from having the test done.

Are there any risks?

Fluorescein angiography is a safe and very helpful test. The chance of adverse effects is low, but as with any medical procedure there are some risks involved. Some patients experience nausea during the angiogram, which usually passes within seconds. Some patients may vomit. Occasionally some fluorescein will leak from a fragile vein, which may cause a localised burning feeling and yellow staining of the skin. The burning usually lasts a few minutes and the staining takes a few days to disappear.

Some patients may experience allergic reactions to the fluorescein dye. The most frequent allergic reaction is a skin rash, which is often itchy and may appear within minutes after the fluorescein injection. More severe allergic reactions are fortunately rare.

The most severe allergic reaction is called anaphylaxis, which is rare but may be life threatening. Very rarely patients may experience breathing difficulty or heart rhythm disturbances, which can be severe and even cause death. If you feel any itching, tingling in the lips or tongue, difficulty breathing or pain during or after the angiogram, let us know immediately. You may require medication to control the reaction, and your condition will be monitored until it has resolved. Delayed reactions are very uncommon, but it is important that you tell us if you experience any delayed effects that you feel may be related to your fluorescein angiogram.

Common reactions

Nausea (2 to 3 per 100)

Vomiting (1 to 2 per 100)

Urticaria/pruritus (3 to 5 per 1000)

Vasovagal reaction – low - reliable estimates not available

Injection site complications – low - reliable estimates not available

Uncommon reactions

Severe reactions – anaphylaxis, severe asthma/bronchospasm, cardiac arrhythmia, myocardial infarction, cardiac arrest, seizure (1:1,900 – 1:18,000)

Death (1:50,000 to 1:222,000)

Other information

It is very important for you to tell us about any health conditions that you have, all the medications that you are taking, and especially any allergies to medications that you have had in the past. It is preferred that you bring this information as a letter from your doctor. If you have had a previous fluorescein angiogram, please let us know. You do not need to fast for this test.

Fluorescein is reported to be safe in pregnancy, but we prefer to avoid this test in pregnant patients if possible. Please advise if you are pregnant or suspect you could be pregnant.

If you have any further questions or concerns, please ask us before you have your angiogram.

CONSENT TO FLUORESCEIN ANGIOGRAPHY

I, _____ have read, or have had read to me, the above information concerning the procedure of fluorescein angiography. I understand this information, and any questions that I have asked have been answered to my satisfaction.

I consent to the procedure being performed _____

Medical Practitioner _____

Date / /

(iii) MCTQ

Munich ChronoType Questionnaire (MCTQ)

Instructions:

In this questionnaire, you report on your typical sleep behaviour over the past 4 weeks. We ask about work days and work-free days separately. Please respond to the questions according to your perception of a standard week that includes your usual work days and work-free days.

Personal Data

Date:	_____
Name:	_____
eMail:	_____
Age:	_____ years
Sex:	female ☐ male ☐
Height:	_____ cm
Weight:	_____ kg
Country:	_____
City:	_____
Postal Code:	_____

Participant ID:

MCTQ, English, Version 2015-01
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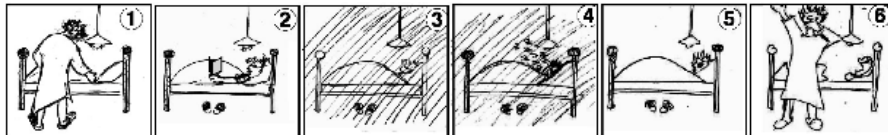
MCTQ

I have a regular work schedule (this includes being, for example, a housewife or househusband):

Yes ☐ I work on 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ day(s) per week.

No ☐

Is your answer "Yes, on 7 days" or "No", please consider if your sleep times may nonetheless differ between regular 'workdays' and 'weekend days' and fill out the MCTQ in this respect.



Please use 24-hour time scale (e.g. 23:00 instead of 11:00 pm)!

Workdays

Image 1: I go to bed at _____ o'clock.

Image 2: Note that some people stay awake for some time when in bed!

Image 3: I actually get ready to fall asleep at _____ o'clock.

Image 4: I need _____ minutes to fall asleep.

Image 5: I wake up at _____ o'clock.

Image 6: After _____ minutes I get up.

I use an alarm clock on workdays: Yes ☐ No ☐

If "Yes": I regularly wake up BEFORE the alarm rings: Yes ☐ No ☐

Free Days

Image 1: I go to bed at _____ o'clock.

Image 2: Note that some people stay awake for some time when in bed!

Image 3: I actually get ready to fall asleep at _____ o'clock.

Image 4: I need _____ minutes to fall asleep.

Image 5: I wake up at _____ o'clock.

Image 6: After _____ minutes I get up.

My wake-up time (Image 5) is due to the use of an alarm clock: Yes ☐ No ☐

There are particular reasons why I cannot freely choose my sleep times on free days:

Yes ☐ If "Yes": Child(ren)/pet(s) ☐ Hobbies ☐ Others ☐, for example: _____

No ☐

Participant ID:

MCTQ, English, Version 2015-01
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(iv) DPIA

Royal Victoria Eye & Ear Hospital

Data Protection Impact

Assessment Template (DPIA)



Privacy Impact Assessment

Section 1: Background Information

DPIA Reference #

Threshold Assessment Reference #	
Project Name	Analysis of circadian differences in inner blood-retina barrier function
Project Type <i>Note: If your selection of choice is a non-IT based project from the list, please complete section 2. For IT based projects, skip section 2.</i>	Clinical Trial
Organisation/Department	RVEEH / TCD Genetics Department
Assessment Completed By	Alan Hopkins
Job Title	Research Fellow
Date completed	30/04/2019
Phone/Mobile	0872622749
E-mail	ahopkin@tcd.ie

Project/Change Outline: *This involves a brief summary of the project described in layman's terms. If an assessment has already been produced as part of Project Initiation Document (PID) or Business Case (BC) or Research Application (RA) etc., it should be referenced here.*

The retina at the back of the eye allows you to perceive light and to convert light into a form of energy that allows our brain to form images. Essentially, the retina can be considered as an accessible part of the brain. Similar to the brain, the retina requires a constant supply of oxygen enriched blood and has an intricate network of blood vessels. These blood vessels are easy to see when an eye doctor examines your eye. The study being proposed will allow us to determine how these blood vessels may differ in integrity at various times of the day and how this might relate to the development of the common form of blindness in the elderly, namely, age-related macular degeneration (AMD). We hypothesize that discrete changes in these blood vessels with age, may pre-dispose some people to developing AMD during their lifetime. In understanding the basic mechanisms underlying this disease, we may be better able to develop new forms of therapy.

This project has previously been approved in healthy controls (by ethics committee RVEEH) aged 18 to 30. Here we look to extend it to testing patients with AMD. The completed healthy control subjects has had no adverse outcomes and shows significant and important results.

Project Objective: *This outlines the rationale for a DPIA. This could be the project objective or the purpose (e.g. new IT system implemented or data survey for clinical trial).*

To assess circadian regulation of iBRB integrity in three groups:
healthy subjects aged 18-30 years old (30 participants) - Complete
individuals diagnosed with dry AMD (100 patients)

-at various stages of disease progression (AREDS scale 2-4).
 healthy age matched controls (100 participants)
 Recruitment, both male and female in each of these groups
 Assessment of each person's individual chronotype by the Munich Chronotype Questionnaire (MCTQ) to determine most appropriate timings for assessment
 Make a full assessment of circadian iBRB integrity in each group
 -Imaging using qFFA and OCT at both morning and night.
 -Bloods collection and analysis for:
 Inflammatory status
 Serum and plasma to correlate cortisol levels
 Analysis of systemic biomarkers:
 Participants with dry AMD will have DNA isolated and will subsequently be genotyped for AMD risk variants.

Data analysis

Analyse FFA and OCT data
 Analysis of inflammatory status
 Analysis of blood biomarkers (plasma/serum)
 Stratify data according to AMD risk variants to assess if disease progression and risk variant are potential linked to changes in retinal permeability.
 Preparation of data for publication and dissemination

Why is Data Collection Required? For example, sustained patient care, patient treatment, patient administration, research, audit, reporting, staff administration etc.

Research

What is the Privacy Impact on Project? How will personal data processing affect and impact on the privacy rights of data subjects? Provide a brief summary of what you feel these could be.

All data collected will be stored, anonymised, coded and password protected. All privacy rights of the data subjects will be respected and GDPR compliant.

Has a DPIA been previously developed? If response to this question is Yes, please provide details of any previous DPIA or other form of personal data compliance assessment done on this initiative.

No

Stakeholders: Who is involved in this project/change? Please list stakeholders including roles. Also include full list of internal, external, organisations (public/private/third parties) and groups that may be affected by this system/change.

Research team including:

Prof. Matthew Campbell, TCD, Neurovascular Genetics Department
 Mr Mark Cahill, RVEEH, Consultant VR Surgeon/ PI
 Dr Natalie Hudson PhD., TCD, Postdoctoral research fellow
 Dr Alan Hopkins, RVEEH/TCD, Research Fellow
 Dr Aisling Naylor, RVEEH/TCD, Research Fellow

Patents/ Subjects

AMD patients
 Age matched controls
 Patients groups, e.g. 'MIST'
Organisations
 RVEEH
 TCD

Section 2: Governance.

There is now an obligation imposed on health-based research projects to comply with the requirements of the Health Research Regulation 2018. This section ensures that relevant research infrastructure and governance processes are in place to ensure compliance and accountability. Please answer all questions in this section if your project is based on health research.

Question	Yes	No	Comments
<i>Ethical approval has been sought and given by Research Ethics Committee (REC).</i>	☒	☐	Ethical approval granted for healthy controls currently seeking approval for extension to AMD patients and age matched controls
<i>Research practice proposed is, in the view of the REC, is appropriate.</i>	☒	☐	
<i>Controller (Institution) involved in the health research is qualified to carry out the research concerned.</i>	☒	☐	
<i>Controller (Institution) who will be carrying out the health research concerned is independent of entity funding or otherwise supports, the health research.</i>	☒	☐	
<i>Roles of the Investigator(s), Sponsor(s), Researcher (s) have been defined and are clearly understood.</i>	☒	☐	
<i>Project will operate under specific code of practice or Protocol (Provide COP and/or Protocol details in the comments section).</i>	☒	☐	
<i>Rules around data sharing have been defined, outlined and explicitly agreed.</i>	☒	☐	

<i>Personal data to be used for project will comply with Data Minimisation principle.</i>	☒	☐	
<i>Unless explicitly stated, personal data by default, will be pseudonymised before processing takes place.</i>	☒	☐	
<i>Research personnel are familiar with the data protection principles and have received appropriate data protection training.</i>	☒	☐	
<i>Measures have been taken to ensure that participation of individuals in the health research will be informed and voluntary.</i>	☒	☐	
<i>Will the project require obtaining consent from participants?</i>	☒	☐	
<i>Has explicit consent has been obtained from participants, prior to start of research, for the processing of personal data for the purpose of specified health research, either in relation to an area or more generally in that area or a related area of health research, or part thereof</i>	☐	☐	
<i>Is the legal basis for processing of personal data based on “public interest”?</i>	☒	☐	
<i>Does public interest in carrying out the research significantly outweigh the public interest in requiring the explicit consent of the data subject?</i>	☒	☐	All investigations and data acquisition are explained and obtained with voluntary consent
<i>Has a Declaration of Consent been applied for?</i>	☐	☒	
<i>Has a Declaration of Consent been approved? Enter reference # in comments</i>	☐	☒	
<i>Data protection implications on the rights and fundamental freedoms of data subjects are known and understood.</i>	☒	☐	
<i>Measures are in place to protect the security of the personal data concerned.</i>	☒	☐	Password protected, anonymised and stored in secure facilities
<i>Adequate measures and safeguards have been taken to protect the privacy of individuals participating in the health research and the confidentiality of their personal data.</i>	☒	☐	

<i>Technical and organisational measures designed to ensure that processing is carried out in accordance with the Data Protection Regulation, together with processes for testing and evaluating the effectiveness of such measures.</i>	☒	☐	
<i>Controls to limit access to personal data in order to prevent unauthorised consultation, alteration, disclosure or erasure of personal data have been put in place.</i>	☐	☐	
<i>Arrangements to pseudonymise personal health data are in place.</i>	☒	☐	
<i>Arrangements to anonymise, archive or destroy personal data once the health research has been completed are in place.</i>	☒	☐	
<i>Arrangements to ensure that personal data are processed in a transparent manner are identified and in place.</i>	☒	☐	

Section 3: Personal Data to be Processed

What data is being collected, shared or used? (Use a chart, or workflow diagram to explain where necessary).			
	Data Type	<i>Explanation – Collection of personal data must be fair, justifiable and reasonable. These must be specified here – consider which data items you could remove, without compromising the overall needs of the project?</i>	
Information that identifies the individual and their personal characteristics	Name	☒	Personal details taken for assessment of sleep wake cycle.
	Address	☐	
	Postcode	☐	
	Dob	☒	
	Age	☒	
	Sex	☒	
	Gender	☐	
	Racial/Ethnic origin	☒	
	Tel no.	☐	
	Physical description	☐	
	Mobile/home phone no.	☐	
	Email address	☒	

	Yes	N/A	Justifications
Will information relating to an individual's physical or mental health or condition be processed?	☒	☐	Physical health details required as may affect results if unaware of same.
Will data concerning health or biometric information relating to an individual be processed?	☒	☐	Ophthalmic investigations FFA and OCT and bloods will be analysed post anonymisation
Will personal data relating to an individual's sexual life be processed?	☐	☒	
Will processing involve personal data relating to the family life of a natural person including social circumstances.	☐	☒	
Will processing involve personal data relating to any offences or crime committed or alleged to be committed by a natural person.	☐	☒	
Information relating to criminal proceedings, outcomes and sentences regarding a natural person.	☐	☒	
Information which relates to a natural person's academic achievement (e.g. details of qualification and professional training).	☐	☒	
Information which relates to a natural person's employment and career history.	☐	☒	
Information relating to the financial affairs of a natural person.	☐	☒	
Information relating to the individual's religion or other beliefs.	☐	☒	
Information relating to the individual's membership of a trade union.	☐	☒	

Section 3: Assessment of Privacy Risks

	Question	Response	Required Action E.g. Seek Information Governance advice
Lawfulness fairness and Transparency?	Outline the legal basis for personal data processing. <i>This should include which lawful conditions for processing under Articles 6 and 9 of GDPR and the common law duty of confidentiality.</i>	“the data subject has given consent to the processing of his or her personal data for one or more specific purposes” All those involved in the study are aware of their duty of confidentiality and the need for consent to be adhered to.	
	a - Is personal data processing likely to interfere with the ‘Right to Private Life clause’ under Article 8 of the European Convention on Human Rights (ECHR)?	No	
	b – Has a social need for processing been identified?	Yes, help develop potential treatment for common ocular diseases	
	c- Are planned response actions proportionate in response to social need?	Yes	
	It is important that patients affected by the project initiative are informed as to what is happening with their personal information. Is this covered by fair processing information already provided to individuals or is new or revised communication needed?	Covered in consent	
	If you are relying on consent to process personal data, how will consent be obtained and recorded, what information will be provided to demonstrate compliance with the consent process and what will you do if	Medical doctor will discuss investigations and information to be collected prior to the day of investigation. On	

	permission is withheld or given but later withdrawn?	the day this again will be discussed and if happy to proceed written consent must be obtained prior to involvement. Subjects are free to refuse or withdraw consent at any time and this will be respected.	
Purpose Limitation	Does project involve use of existing personal data for new purposes?	No	
	Are potential new purposes likely to be identified as project scope expands?	Yes, however we state these circumstances in consent and should they not be included in our consent then we would obtain consent where appropriate	
Adequacy	Does information meet defined criteria for data quality?	Yes	
Data Accuracy	Can personal data be amended where required or necessary to ensure currency and accuracy?	Yes	
	What is the guarantee that personal data obtained from individuals or other organisations is accurate?	All personal data comes from volunteers	
Data Retention	What are the retention periods for the personal data and how will this be implemented?	For the duration of the study	
	Where required, what measures are in place to ensure pseudonymisation of personal data?	All samples collected assigned an individual code so as to avoid potential viewing of subject's name during analysis	

	Can pseudonymised data be linked back to source data?	Yes, PI and research fellow have ability to link to source data but can only do so under specific circumstances	
	Are there any exceptional circumstances for retaining certain personal data for longer than is necessary?	Yes, should any unexpected delay occur in the study by which analysis is not completed yet.	
	How will personal data be fully anonymised or destroyed after it is no longer necessary or fit for purpose?	Can be fully anonymised by deleting the encryption key held by PI and research fellow	
	Will personal data be destroyed internally or externally?	Will be destroyed internally	
Rights of the individual	What measures are in place to action requests from individuals (or someone acting on their behalf) for access to their personal information held?	Contact details of the research fellow provided to all subjects who are free to contact at any time and all queries will be responded to.	
Technical & Organisational measures	What procedures are in place to ensure that all staff with access to the patient data have received appropriate data protection / information governance training?	Comply with all training provided by host institutions. Training has been provided by TCD in this area.	
	If using an electronic system to process subject access requests, what security measures are in place?	Password protected, anonymised and held in secure locations	
	How will the information be provided, collated and used?	Collected via a survey which is anonymised and collated using a digital spreadsheet programme.	

	What security measures are in place to facilitate transfer of identifiable information?	Transferred in secure location from acquisition devices to dedicated study hard drive in the host institution, where this data is securely held.	
Data Disclosure & Transfer	Will personal data be disclosed internally/externally in identifiable form and if so to whom, when, how and why?	No	
	Will personal data be transferred to a country outside of the European Economic Area? If yes, what arrangements are in place to safeguard the personal data during transit / transfer?	No	
	Will data transfer take place on a regular basis or occasionally?	No	
Consultation	Who is/are the main point(s) of contact on privacy? <i>Nominated entities should be reachable to identify privacy related risks and how will this be achieved? Identify both internal and external stakeholders.</i>	Between subjects and Research fellows Natalie Hudson and Alan Hopkins	
	Following the consultation – what privacy risks have been raised? E.g. Legal basis for collecting and using the information, security of the information in transit etc.	None	
	List any national guidance/code of practice applicable to the initiative being referred to.	GDPR compliance	