



THE ROLE OF LIPID TOXICITY AND PARA- INFLAMMATION AS POTENTIAL MECHANISMS OF AGE RELATED MACULAR DEGENERATION

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BY

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إهداء إلى

من كانوا يضيئون لي الطريق

ويساندوني ويتنازلون عن حقوقهم لإرضائي و لأعيش في هناء

للاستاذ : سالم حمد الراشد و للاستاذة : سكينه غلوم الصالح

أحبكم حبا لو مر على أرض قاحلة

لتفجرت منها ينابيع المحبة

Abstract

Age-Related Macular Degeneration (ARMD) is the most common cause of legal blindness in the elderly in the western world. One of the earliest signs of aging is the accumulation of lipid rich debris within and underneath the Retinal Pigmented Epithelium (RPE) cells, known as “drusen”. These deposits are considered one of the associated factors in ARMD progression. The sequels of bio-chemical, cellular and molecular events leading to the development of ARMD and the formation of drusen are still poorly understood - mainly because it occurs late in life as well as the lack of appropriate cell and animal models. Understanding the molecular process in the formation of drusen has a great impact in understanding the disease pathology. Here it is hypothesized that that RPE lipo-toxicity (the increased content of lipids within the RPE cell) could be a major factor affecting both the molecular mechanisms and the metabolic responses of the RPE cells leading to changes associated with ARMD pathology. To investigate this phenomenon, aged ARPE-19 cultures were induced to long term lipid loading (60 days) with a free fatty acids (FFAs) mixture that reflects the FFA’s composition in the western diet. This intervention of FFAs ensures the increase of the intra-cellular lipid level within the ARPE-19 cells. What is interesting is that this increase of intra-cellular lipids was found to correlate with a destruction of the ARPE-19 monolayer integrity, an increase in VEGF-A secretion in media and most importantly the production of sub-RPE deposits positive for apolipoprotein E (apoE), vitronectin and Amyloid beta 1-42 ($A\beta$ 1-42), all of which are

prominent constituents of drusen in ARMD patients. These findings supported our hypothesis for lipo-toxicity model of ARMD.

It has been suggested that ARMD is not a classical inflammatory disease, but a product of prolonged para-inflammatory response driven by the over-activation of the complement system. To investigate this statement, the effect of long term complement activation in the presence and absence of lipid loading were studied using a minimum of $n=3$ of the produced cell culture model. Long term (60 days) complement activation intervention showed no effect on ARPE-19 cell culture viability and monolayer integrity unlike the effect seen with chronic lipid loading while lipid loaded cultures, that had their complement activated, had reduced intra-cellular lipid accumulation and intact monolayer integrity and TJ presentation. These observations suggest that the complement system plays a secondary modulating response role to a primary destructive initiator “lipid loading”. Complement activation was found to prevent intra-cellular lipid accumulation and loss of barrier function in RPE cells through shifting intra-cellular lipid accumulation in a lipoprotein particles form which would be expected to be cleared by passing across Bruch’s membrane to the choroidal circulation to be cleared outside the BRB. In this hypothesis it is also suggested the chronic lipo-toxicity conditions might trigger a chronic vicious cycle of para-inflammation driven by the complement activation eventually leads to tissue damage and vision lost.

These findings presented in this thesis suggest the use of aged ARPE-19 cell culture can be a promising model for ocular aged related diseases study

including drusen deposition mechanisms, while the use of ARPE-19 lipo-toxicity model will facilitate the analysis of molecular and cellular characteristics of ARMD pathogenesis, augmenting the therapeutic strategies for dry ARMD.

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr Alex Foss, for the patient guidance, encouragement and advice he has provided throughout my time as his student. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly.

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Poster presentation

Fatema Alrashed, Ian Kerr, Alex Foss and Andrew Bennett. Use of ARPE-19 cell line as an in-vitro model for age related macular degeneration. ARVO annual meeting, 2014, Orlando, Florida

Fatema Alrashed, Ian Kerr, Alex Foss and Andrew Bennett. The role of complement system in age related macular degeneration (ARMD) pathology, a friend of foe? Immunology showcase, 2015, Nottingham, UK.

Oral presentation

Fatema Alrashed, Ian Kerr, Alex Foss and Andrew Bennett. The role of complement system in age related macular degeneration (ARMD) pathology, a friend of foe? Immunology showcase, 2015, Nottingham, UK.

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Declaration

I hereby declare that all work in this thesis was performed entirely by me (unless otherwise stated). The work was carried out while I was a PhD student in the School of Life Sciences, University of Nottingham, Medical School, Queen's Medical Centre. All the experiments were performed by me, under the supervision of Dr Andrew Bennett.

Fatema S. Alrashed



List of Abbreviations

Activation Function (**AF-1**)

Adherents Junctions (**AJ**)

Age-related macular degeneration (**ARMD**)

Aryl hydrocarbon receptor (**AHR**)

Alpha-mannosidase (**α -Mann**)

Alzheimer's disease (**AD**)

Amyloid-Beta (**A β**)

Amyloid precursor protein (**APP**)

Angiotensinogen (**AGT**)

Apolipoprotein A-1 (**ApoA-1**)

Apolipoprotein E (**ApoE**)

Arylsulfatase B (**ASB**)

ATP-binding cassette transporter (**ABCA1**)

B cell lymphoma-6 protein (**BCL-6**)

Beta- glucuronidase (**GUSB**)

Blood Retinal barrier (**BRB**)

Brahma-related gene (**BRG1**)

Bruch's membrane (**BrMs**)

cAMP response element-binding protein (**CREB**)

Catenin beta-1 (**CTNNB1**)

Cathepsin D (**CatD**)

Carboxyethylpyrrole (**CEP**)

Cellular retinal aldehyde binding protein (CRALBP)

Cholesteryl ester transfer protein (**CETP**)

Choroidal Neovascularization (**CNV**)

Cluster of Differentiation 36 (**CD36**)

Coefficient of variation (**CV**)

Complement Component 3 (**C3**)

Complement Component 5 (**C5**)

Complement Factor B (**CFB**)

Complement Factor H (**CFH**)

Diabetic Retinopathy (**DR**)

DNA binding domain (**DBD**)

Docosahexaenoic acid (**DHA**)

Fatty Acid Translocase (**FAT**)

Free Fatty Acids (**FFAs**)

Fatty Acid Binding Proteins (**FABPs**)

Foetal Bovine Serum (**FBS**)

Fold Change (**FC**)

Ganglion cell layer (**GCL**)

Gene ontology (**GO**)

Genome-Wide Association Studies (**GWAS**)

Geographic Atrophy (**GA**)

Heme Oxidase-1 (**HO-1**)

High Density Lipoprotein (**HDL**)

Human Serum (**HS**)

Immunocytochemistry (**ICC**)

Ingenuity Knowledge Base (**IPKB**)

Ingenuity Pathway Analysis (**IPA**)

Inner limiting membrane (**ILM**)

Inner nuclear layer (**INL**)

Inner plexiform layer (**IPL**)

Interleukin (**IL**)

LDLR-related protein (**LRP**)

Lecithin—cholesterol acyltransferase (**LCAT**)

Ligand Binding Domain (**LBD**)

Liver X Receptors (**LXRs**)

Low Density Lipoprotein (**LDL**)

Low Density Lipoprotein Receptor (**LDLR**)

Mannan-Binding Lectin (**MBL**)

Membrane Attack Complex (**MAC**)

Metallothionein (**MTs**)

Mitogen-Activated Protein Kinases (**MAPK**)

N-retinylidene-N-retinyl ethanolamine (**A2E**)

Nerve fibre layer (**NFL**)

Neurogenin 1(**NEUROG1**)

Neuropilins (**NP-1**)

Neuroretina (**NR**)

Nonvascular (**NV**)

Nitric Oxide (**NO**)

Nottingham Arabidopsis Stock Centre (**NASC**)

Nuclear Factor- κ B (**NF- κ B**)

Nuclear Receptor (**NR**)

Outer segments of photoreceptor cells (**POS**)

Outer nuclear layer (**ONL**)

Outer plexiform layer (**OPL**)

Parkinson disease (**PD**)

Peroxisome Proliferator-Activated Receptor (**PPARs**)

Peroxisome proliferator-activated receptor alpha (**PPAR α**)

Peroxisome proliferator-activated receptor delta/beta (**PPAR δ/β**)

Peroxisome proliferator-activated receptor gamma (**PPAR γ**)

Phospholipase C (**PLC**)

Phosphatase and tensin homolog (**PTEN**)

Placental growth factor (**PlGF**)

Protein kinase B (**PKB**)

PPAR responsive element (**PPRE**)

Polyunsaturated Fatty Acids (**PUFAs**)

Reactive Oxygen Species (**ROS**)

Retinal Microvascular Endothelial cells (**RMECs**)

Retinal pigment epithelium (**RPE**)

Retinal pigment epithelia specific protein 65 kDa (**RPE65**)

Retinoid X Receptors (**RXR**)

Reverse Cholesterol Transport (**RCT**)

RNA integrity number (**RIN**)

Robust Multichip Analysis (**RMA**)

Principal components analysis (**PCA**)

Scavenger receptor class B member 1 (**SR-BI**)

Scavenger receptor class B member 2 (**SR-BII**)

Single Nucleotide Polymorphisms (**SNP**)

Sterol regulatory element-binding protein (**SREBF**)

The corrected total cell fluorescence (**CTCF**)

Thiazolidinedione (**TZD**)

Trans-epithelial electrical resistance (**TEER**)

Transforming Growth Factor, Beta 1 (**TGFB1**)

Orthodenticle homeobox 2 (**OTx2**)

Outer blood retinal barrier (**oBRB**)

Inner blood retinal barrier (**iBRB**)

Tight junction (**TJ**)

Vascular Endothelial Growth Factor (**VEGF**)

Very Low Density Lipoprotein (**VLDL**)

Intermediate Density Lipoprotein (**IDL**)

Esterified Cholesterol (**EC**)

Un-esterified Cholesterol (**UC**)

Vascular Cell Adhesion Molecule-1 (**VCAM-1**)

VEGF receptor 1 (**VEGFR 1**)

VEGF receptor 2 (**VEGFR 2**)

Waist to Hip Ratio (**WHR**)

Western Blot (**WB**)

Volume (**vol**)

1

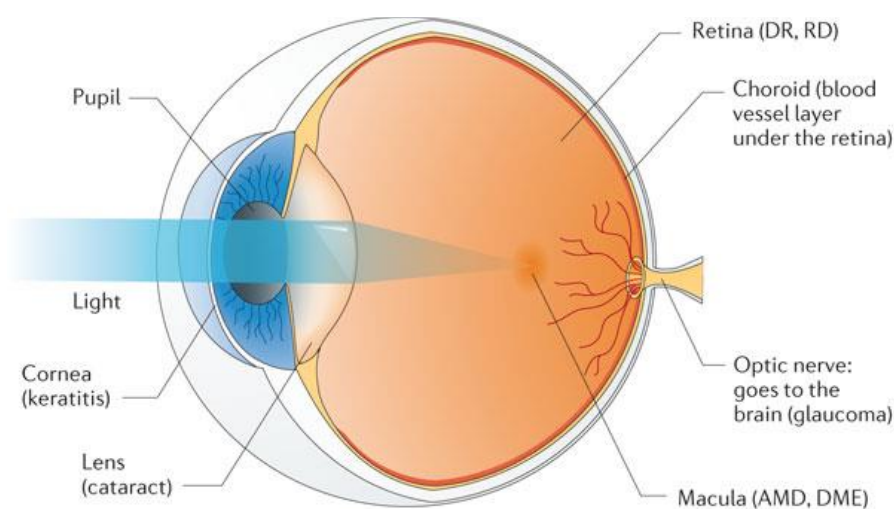
INTRODUCTION

1.1. The Human Eye

The human eye is a highly complex organ that detects photons, processes them and transmits them to the brain to produce vision. There are four main parts in the human eye; The **Protective Structures** of the Eye which consist of the lids and the sclera. The **anterior chamber** is the space in front of the eye which makes up the space between the posterior surface of the cornea and the anterior surface of the iris; it consists of the cornea, the aqueous humor, the iris, the crystalline lens and ciliary muscle. The **posterior chamber** is the space between the posterior surface of the iris and the anterior surface of the lens and is avascular. It consists of the retina which is a thin tissue that contains blood vessels and light-sensitive photoreceptors known as rods and cones and the vitreous humor which is a gel-like structure that fills the posterior portion of the globe. The **visual system** offers pathways to the brain that facilitate the transport of the neural signal that was processed by the retina and it consists of

the optic nerves and optic tracts, the lateral geniculate nucleus and the visual cortex.

The cornea, lens, iris and ciliary body all work together to focus and transmit light onto the retina. Vision is initiated at the retina where photo-receptor cells induce changes in this specialized nerve. The image is transduced into neural impulses which are subsequently relayed to the optic nerve to the brain for processing, where the neuronal electrical signal is processed and consciously interprets as vision **Figure 1.1**.



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Figure 1. 1 Schematic diagram of the structure of the human eye. **Cornea;** Clear outer part of the eye's focusing system located at the front of the eye. **Iris;** Coloured part of the eye that regulates the amount of light entering the eye. **Pupil;** Opening at the centre of the iris that allows light into the eye. **Lens;** Clear part of the eye behind the iris that helps to focus light or image onto the retina. **Vitreous gel;** Clear gel that fills the inside of the eye. **Retina;** Covering on the back of the eye which contains receptors for light detection, similar to the film in a camera. **Macula/Fovea;** Small sensitive area of the retina that gives central detailed vision. **Optic nerve;** Transfers information collected by the receptors in the retina to the brain, where the image is registered as seen. The figure and information are provided from the following article: Ophthalmic drug discovery: novel targets and mechanisms for retinal diseases and glaucoma, Kang Zhang, Liangfang Zhang & Robert N. Weinreb. Nature Reviews Drug Discovery 11, 541-559 (July 2012) doi:10.1038/nrd3745.

1.2. Retina and Retinal Pigment Epithelium

The retina is a light sensitive tissue on the border of the vitreous chamber of the eye and is part of the central nervous system. It receives light, transforms it into image-forming signals and transmits the signals through the optic nerve to the brain. The retina consists of two primary layers: the neurosensory retina and an outer epithelium - the Retinal Pigment Epithelium (RPE)

1.2.1. Structure of Retina

The light-sensitive neurosensory retina is composed of inner and outer layers containing several different cell types **Figure1.2**. The rods and cones of the photoreceptors make up the outer neurosensory layer, while the inner neurosensory retina is composed of many other cell types including Müller cells, ganglion cells, bipolar cells, horizontal cells, amacrine cells and other non-neuronal cells. A histological side section of the retina shows nine organized distinctive layers as shown in Figure 1.2: (1) the RPE; (2) the outer segments of photoreceptor cells (POS); (3) the outer nuclear layer (ONL), a layer of nuclear bodies of rod and cone cells; (4) the outer plexiform layer (OPL); (5) the inner nuclear layer (INL), a layer of nuclear bodies of bipolar cells, horizontal cells and amacrine cells; (6) the inner plexiform layer (IPL), a layer where horizontal cells synapse retinal ganglion cells; (7) the ganglion cell layer (GCL); (8) the nerve fiber layer (NFL), composed of ganglion cell axons which go on to form the optic nerve; and (9) the inner limiting membrane (ILM), the boundary between the retina and the vitreous, formed by astrocytes and the end feet of the Müller cells

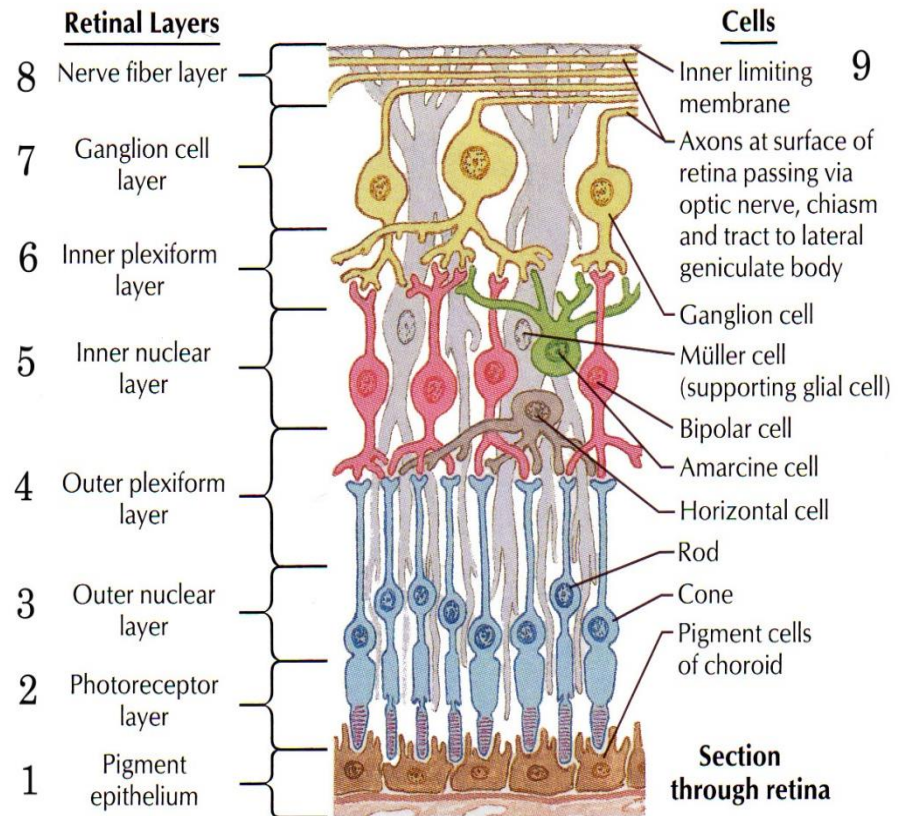


Figure 1. 2 Schematic structure of the retina. Nine layers: (1) RPE, retinal pigment epithelium; (2) POS, photoreceptor outer segments; (3) ONL, outer nuclear layer; (4) OPL, outer plexiform layer; (5) INL, inner nuclear layer; (6) IPL, inner plexiform layer; (7) GCL, ganglion cell layer; (8) NFL, nerve fiber layer; (9) ILM, inner limiting membrane. Six major cell types: photoreceptor cell (cone and rod); GC, ganglion cell; AC, amacrine cell; BC, bipolar cell; HC, horizontal cell; MC, Müller cell. Figure is obtained from http://www.corpshumain.ca/en/Vue_en.php (copyright is released for public use).

1.2.2. Structure and Function of Photoreceptors

Photoreceptor cells are a specialized type of neurons capable of photo-transduction; they absorb and convert photons to neural signals, which are then processed through the network of bipolar, horizontal, amacrine and ganglion cells. There are two main types of photo-receptor cells that are found in vertebrate retina - rods and cones- essentially named for their shape. They contribute to the image forming visual system by collaboration as each type

functions differently. Their outer segment contains light sensitive visual pigment molecules called “opsins” in stacked disks (in rods) or invaginations (in cones) **Figure 1.3.** Cones provide the ability to discern colour and the ability to see fine detail as they are more concentrated in the central retina while rods are mainly responsible for peripheral vision and vision under low light conditions and are more prevalent in the mid-peripheral and peripheral retina. The human retina contains approximately 120 million rod cells and 6 million cone cells. Ten per cent of the outer segment of the rods is shed into the retinal pigment epithelium (RPE) every day; the shed out segment discs are actively taken up by the RPE and this imposes a large phagocytic load on these cells. One of the most common conditions that can affect the retina is ARMD, in which there is a loss of vision in the centre of the visual field (Klein, 2007, Ambati and Fowler, 2012, Bressler et al., 1988). Degeneration of RPE cells in ARMD has been suggested to begin with impaired clearance of cellular waste material within the RPE. This leads to a state of chronic inflammation in the eye, and the formation of abnormal deposits called “drusen”, which are believed to impair the function of RPE cells (Buschini et al., 2011, Ambati and Fowler, 2012, Stefánsson et al., 2011).

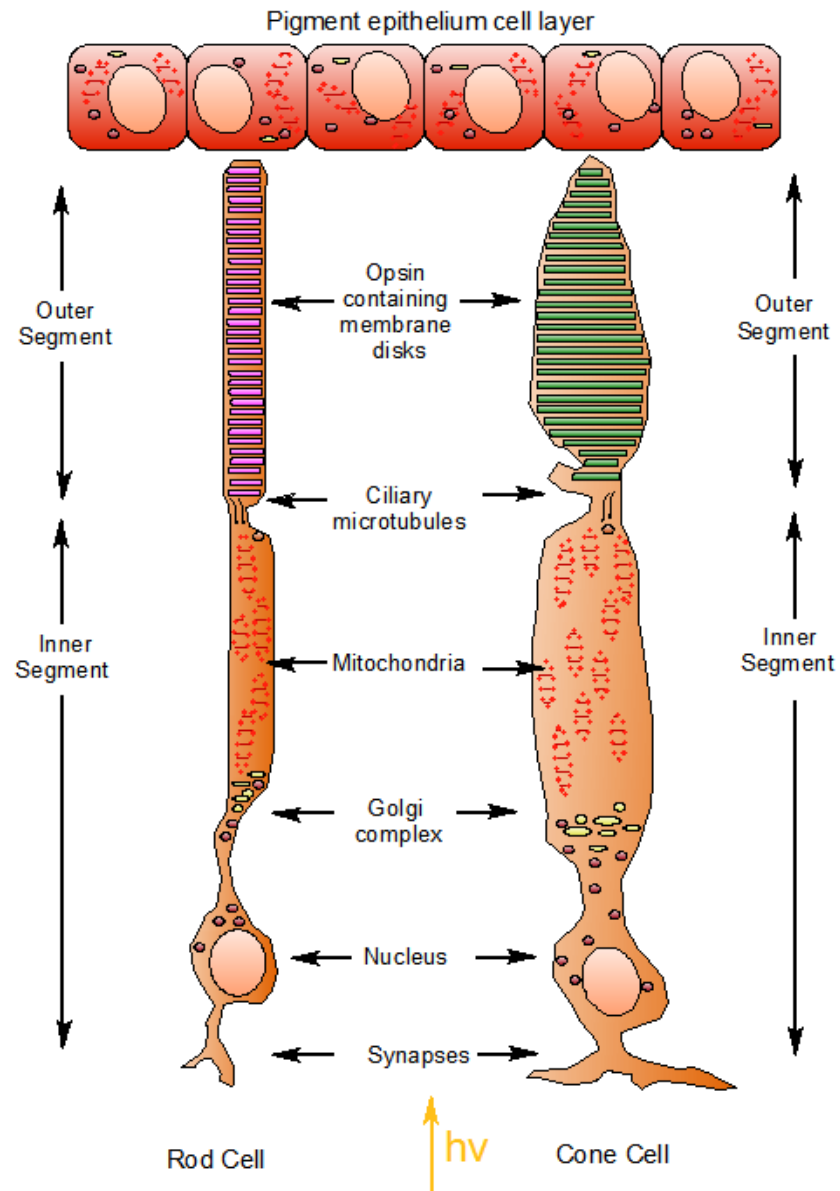


Figure 1. 3 Schematic diagram of rod and cone cells. Rod (left) and cone (right) are two primary types of photoreceptors in vertebrate retina. Each rod and cone photoreceptor cell consists of an inner and outer segment joined by connecting cilium. The nucleus is situated in the outer nuclear layer of the retina and axons pass into the outer plexiform layer where they form synaptic terminals with bipolar cells and interneurons. The outer segment of the receptor cells contain the light sensitive visual pigment molecules called “opsins” in stacked disks (rods) or invaginations (cones). The receptors absorb and convert photons to neural signals, which are then processed through the network of bipolar, horizontal, amacrine and ganglion cells. The output axons of the ganglion cells form the nerve fiber layer that collects at the optic nerve to exit the eye. $h\nu$ (yellow arrow); light exposure. Figure is licensed by the author using the same ganfyd-Attribution-NonCommercial-RegisteredMedical-ShareAlike 1.0 term.

1.2.3. Function of the Retinal Pigment Epithelium

The RPE is a monolayer of polarized pigmented cells with apical surfaces associated with the photo-receptor cell of the neuroretina and basal sides firmly attached to the underlying Bruch's membrane. The RPE cells have a cobblestone shape which is densely packed and joined by tight junctions. They perform multiple and essential functions in the maintenance of the normal visual process. These processes include:

- Reduction of light scatter within the eye and protection against photo oxidation.
- Trans-epithelial transportation of nutrients and metabolic end products between photo-receptors and the choriocapillaris.
- Stabilizing the ion composition in the sub-retinal space
- Maintenance of the photo-receptor as it is the principal site for 11-cis retinal regeneration in the visual cycle
- Phagocytosis of shed rod outer segments

The maintenance of photo-receptors is of great importance, as these light sensitive organs rely on the RPE for most of their required metabolism, such as the regeneration of 11-cis-retinal from all-trans-retinal (the visual cycle), and the phagocytosis, digestion and recycling of the lipid rich photoreceptor outer segments. They are also responsible for the secretion of growth and signalling factors in maintaining the structural integrity of photo-receptors and choriocapillaris endothelium and the secretion of immunosuppressive factors to establish the immune privilege **Figure1.4.**

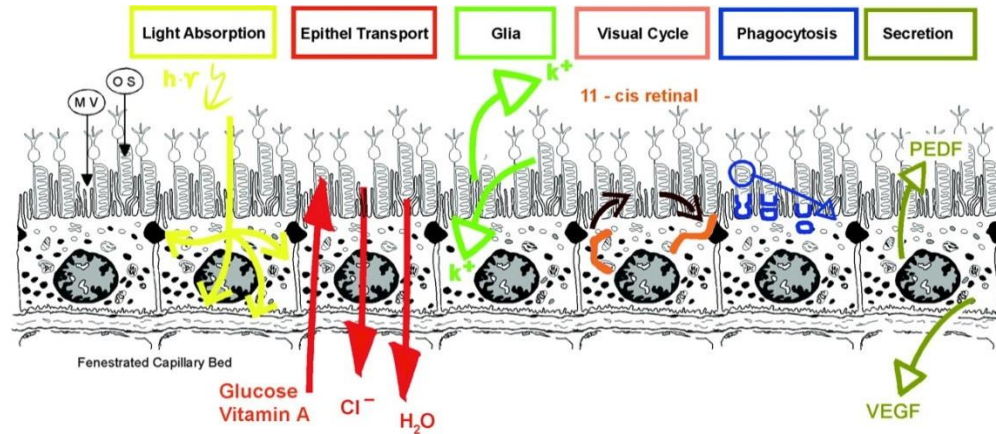


Figure 1. 4 Schematic Summary of RPE functions. The RPE is a monolayer of pigmented cells forming a part of the blood/retina barrier. It plays a critical role in the normal visual process with its function in light absorption (yellow), transepithelial transportation of water and nutrition's (red), ion buffering(light green), the retinal cycle/visual cycle (orange), photoreceptor outer segment phagocytosis (blue) and secretion of important signalling proteins such as VEGF and PEDF (dark green). Hv= light, K⁺= Potassium ions, Cl⁻ = Chloride ion and H₂O = Water. Reprinted with permission from Physiol. Rev. 85 (2005) Strauss,O. The Retinal Pigment Epithelium in Visual Function: 845-881

1.2.3.1. Human retinal pigment epithelial cell line (ARPE-19)

During the last decade, the ARPE-19 cell line proved to be one of the most popular cell lines used in RPE cell research. Originally the cells were extracted from the eye of a 19-year old male donor who underwent spontaneous immortalisation where they maintained a normal number of chromosomes (Dunn et al., 1996). RPE specific markers included retinal pigment epithelia specific protein 65 kDa (RPE65), orthodenticle homeobox 2 (OTx2) and cellular retinal aldehyde binding protein (CRALBP) and they have all been shown to be expressed in the ARPE-19 cell line at both the mRNA and protein level, respectively (RK et al., 2005). ARPE-19 cells have been widely used in studies on oxidative stress, retinal pathogenesis, signalling and inflammatory pathways as well as in drug and toxicity related studies (Ablonczy et al., 2011). More importantly they have also been commonly used as a model for the outer blood retinal barrier (OBRB) as they were

demonstrated to have structural and functional properties such as similar monolayer morphology and tight junction (TJ) formation which are characteristic of *in vivo* RPE cells (Dunn et al., 1996, Dunn et al., 1998, Hamilton et al., 2007).

1.2.4. The structure and function of Bruch's membrane (BrMs)

Bruch's membrane is a tri-layer structure situated between the RPE and the choriocapillaris. It consists of two layers of collagen; an outer layer connected to the basement membrane of the choriocapillaries of the choroid and an inner layer facing the RPE cells that sandwich a centre of insoluble elastin. An important function of Bruch's membrane (BrMs) is to provide a mechanical separation between the leaky, branching vasculature of the choroid and the ordered, non-vascularized packing of the photo-receptors. If the barrier properties of BrMs are damaged, the blood vessels in the choroid can grow into the retina, causing damage to the photo-receptors and ocular disorders such as neovascular age related macular degeneration (ARMD) (described below).

1.3.4.1. The use of synthetic membranes in *in-vitro* models

The presence of BrMs is important for the RPE cells' polarization and for the cells to adhere and form a monolayer; therefore artificial membrane has been used as an alternative *in-vitro* solution.. However, the artificial membrane must be bio-compatible and should possess comparable properties to the native Bruch's membrane, such as similar mechanical rigidity, sufficient permeability to nutrients and good cell adherence. In this study we used Millicell-PCF or Millicell-HA culture inserts (Millipore Millicell-PCF Culture plate inserts, PIHA 01250, PIHP01250). Both cell culture inserts had a pore size of 0.4 –

0.45 μm and a filter diameter of 12mm. The inserts were used in a 24-well plate. To further imitate both the ultra-structural and mechanical properties of a natural biological substrate of BrMs they were coated with 10 $\mu\text{g cm}^{-2}$ purified mouse laminin. One of the advantages of using Millipore inserts is to maintain the constancy of product and they are easily available and relatively cheap.

1.3. The Blood Retinal Barrier (BRB)

The Blood Retinal Barrier is located in the eye and provides a selective barrier for regulation of the retinal milieu and for maintaining the specialised environment of the neural retina. This barrier is similar to the blood–brain barrier (BBB), is particularly tight and restrictive and is a physiological barrier that regulates ion, protein and water flux into and out of the retina (Cunha-Vaz et al., 1966, Shakib and Cunha-Vaz, 1966). The blood–retinal barriers include two main barrier systems; the inner blood–retinal barriers and the outer blood–retinal barriers. The inner layer (iBRB) is composed of the Retinal Microvascular Endothelial cells (RMECs) and the outer layer (oBRB) comprises the Retinal Pigment Epithelial (RPE) cells. Together, the BRB layer plays a fundamental role in the micro-environment of the retina and retinal neurons. It regulates fluids and molecular movement between the ocular vascular beds and retinal tissues and prevents leakage into the retina by macro-molecules and other potentially harmful agents (Cunha-Vaz and Coscas, 2010). The barrier property of the BRB is because of the presence of ‘tight junctions’ (TJs) which seal the paracellular space from the outer environment. The TJs are formed by interaction between membrane proteins in the adjacent cells in the BRB.

1.3.1. Inner Blood Retinal Barrier (IBRB)

The iBRB is structurally similar to the Blood Brain Barrier (BBB) with the RMECs surrounded by retinal pericytes and macroglial cells (Hosoya and Tomi, 2005). The RMECs form complex tight junction which function to prevent the free diffusion of substances between circulating blood and neural retina (Campbell and Humphries, 2012). The selectively permeable TJs in RMECs ensure correctly regulated hemodynamics (Pournaras et al., 2008).

1.3.2. Outer Blood Retinal Barrier (OBRB)

The oBRB is formed by a monolayer of RPE cells. The oBRB faces the photo-receptor layer of the eye on its apical side. The BrMs separates the RPE from the choriocapillaries on the basal side. The long apical microvilli of RPE cells surround the light sensitive outer segments of the photo-receptor cells establishing a complex of close structural interaction (Strauss, 2005). The main function of oBRB is to regulate the passage of ions and macro-molecules into the photo-receptor layer and to transport water and metabolic end products from the sub-retinal space to the blood. Breakdown of the oBRB in conditions such as ARMD may lead to degradation of the photo-receptor cell layer (Dorcarmo et al., 1998, Omri et al., 2013).

1.3.2.1. Tight junctions

The interaction between protein complexes of adjacent RPE cells gives rise to two types of membrane barrier junctions – Tight Junctions (TJ) and Adherens Junctions (AJ). Among other components, TJs are required in the oBRB to regulate the trans-epithelial movement of ions and other molecules into the paracellular space (Balda and Matter, 2008). TJs provide a continuous

seal around the apical region of the lateral membranes of adjacent cells (Anderson and Van Itallie, 1999). When viewed under the microscope using freeze fracture electron microscopy, TJs appear as a ‘necklace’ of strands encircling the cells (Rizzolo, 2007, Mitic and Anderson, 1998). Damage to the TJs or AJs was shown to increase back leakage and a loss of net transport leading to retinal degeneration (Xu et al., 2011, Rizzolo, 2007, Jin et al., 2002). However, their function is not merely limited to their barrier properties. TJs play an important role in vesicle targeting, cytoskeletal dynamics’ proliferation and transcription signalling while also defining cell polarity (Anderson and Van Itallie, 2008)

1.4. Age-Related Macular Degeneration (ARMD)

ARMD is the leading cause of blindness in the elderly in the developed world (Bressler et al., 1988). The disease affects the macula, which is the central part of the retina in the back of the eye which is responsible for sharp-sightedness. People affected by ARMD gradually lose their central vision which is essential for common daily activities such as reading and driving. Studies showed that ARMD is associated with a 60% reduction in the quality of life for those having this condition (Brown et al., 2005).

1.4.1. Classification

ARMD is a disease strongly associated with aging. As people grow older, age-related changes occur to the retina in general. Yellowish focal deposits, called drusen, appear between the RPE and BrMs. The appearance of drusen is considered an early sign of developing ARMD. ARMD is classified by the size and extent of drusen, the manifestations of geographic atrophy, and

neovascular changes as shown in **Figure 1.5**. Early ARMD is characterized by the presence of fewer than twenty medium size drusen or retinal pigmentary abnormalities **Figure 1.5A**. At this stage, blurred vision and abnormal dark adaptation may occur, but the visual loss is generally mild. Intermediate ARMD is characterized by the presence of at least one large druse, numerous medium size drusen, or geographic atrophy that does not extend to the centre of the macula, **Figure 1.5B**. Advanced ARMD includes non-neovascular (dry) and neovascular (wet) forms. Advanced non-neovascular ARMD (dry ARMD) is characterized by geographic atrophy extending to the centre of the macula, shown in **Figure 1.5C**. Patients having the advanced form of dry ARMD start to lose their central vision and experience paracentral visual scotomas known as “blind spots”. The process of disease development from the early stage to advanced dry form usually takes years. Advanced neovascular ARMD (wet ARMD) is characterized by choroidal neovascularization (CNV) **Figure 1.5D**. People with the advanced form of neovascular ARMD can have sudden, severe vision loss which can occur within days to weeks resulting from sub-retinal haemorrhage. Although neovascular ARMD only represents less than 15% of ARMD incidence, it is responsible for more than 80% of debilitating visual loss in ARMD (Cachulo et al., 2011).

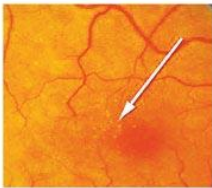
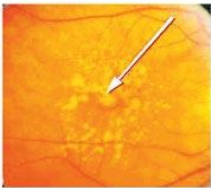
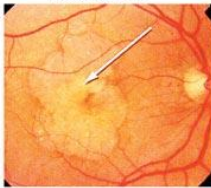
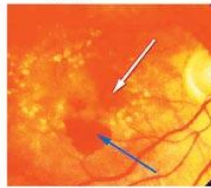
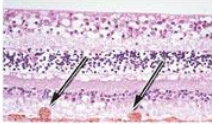
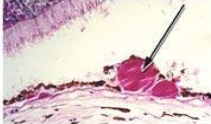
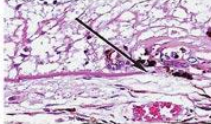
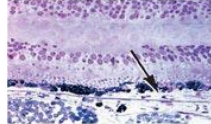
	A Early AMD	B Intermediate AMD	C Advanced Non-neo-vascular AMD	D Advanced Neovascular AMD
Fundus				
Histopathological Features				
Clinical Features	Presence of a few medium-size drusen Pigmentary abnormalities such as hyperpigmentation or hypopigmentation	Presence of at least one large druse Numerous medium-size drusen Geographic atrophy that does not extend to the center of the macula	Drusen and geographic atrophy extending to the center of the macula	Choroidal neovascularization and any of its potential sequelae, including subretinal fluid, lipid deposition, hemorrhage, retinal pigment epithelium detachment and a fibrotic scar
Current Management	Lifestyle and dietary modifications (e.g., cessation of tobacco use, increased dietary intake of antioxidants, control of blood pressure and body-mass index)	Supplementation according to the Age-Related Eye Disease Study Lifestyle and dietary modifications	Supplementation according to the Age-Related Eye Disease Study, if the other eye has early or intermediate AMD Lifestyle and dietary modifications	Supplementation according to the Age-Related Eye Disease Study, if the other eye has early or intermediate AMD Lifestyle and dietary modifications Antiangiogenic therapy (e.g., intravitreal injection of antiangiogenic or angiostatic agents) Laser therapy (ocular photodynamic therapy or argon-laser photocoagulation)

Figure 1. 5 Classification of ARMD. Column A shows the fundus with a medium-size drusen (white arrow) in early ARMD, column B shows the fundus with a large druse (white arrow) in intermediate ARMD, column C shows the fundus with geographic atrophy (white arrow) in advanced non-neovascular ARMD, and column D shows the fundus with subretinal hemorrhage (blue arrow) and choroidal neovascularization (white arrow) in neovascular ARMD. Text reprinted from The New England Journal of Medicine (2008) Rama D Jager et al. Medical Progress: Age-Related Macular Degeneration 358:24, 2606-2617, with permission from NEJM. The images are provided by the National Eye Institute, National Institute of Health (NEI/NIH)

1.4.2. Risk Factors

ARMD is a multifactorial disease, where age, gender, race, environmental and personal behaviours such as smoking, obesity and bad diet, have all been found to be associated with disease advancement and susceptibility. Among the environmental risk factors, oxidative processes appear to play a common role and contribute to ARMD pathogenesis.

1.3.2.1. Age

The aging process allows time for free radical oxidative damage to be accumulated in the body and is the most important risk factor for ARMD. With age the risk of ARMD onset increases (Del Priore et al., 2002). As people grow older, age related changes occur to the retina; these changes include the build-up of lipid waste deposits “drusen”, pigmentary abnormalities and thickening of BrMs. These changes contribute to the increased risk of developing ARMD. A study carried by Owen et al (2012), showed that people with age 90 years and up have 8- to 10-fold higher risk of ARMD onset compared to people with age 50 years **Figure 1.6** (Owen et al., 2012)

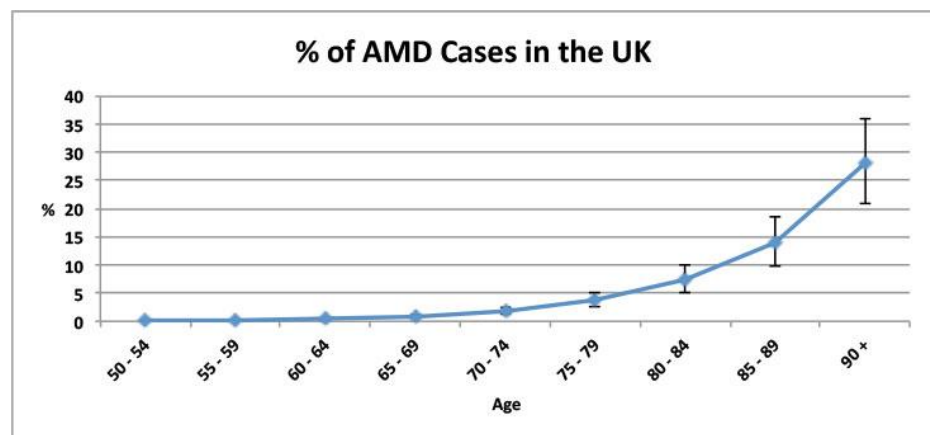


Figure 1. 6 Prevalence of ARMD by age. Statistics taken from the *British Journal of Ophthalmology* .The estimated prevalence and incidence of late stage age related macular degeneration in the UK. Br J Ophthalmol. 2012 May ;96(5):752-6. doi: [10.1136/bjophthalmol-2011-301109](https://doi.org/10.1136/bjophthalmol-2011-301109). Epub 2012 Feb 13 .

1.3.2.2. Smoking

Smoking is a major risk factor in ARMD susceptibility. Several studies, including cross-sectional studies (Thornton et al., 2005), cohort studies (Cong et al., 2008) and case-control studies (Tamakoshi et al., 1997), have all confirmed a strong association between current smoking and ARMD. The risk of ARMD for smokers (current or past) was higher compared to non-smokers (odds ratio 1.06 – 4.96) (Christen et al., 1999, Seddon et al., 2011). Although the pathogenesis of ARMD and the mechanism of action of smoking on the eye are not fully understood, it has been suggested that its effect involves more than one mechanism which ranges from the accumulation of oxidative damage in the retina (Beatty et al., 2000) to reducing the choroidal blood flow in the eye (Solberg et al., 1998).

1.3.2.3. Genetic Factors

In addition to environmental factors, the genetic background was also found to influence susceptibility to ARMD. Several genes have now been implicated as possible risk factors for ARMD involving single nucleotide polymorphisms (SNP) in which one amino-acid within the protein is substituted for another, with a strong correlation with genes involved in immune modulation and the complement system (van de Ven et al., 2012)

Table 1.1. Complement system protein *factor H (CFH)* (Klein et al., 2005, Narayanan et al., 2007, Despriet et al., 2006), *factor B (CFB)* (Mantel et al., 2013, Hughes et al., 2011), and *factor 3 (C3)* (Maier et al., 2008, Luo et al., 2011) appear to exhibit the strongest associations with the risk of developing ARMD **Table 1.1.** For example a SNP in the variant Tyr402His (rs1061170) in CFH, was found to be a significant determinant of the disease with odds ratios

(OR) ranging from 3.3 to 10.1 for early stage ARMD and 6.5 - 10.1 for advanced stage ARMD (determined by geographic atrophy or neovascularization) (Kuo et al., 2013). This SNP in Tyr402His reduces the affinity of CFH for C3b (explained in greater details in **Section 1.3.3.**) and may also alter the ability of CFH to recognize specific glycosaminoglycans “polyanionic surface markers”. This change results in the reduced ability of CFH to regulate the activity of complement proteins on critical surfaces, such as the retina, and may lead to an increased inflammatory response at the macula.

Mutations in genes modulating the immune response are not the only genes found to be associated with the susceptibility of ARMD; genes involved in lipid metabolism were also found to be associated. A study carried out by McKay et al. (2011) investigated a pool analysis of fifteen studies and found associations between *APOE* allele's $\beta 4$ and $\beta 2$ with late ARMD regardless of gender and environmental risks (McKay et al., 2011). These findings were confirmed by a more recent, meta-analysis of early onset ARMD which suggested a similar association with *APOE* (Holliday et al., 2013). Even though more and more genes have been identified as associated with ARMD, no direct effect has been reported from any genetic polymorphism to the onset of the disease, as in other complex diseases, the role of genetics is thought to be the result of the combined effects exerted by multiple gene variants and their interaction with environmental factors. Therefore, the interaction between the environment and genes cannot be ignored. Environmental components could trigger the ARMD phenotype in genetically predisposed individuals.

Table 1. 1 Allele Frequency and Odds Ratio for ARMD Susceptible Genes

Gene	ARMD Subject	Minor Allele Frequency		Odds Ratio*		Reference
		Control	ARMD	Homozygous	Heterozygous	
CFH rs1061170	all ARMD†	0.46	0.97	3.33	2.45	Haines, et al, 2005
	all ARMD†	/	/	2.25-2.82#	/	Hageman, et al, 2005
	all ARMD‡	0.382	0.595	6.43	1.76	Rivera, et al,2005
	early ARMD‡	0.28	0.4	3.9	/	Recalde, et al, 2008
	mid and advanced ARMD‡	0.355	0.549	/	/	Edwards, et al, 2005
	mid and advanced ARMD‡	/	/	6.3	3.1	Yates, et al, 2007
	dry ARMD‡	0.371	0.634	7.5	2.3	Seddon, et al, 2006
	wet ARMD‡	/	/	5.57	3.45	Haines, et al, 2005
	wet ARMD‡	0.371	0.608	6.5	2.3	Seddon, et al, 2006
	wet ARMD‡	0.28	0.47	7.7	/	Recalde, et al, 2008
	advanced ARMD‡	/	/	7.4	4.6	Klein, et al, 2005
	advanced ARMD‡	0.31	0.609	10.05	2.66	Jakobsdottir, et al, 2005
	advanced ARMD‡	0.359	0.615	7.6	2.7	Maller, et al, 2006
C3 rs2230199	all ARMD†	0.21	0.29	1.55#	/	Spencer, et al, 2008
	mid and advanced†	0.20	0.285	2.6	1.7	Yates, et al, 2007
	advanced ARMD†	0.21	0.31	3.26	1.61	Maller, et al, 2007

*Odds ratio is defined as the odds of ARMD in individuals carrying risk genotypes (homozygous or heterozygous) vs. the odds of ARMD in individuals carrying non-risk genotype. †Caucasian population ‡Indian population ‡‡Chinese population #Odds ratio for risk allele

Table 1. 2 Allele Frequency and Odds Ratio for ARMD Susceptible Genes (Continued)

Gene	ARMD Subject	Minor Allele Frequency		Odds Ratio*		Reference
		Control	ARMD	Homozygous	Heterozygous	
LOC387715 rs10490924 10q	all ARMD†	0.196	0.417	7.61	2.82	Rivera, et al, 2005
	all ARMD†	0.247	0.397	6.09	1.35	Yang, et al, 2006
	all ARMD‡	0.358	0.619	8.24	2.2	Kaur, et al, 2008
	all ARMD†	0.199	0.426	8.1	/	Fritsche, et al, 2008
	mid and advanced ARMD†	/	/	2.4	2.4	Yates, et al, 2007
	wet ARMD‡‡	/	/	11.14	1.66	DeWan, et al, 2006
	wet ARMD†	0.328	0.526	61.91	5.32	DeAngelis, et al, 2007
	wet ARMD†	0.21	0.49	11.8	/	Recalde, et al, 2008
	advanced ARMD†	0.193	0.475	10.57	2.72	Jakobsdottir, et al, 2005
HTRA1 rs11200638	all ARMD†	0.252	0.403	6.56	1.86	Yang, et al, 2006
	all ARMD‡	0.345	0.587	6.69	1.63	Kaur, et al, 2008
	all ARMD†	0.199	0.426	2.85	/	Fritsche, et al, 2008
	wet ARMD‡‡	/	/	10.0	1.6	DeWan, et al, 2006
	wet ARMD†	0.317	0.522	98.41	6.05	DeAngelis, et al, 2007
	exudative ARMD‡‡	0.45	0.73	1.73	7.56	Tam, et al, 2008

*Odds ratio is defined as the odds of ARMD in individuals carrying risk genotypes (homozygous or heterozygous) vs. the odds of ARMD in individuals carrying non-risk genotype. †Caucasian population ‡Indian population ‡‡Chinese population #Odds ratio for risk allele

1.3.2.4. Diet and obesity

The increase in body weight and the high consumption of saturated fat have been reported as elevating the susceptibility to ARMD. This relation was recognised following two major studies; The Beaver Dam Eye Study (Klein et al., 2007) and The Blue Mountain Eye Study (Cumming et al., 2000). Both these studies highlighted a robust correlation between high-density lipoprotein cholesterol and ARMD, while a protective role was reported from frequent consumption of mono- and polyunsaturated fats (i.e. fish and vegetable fats) .These results indicated that consumption of certain types of fat rather than total fat intake may influence the risk of ARMD (Dasari et al., 2011, Seddon et al., 2001, Chua et al., 2006, Gopinath et al., 2014, Cumming et al., 2000). Histopathological studies have shown that fatty acids and cholesterol accumulate with age in Bruch's membrane. This deposition may interfere with the normal function of the RPE and promote ARMD development. In addition, a high serum lipid was found to promote the process of atherosclerosis - a disease that shares similar lipid deposition to ARMD (Mullins et al., 2000).

1.4.3 Mechanisms

The pathological mechanism of ARMD is still not clear, but it is not due exclusively to the normal aging process. Genetic and environmental influences suggest that inflammation and oxidative stress play a role in ARMD pathogenesis.

1.4.3.1. Inflammation

Genetic studies have shown that mutations in complement genes such as CFH and C3 are found to be associated with increased ARMD risk. Both CFH and C3 are implicated in the complement cascade activation through the “alternative pathway”, and are, thus, associated with the inflammatory process in ARMD pathogenesis (Despriet et al., 2006, Silva et al., 2012). The complement system is a powerful arm of the innate immune system, immediately participating in the recognition, trafficking, and elimination of pathogens and unwanted host material (Scholl et al., 2008). It consists of several inactive zymogens that, upon activation, assemble with other components and are converted into active proteases of effector compounds. The complement cascade can be divided into three stages: initiation of the complement activation, amplification by activation of C3 and C5 convertases and the effector function of the terminal pathway known as “membrane attack complex (MAC)”. This process is tightly controlled by multiple complement regulators and inhibitors and can be activated through three main pathways each with its own specific triggers;

(1) Classical pathway “initiated by antibody-antigen complexes”,

(2) Alternative pathway “initiated by the interaction of regulatory components by structures on microbial surfaces”, and

(3) Mannan-binding lectin (MBL) pathway “initiated by binding of MBL to carbohydrates”(Luo et al., 2011).

The “alternative cascade” is activated when complement component C3 is cleaved to C3a and C3b, C3b which, in turn, form a bimolecular complex with CFB C3bBb, also called C3 convertase. This enzyme complex cleaves C3 to C3b in a loop and thus amplifies the cascade **Figure 1.7**. CFH is the most important regulatory protein of the alternative pathway (Kopp et al., 2012, Ferreira et al., 2010). CFH inhibits this cascade by binding to C3b and accelerating the dissociation of C3 convertase and thus prevents complement activation on host cells (Narayanan et al., 2007, Fritsche et al., 2010). In a defective SNP of CFH, the ability of CFH to inhibit the complement cascade is compromised. It has been suggested that this dysregulation can cause the chronic inflammation seen in ARMD. A new concept for ARMD pathology has recently been introduced. This concept is based on inefficient or defective transport/diffusion of nutrients from the choroids via the Bruch’s membrane to the photo-receptors, or inappropriate reverse transport of waste products from photo-receptors to the choroid (Zarbin, 2004). Consequently debris deposits may accumulate in a sub-RPE form, providing an activator surface that allows complement activation. Several constituents of drusen could activate the complement cascade locally. For instance, in ARMD patients, amyloid- β is present in drusen (Dentchev et al., 2003, Johnson et al., 2002); amyloid is also a known activator of complement cascade as well as a potent initiator of inflammation. It is also believed that a defective or inefficient complement regulation may cause further amplification of the complement cascade and inflammation. Both concepts may explain how inappropriate control and inhibition of spontaneous or triggered complement activation cause progression

and amplification of the complement cascade and consequently the generation of inflammatory activation compounds in the form of C3a and, in particular, of the potent inflammatory marker C5.

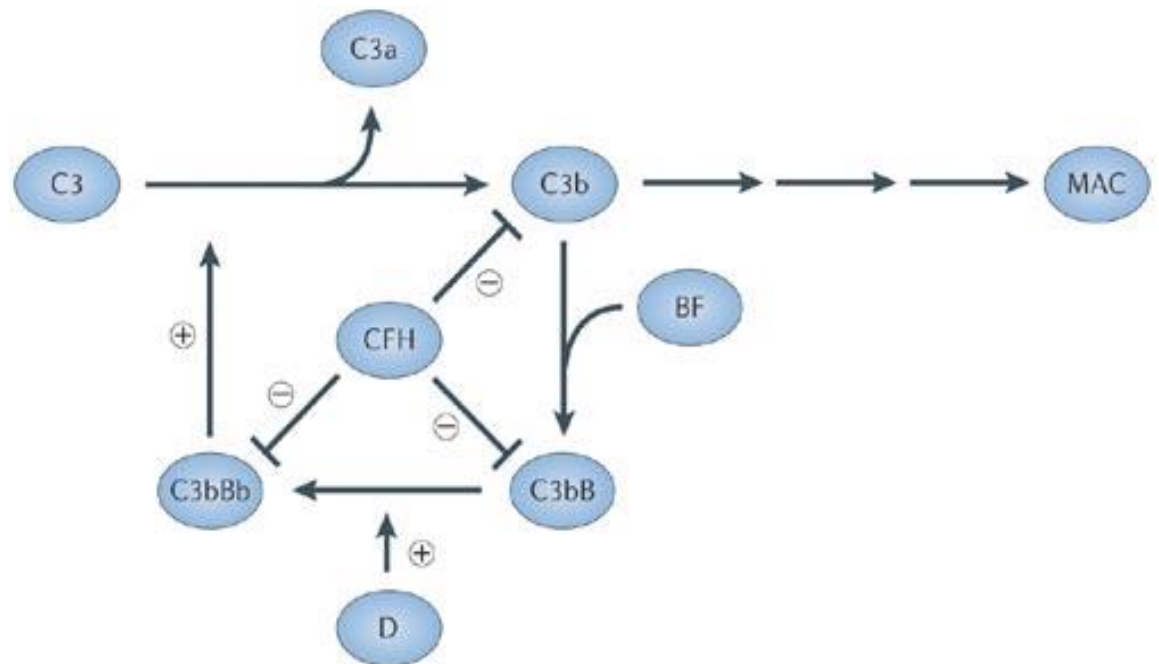


Figure 1. 7 The alternative complement pathway and the role of complement factors B and H. Hydrolysis of complement factor 3 (C3) generates an active fragment C3b, which binds with factor B (BF) and is then hydrolyzed to formed C3bBb complex. The C3bBb complex is a C3 convertase which cleaves C3 to C3b and amplifies the complement cascade. Complement factor H (CFH) negatively regulates the cascade by inhibiting the binding of C3b with BF and accelerating the decay of C3bBb. Reprinted by permission from Macmillan Publisher Ltd: Nature Reviews Neuroscience, 7, Amir Rattner and Jeremy Nathans, Macular degeneration: recent advances and therapeutic opportunities,860-872, copyright (2006).

1.4.3.2. Oxidative stress

Oxidative stress is involved in many human disease processes, especially age-related disorders. The term “oxidative stress” refers to a state characterized by excessive production of reactive oxygen species (ROS) and/or reduction in antioxidant defences, which generates an imbalance between ROS production and removal. Reactive oxygen species include free radicals,

hydrogen peroxide, and singlet oxygen, see **Figure 1.8**. ROS plays an important role in the regulation of many physiological processes involved in intracellular signalling (Finkel, 2012), but they can also induce serious damage to biomolecules.

Oxidative stress in retina comes from several sources; oxygen consumption by retina is much greater than other tissues, which provides a rich source of ROS. High levels of cumulative irradiation (Ham et al., 1978) and abundant retinal chromophores, such as rhodopsin, melanin, lipofuscin and cytochrome c oxidase, can cause photo-chemical damage to the retina and RPE by the generation of ROS (Beatty et al., 2000, Organisciak et al., 1996, Sarna, 1992). Retina is very rich in membranes containing polyunsaturated fatty acids (PUFAs). PUFAs account for about 50% of the lipid bilayer of rod outer segment membranes which are also rich in Docosahexanoic acid (DHA) (Stone et al., 1979) DHA contains six double bonds and this structure is particularly susceptible to free radical damage because their conjugated double bonds are convenient sources of hydrogen atoms, and contain one electron. The lipid radical then combines with oxygen to form lipid peroxy radicals and lipid peroxides, which can achieve a steady state only by stealing electrons from other polyunsaturated fatty acids, thus creating a cytotoxic cascade of reactions that consume valuable PUFAs and produce damaged molecules leading to loss of membrane function and structural integrity (Marchette et al., 2014, Njie-Mbye et al., 2013, Liu et al., 2010).

The relationship between oxidative stress and the development of ARMD is supported by epidemiologic evidence that multiple oxidative related factors are associated with the risk of ARMD. These factors include age, smoking history and light exposure, in which oxidation clearly plays a common role. Studies of oxidation markers have provided further evidence of the association between oxidative damage and ARMD. A direct molecular link between oxidative damage and the neovascularization seen in ARMD was established by the finding that carboxyethylpyrrole (CEP), an oxidative protein modification generated from docosahexaenoate (DHA)-containing phospholipids, is elevated in Bruch's membrane and drusen from ARMD patients as well as in the plasma of ARMD donors (Gu et al., 2012, Ebrahim et al., 2006, Gu et al., 2009).

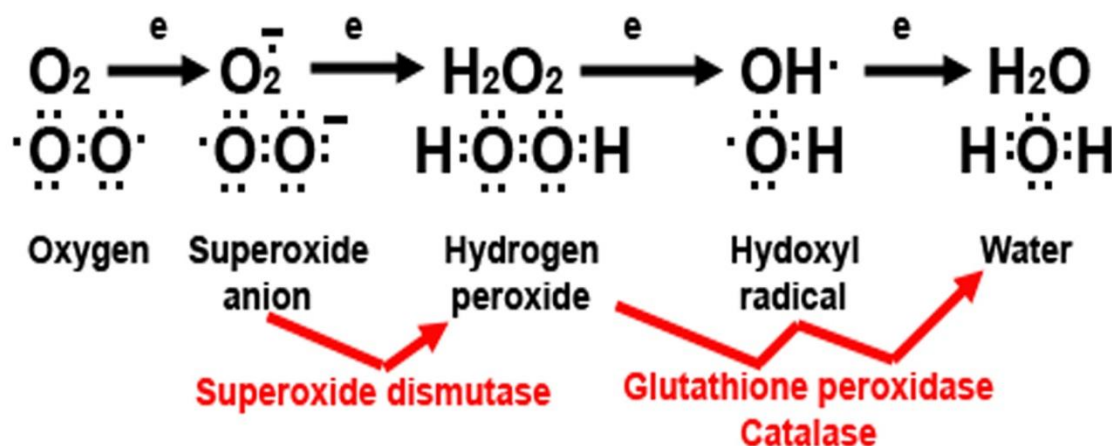


Figure 1. 8 Generation of reactive oxygen species. Reactive oxygen species include superoxide anion radical, hydrogen peroxide, hydroxyl radical and singlet oxygen. Superoxide anion is formed by oxygen accepting an electron, and can in turn react with an electron and two protons to form hydrogen peroxide, which degrades to water and very reactive hydroxyl radicals. Hydroxyl radicals can be neutralized by accepting an electron and a proton to form water. Reactive singlet oxygen is formed when chromophores absorb energy and transform the energy to oxygen molecule.

1.4.3.3. Lysosomal enzymes dysfunction

The RPE plays an important role in phagocytosis and the renewal of the photo-receptor outer segments. In phagocytic cells, the endolysosomal system is a crucial regulator of both phagocytosis and autophagy, which is the process by which damaged proteins or organelles within the cell are collected, degraded, and removed (Lakkaraju, 2012). To maintain healthy functions and integrity in the retina, the RPE cells are equipped with a highly efficient lysosomal system to keep both phagocytosis and autophagy in balance (Kevany and Palczewski, 2010, Mitter et al., 2012). An increased level of lipofuscin accumulation was shown to correlate with advanced age as well as ARMD onset. This accumulation is thought to result from the partially digested rod outer segments indicating a dysregulation in the lysosomal system (Lakkaraju et al., 2007). The physiological balance between various interlinked pathways (e.g. vascular growth factor pathways, lipid pathways and oxidative stress pathways) is, therefore disturbed in the ARMD which may impair the autophagy process.

Several ways exist for internalization of materials into the lysosomal compartment; large particles form autophagosomes which fuse with the lysosome (Korolchuk and Rubinsztein, 2011, Wang and Klionsky, 2003, Klionsky and Emr, 2000) while small particles can enter through a chaperone-mediated transport autophagy (Dice, 2007), or through invaginations of the lysosomal membrane (Marzella et al., 1981). The lysosomes contain a wide variety of hydrolytic degradative enzymes, including α -mannosidase (α -Mann), β -glucuronidase (GUSB), arylsulfatase B (ASB) (Verdugo and Ray, 1997) and

the cathepsin D (CatD) which is the most important among these and is involved in opsin proteolysis (Hjelmeland et al., 1999). Boulton et al (1994) reported an increase in CatD and acid phosphates with age (Boulton et al., 1994) and Cingle et al (1996) reported a decrease in α -Mann activity (Cingle et al., 1996). Comparison of many research studies proved to be difficult. This could be a result of differences in tissues or species analysed or different methodologies used in measuring enzyme activity (Verdugo and Ray, 1997). It has been hypothesized that the age-related decrease in the lysosomal enzyme activity would lead to accumulation of lipofuscin (Beatty et al., 2000). However, work done by Brunk and Terman (2002), suggested that the accumulation of lipofuscin may decrease the lysosomal enzyme activity (Brunk and Terman, 2002). Recently it has been shown that N-retinylidene-N-retinylethanolamine (A2E), which is an important component of the RPE lipofuscin, is a strong inhibitor of the major lysosomal catabolic systems (Bergmann et al., 2004)

1.4.4. Current Management

There is no cure for the dry form of ARMD (Buschini et al., 2015, da Cruz, 2008, Alvarez Palomo et al., 2015). However, several treatments are available for the wet form of ARMD that may prevent severe vision loss or slow the progression of the disease. Anti-angiogenic drugs (anti-VEGF), photodynamic therapy with verteporfin (PDT-V) and the use of monoclonal antibodies therapies were found to be very effective for wet ARMD. Currently, bevacizumab (Avastin) is the most widely used anti-VEGF agent throughout the world because of its significantly lower cost and similar efficacy compared

to Ranibizumab (Lucentis). Another new promising drug is Aflibercept (known as VEGF-Trap) which is a human recombinant fusion protein which consists of the extra-cellular domains of VEGF receptor 1 and 2 (VEGFR-1 and -2) fused with the Fc portion of IgG1 (Heier et al., 2012). It binds to VEGF-A, VEGF-B, and placental growth factor (PlGF). It has a higher affinity for VEGF compared to other anti-VEGFs, including bevacizumab and ranibizumab (Hanout et al., 2013, Agarwal et al., 2015, Amoaku et al., 2015).

1.5 Lipids and Age-related Macular Degeneration

Lipids and lipid-soluble compounds are essential constituents of the cells and tissues and defects in their synthesis, intra-cellular and extra-cellular transport, and turnover underlie a variety of significant, common, and often severely debilitating eye diseases. Lipid particles accumulate underneath the RPE and between Bruch's membrane in the exact location and prior to the development of basal deposits or drusen (Abdelsalam et al., 1999). This observation has led to the suggestion that these lipid particles contribute to drusen formation during the development of ARMD.

1.5.1 Plasma Lipids and ARMD

Sub-RPE deposits found in ARMD patients were found to be rich in lipoproteins (Wang et al., 2010b). Lipoproteins are a biochemical assembly that contains both proteins' case coating and a lipid core, which enables lipids to be carried in the blood stream. There are several different types of lipoprotein, including HDL (high density lipoprotein), LDL (low density lipoprotein), VLDL (very low density lipoprotein), IDL (Intermediate density

lipoprotein), and chylomicrons .The ratio between the proteins to lipids determines the class of lipoprotein which is summarized in **Table 1.3**. Since lipoproteins are mostly synthesized in the liver, the plasma has been implicated as the source of these lipoproteins (Curcio et al., 2001, Holz et al., 1994). A study carried by Hamimovici et al, (2001) found that the backbone structure and size of these lipid particles, which are found in ARMD patients, suggest that they were most closely related to LDL or VLDLs. This has prompted a search for an association between serum lipid and ARMD(Haimovici et al., 2001).

Table 1. 3 Proteins to lipids ratio deference between different lipoprotein classes

Lipoprotein class	Density (g ml ⁻¹)	Diameter (nm)	% Protein	% Cholesterol	% Phospholipid	% Triglycerides
HDL	1.063-1.210	5-15	33	30	29	8
LDL	1.019-1.063	18-28	25	50	21	4
IDL	1.006-1.019	25-50	18	29	22	31
VLDL	0.95-1.006	30-80	10	22	18	50
Chylomicrons	<0.95	100-1000	<2	8	7	84

To date, epidemiological studies on the association between serum lipid and ARMD risk have been inconsistent (van Leeuwen et al., 2004, Tan et al., 2007). Part of this variability may have resulted from reporting different forms and stages of ARMD with different lipid profiles. For example, in a study by Van Leeuwen et al, it was reported that elevated HDL, but not total cholesterol,

was associated with an increased risk of ARMD (Hyman et al., 2000, Abalain et al., 2002, van Leeuwen et al., 2004), while non-neovascular ARMD was found to be unrelated to cholesterol level in a study by Hyman et al (Hyman et al., 2000) .No difference in total cholesterol, triglycerides, phospholipids, high- and low-density lipoprotein-cholesterol (HDL-C and LDL-C) concentrations were observed between ARMD patients and controls in the study by Abalain et al. (2002) and there was no significant difference in the concentration of the Lipoprotein a (apoA) between the ARMD and control groups in the work by Nowak et al. (Nowak et al., 2005)

Some correlations between serum lipid and ARMD have been made when investigators looked specifically at high-density lipoproteins. Again, the results have been conflicting. The Rotterdam (van Leeuwen et al., 2004) and Pathologies Oculaires Liees al'Age (Delcourt et al., 2001) studies found an association between ARMD risk and high-density lipoprotein-cholesterol (HDL-C). When separated by disease type, Klein et al. (Klein et al., 2003) showed that higher serum HDL-C at baseline was associated with pure geographic atrophy or advanced non-neovascular ARMD , while Hyman et al. (Hyman et al., 2000) revealed a positive association between HDL level and neovascular ARMD, but not non-neovascular ARMD , and dietary cholesterol level.

On the other hand, other studies have found an inverse correlation with HDL and ARMD risk. Reynolds et al. (Reynolds et al., 2010) demonstrated that elevated HDL is associated with a reduced risk of advanced ARMD, especially the neovascular (NV) sub-type and that higher low density

lipoprotein (LDL) is associated with increased risk of advanced ARMD and the NV subtype. When looking for an association of serum lipids with advanced ARMD in multivariate modelling, Reynolds et al. also found significant trends with a higher quartile of LDL and increasing ARMD risk.

1.5.2 ARMD and the cholesterol connection

Cholesterol is an essential lipid for normal human development, growth and physiology, and is transported around in the bloodstream inside lipoproteins. Cholesterol homeostasis governed by a complex regulation process, in which a balance is maintained between the uptake of exogenous cholesterol from circulating lipoproteins, and the export of cholesterol from the tissue to the circulation, which maintains a constant level in cholesterol in a given tissue. Unesterified (free) cholesterol is the pre-dominant form of cholesterol in the CNS, of which the neural retina part off (Fliesler and Anderson, 1983, Fliesler and Bretillon, 2010) and the increase of cholesterol build up have been associated with the formation of deposits found in several diseases such atherosclerosis plaque (Klein et al., 2013) and Alzheimer's disease (AD) plaque as well as in ARMD drusens. Several studies have linked cholesterol and ARMD; these studies were driven by the cholesterol accumulation in BrMs (Curcio et al., 2001). Further work carried out by Curcio et al established that esterified (EC) and unesterified cholesterol (UC) are significant components of the lipid-rich lesions associated with ARMD (basal linear deposits, BLinD, and soft drusen) where they comprise less than 40% of hard druse volume (Curcio et al., 2011). The use of genome-wide association

studies (GWAS) analysis further supported the findings of Curcio et al. when variants in the cholesterol-related genes were found to be associated with ARMD, suggesting that these variants may play important roles in early ARMD (Cheng et al., 2015, Fritsche et al., 2013, Neale et al., 2010). There are two sources of cholesterol in the neuroretina; endogenous biosynthesis (Zheng et al., 2012) and systemic circulation “diet” (Tserentsoodol et al., 2006b). The majority of cholesterol in human NR (~90%) is unesterified (UC). Moreover, UC is present in the retina at concentrations higher than those of other sterols (Mast et al., 2011, Fliesler and Bretillon, 2010, Omarova et al., 2012). Very little is known about the maintenance of cholesterol homeostasis in the retina (Zheng et al., 2012). Through immuno-chemical localizations analysis conducted in conjunction with quantifications by RT-PCR, a co-localization was found between cholesterol and proteins necessary for efflux, assembly, maturation and uptake of lipoprotein particles such as ATP-binding cassette transporter (ABCA1), Apolipoprotein E (apoE), Apolipoprotein A-1 (apoA-1), Lecithin—cholesterol acyltransferase (LCAT), Cholesteryl ester transfer protein (CETP), Scavenger receptor class B member 1 and 2 (SR-BI and SR-BII), and the cluster of differentiation 36 (CD36) - which also known as also known as fatty acid translocase (FAT) - were reported by several independent laboratories that conducted their work on different mammalian species including humans (Claudepierre et al., 2010, Duncan et al., 2009, Hayes et al., 1989, Kuhrt et al., 1997, Kurumada et al., 2007, Zheng et al., 2012). These findings suggest a role in lipoprotein-mediated cholesterol transport in the retina and the RPE cells, which itself was found to be able to

express and provide an abundant level of apoE for this transport (Tserentsoodol et al., 2006b, Amaratunga et al., 1996, Shanmugaratnam et al., 1997, Zheng et al., 2012).

1.5.3 Apolipoprotein E in ARMD pathogenesis

Apolipoprotein E (apoE) is unique among apolipoproteins, in its special relevance to nervous tissue. ApoE is an amphipathic glycoprotein that mediates the distribution of lipids and cholesterol among different cells including Müller cells, BrM and RPE in the human eye (Wang et al., 2010b, Wang et al., 2012b, Verghese et al., 2013). The gene for apoE (APOE), is located on chromosome 19q13.2; ApoE is a polymorphic with three common isoforms - E2, E3 and E4 - which are coded for by three separate alleles - Apo ϵ 2, Apo ϵ 3 and Apo ϵ 4. These alleles are differentiated on the basis of cysteine-arginine residue interchanges at sites 112 and 158 in the amino-acid sequence. As a result of this polymorphism, six common phenotypes exist: three homozygous phenotypes (ϵ 3 ϵ 3, ϵ 2 ϵ 2, ϵ 4 ϵ 4) and three heterozygous phenotypes (ϵ 2 ϵ 3, ϵ 2 ϵ 4, ϵ 3 ϵ 4) (Utermann et al., 1980).

ApoE plays a central role in serum cholesterol homeostasis through its ability to bind cholesterol and other lipids, where it mediates their transport in or out of cells (Mahley et al., 2006). For examples, in the macrophages, it serves as the ligand that mediates the uptake of lipoprotein particles from cells via the low density lipoprotein receptor (LDLR) and the LDLR-related protein (LRP) and transports it back to the liver, where the cholesterol can be excreted as bile salts - a process known as reverse cholesterol transport (RCT) (Basu et

al., 1983, Mahley et al., 2006). During nerve cell loss, large amounts of lipids are released from degenerating cell membranes and this release of lipids is believed to stimulate astrocytes in synthesising apoE. ApoE then binds these excess lipids and distributes them appropriately for re-use in cell membrane bio-synthesis (Klaver et al., 1998). An observation made by Ishida *et al* (2004) identified the presence of apoE and lipids at the inner aspect of the RPE and this observation, along with the work of other research groups, identified that the RPE cells are capable of producing their own apolipoprotein particles (Amaratunga et al., 1996, Li et al., 2006, Anderson et al., 2001, Ishida et al., 2004). This finding prompted the idea that this apolipoprotein may also facilitate the efflux of lipids from the RPE into the adjacent BrM and the possibility of the presence of a pathway for RPE cell-secreted lipids to cross BrM, where lipid wastes of the partially digested or undigested photo-receptor outer segments are secreted across the basal surface in association with ApoE. Therefore, it is suggested that age and/or disease-related disruption of normal ApoE function may result in the accumulation of lipoproteins at the interface between the RPE and BrM. This is consistent with observations that lipid deposits in drusen are largely composed of cholesteryl esters and unsaturated fatty acids. These findings are also consistent with the view that ApoE plays an important physiological role in the maintenance of macular health. Although genetic evidence establishes that APOE's polymorphism, specifically the $\epsilon 4$ allele of apolipoprotein E (apoE), is a risk factor strongly associated with Alzheimer's Disease (AD) (Strittmatter et al., 1993a, Strittmatter et al., 1993b, Jones et al., 2011), Parkinson's Disease (PD) (Vefring et al., 2010, Gao et al.,

2011, Huang et al., 2006) and ARMD (Malek et al., 2005, Li et al., 2006, Bojanowski et al., 2006), but the functions of apoE in the central nervous system remains unclear.

1.4.3.1 ApoE and A β interaction

One mechanism by which apoE might be involved in the pathology in neuro-degenerative diseases, such as AD and ARMD, is by modulating the activity of amyloid- β (A β). A β is derived by the proteolytic processing of amyloid precursor protein (APP). The amyloid cascade hypothesis sustains that aggregation of A β into amyloid deposits of neuro-degenerative diseases, such as AD and ARMD, induces toxic damage. In transgenic mice, apoE was found to influence the deposition of A β into both diffuse and compacted plaques (LaDu et al., 1997, Hartman et al., 2002), while *in vitro* studies demonstrated that apoE forms stable complexes with A β , alters the aggregation of various A β peptides, inhibits A β -induced neurotoxicity and inflammation and promotes clearance of A β , partially via apoE receptors (Youmans et al., 2012, Strittmatter et al., 1993b, Yang et al., 1997, Verghese et al., 2013, Tai et al., 2013).

1.4.3.2 ApoE and A β transport

Studies in both humans and transgenic mice indicated that A β levels aggregates found in AD plaques or ARMD drusen are ApoE isoform-dependent ($\epsilon 4 > \epsilon 3 > \epsilon 2$) (Yang et al., 1997, Martinelli et al., 2009, Hu et al., 2015, Cezario et al., 2015), and this suggests that a general mechanism of ApoE in modulating A β metabolism, aggregation, and deposition. This, in turn,

may be driven by apoE's ability to act as a chaperone and binds to different forms of A β . In the brain, apoE-containing lipoproteins were found to act as efficient acceptors by binding directly to A β secreted from neurons, thus playing a major role in the clearance or aggregation of A β in an isoform-dependent manner in the CNS (Youmans et al., 2012, Verghese et al., 2013). The deletion of the mouse APOE gene was found to eliminate deposition of fibrillar A β in amyloid model mice (Bales et al., 1997, Dorey et al., 2014, Liu et al., 2013). Given that ApoE is co-deposited with A β in drusen, it is possible that ApoE promotes A β aggregation and deposition in an isoform-dependent manner in the retina as well. The exact mechanisms by which ApoE isoforms differentially regulate A β aggregation and deposition require further investigation.

Though the role of apoE in ARMD pathology is not completely established, this apolipoprotein has several functions that may affect the course of this disease. ApoE has anti-angiogenic (Browning et al., 1994), anti-inflammatory (Dorey et al., 2014, Wang et al., 2012b, Liu et al., 2013, Kelly et al., 1994), and anti-oxidative effects (Tangirala et al., 2001, Fernández-Robredo et al., 2013). These are all considered as atheroprotective attributes of apoE, which are also important in protecting against the progression of ARMD. The expression of apoE is transcriptionally regulated by the ligand-activated nuclear receptors, peroxisome proliferator-activated receptor gamma (PPAR γ) and liver X receptors (LXRs) (Chawla et al., 2001).

1.6 Hypotheses

ARMD is a multi-factorial disease in which the relative contributions of genetic and environmental factors remain unknown. In this work we hypothesised that the degeneration of RPE cells in ARMD, begin with the impaired clearance of cellular lipid waste that accumulates within the RPE cells. This lipid build-up is thought to primarily interfere with the fluid efflux from the RPE across BrM's, generating a state of chronic stress in the RPE cells that eventually lead to the formation of abnormal deposits called drusen. Drusens formation is thought to impair the function of RPE cells, and believed to trigger complement activation as a secondary response. Complement activation expression is initially intended to protect the RPE from lipo-toxicity damage. Yet, within this paradigm it is suggested that this chronic activation of the complement system will eventually lead to a state of para-inflammation that might lead to further tissue damage.

1.7 Project Aims

The research presented in this thesis will focus on the effects of RPE lipo-toxicity as a potential *in vitro* model for ARMD, and how complement activation modulate a secondary response. These effects will be investigated using an *in vitro* model of human retinal pigment epithelial cells grown on lamnin coated filters under serum free conditions. Specifically the following will be determined;

- I. The effect of treatment in capturing certain aspects of ARMD pathologies i.e the effect of lipo-toxicity on tight junction expression,



vascular endothelial growth factor (VEGF) secretion and sub-RPE deposits' formation.

- II. The effects of complement activation, which is elevated in ARMD, on RPE function as determined by TJ expression, VEGF level and sub-RPE deposits constitutes.
- III. A genomic wide investigation on the effect of the two treatments.



2

MATERIALS AND METHODS

2.1. Chemicals and Reagents

Unless otherwise specified all chemicals were obtained from Sigma Aldrich (St. Louis, MO/USA). Palmitic acid and oleic acid were purchased from Tocris Cookson (Bristol, UK). All water used was deionised (dH₂O) unless state otherwise. All phosphate buffered saline (PBS) was at pH 7.4 without calcium and magnesium salts. All cell culture reagents were purchased sterilised or sterilised prior to use either by filtration through a 0.22µm Whatman sterile filter or autoclaving at 121°C at 1bar for 20 min.

2.2. Cell culture

2.2.1. Cell lines used

2.2.1.1. ARPE-19; Human retinal pigment epithelia (ARPE-19; ATCC/CRL-2302) a spontaneously transformed line of RPE cells derived from the globes of a normal 19 year old male donor in 1986. This cell line maintained a normal number of chromosomes and RPE specific markers expression ie retinal pigment epithelia specific protein 65 kDa (RPE65), Orthodenticle homeobox 2 (OTx2) and cellular retinal aldehyde binding protein (CRALBP) at both the mRNA and protein level, respectively. Those genes are uniquely expressed in mammalian RPE cells. ARPE-19 cells have been widely used in studies on oxidative stress, retinal pathogenesis, signalling and inflammatory pathways as well as in drug and toxicity related studies. When grown on filter support they form stable monolayers, which exhibit morphological and functional polarity.

2.2.1.2. HEPG-2; Human hepatocellular liver carcinoma cell line (HEPG-2; ATCC/ HB-8065), a perpetual cell line which was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a modal chromosome number of 55, and are not tumorigenic in nude mice. The cells secrete a variety of major plasma proteins, e.g., albumin, transferrin, and the acute-phase proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin, and plasminogen. They have been grown successfully in large-scale cultivation systems.

Main use: In this study HepG2 cells are used as positive control for the expression of Apolipoprotein E and B100 (ApoA/B100), Microsomal triglycerides transfer protein (MTTP) and FABp1, 3 and 5.

Culturing Media used Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) containing 10% (v/v) Adult Bovine Serum (Sigma Aldrich), 1% (w/v) penicillin-Streptomycin (Sigma Aldrich) and 2 mM glutamine (Sigma Aldrich).

2.2.2. Initiation of cell culture

Cells stock were removed from liquid nitrogen storage and recovered by rapid thawing quickly to retain viability in a 37°C water bath with gentle swirling while keeping the O-ring and cap out of the water. The vial was removed as soon as the contents were thawed and decontaminated with 70% ethanol. All operations, i.e. seeding and sub-culturing, from this point on were accomplished under aseptic conditions in a biological safety cabinet. The contents of the vial were then transferred to a 75-cm² T-flask containing approximately 10mL fresh medium supplemented 10% FBS serum. Prior to the addition of the cell content, the flask containing the medium was placed in a 5% CO₂ incubator at temperature of 37 °C for at least 15 minutes to provide optimal temperature and pH conditions. The prepared (seed) culture was incubated in a humidified atmosphere of 5% CO₂ at 37°C.

2.2.3. Sub-culturing procedures and determination of cell count

Depending on the rate of cell growth and initial concentration, cells from the mid-exponential phase were harvested and passaged to a fresh medium every two or three days as follows:

First, spent media was removed from the flask and the cells were washed with pre-warmed PBS. Appropriate volumes (e.g. 3 ml for 75 cm² flask) of 0.05 % trypsin/EDTA (Invitrogen, UK) were then added to the flask and incubated at 37°C until the cells detached (4 to 6 minutes). This incubation generally facilitates cell dispersal and so reduces the enzyme exposure period. Cell detachment was checked by an inverted phase-contrast microscope and after complete detachment was observed, the trypsin was then inactivated by adding the double volume of serum-containing media (Culture media) to the flask- approximately 6mL of fresh medium was added.

The cell suspension was removed from the flask and placed in a sterile 25 ml centrifuge tube then centrifuged at 400 g for 5 minutes in Biofuge primo centrifuge. The cell pellets were re-suspended in 10 ml DMEM with 10% fetal bovine serum and then 100 µl aliquot was added to 100 µl trypan blue (Sigma) (1:2) after gentle mixing cells were counted with haemocytometer or using an automated Cedex XS cell counting system. Based on this count a new subculture was prepared with a cell concentration. A suitable amount of cells (1.6×10^5 cells/mL for ARPE-19 cell cultures, 2×10^5 for HepG2 cultures) was finally seeded into 10 ml of the supplemented DMEM/F12 in vented 75 cm²

cell culture flask (Corning, UK). The cells were then incubated in a humidified atmosphere with 5 % CO₂ at 37°C.

2.2.4. Cryogenic Preservation of cell lines

In order to maintain a cell bank reserve, cryogenic preservation of CHO cells was carried out. Cells were harvested in mid-exponential phase of growth with viability higher than 90%, centrifuged and re-suspended in a cryopreservation medium to a concentration of approximately 1×10^6 cells per mL. The cryopreservation medium contained 70% fresh medium, 20% FBS and 10% of a cryoprotective agent, DMSO (dimethyl sulphoxide). FBS Serum was added to the fresh medium to improve post-freezing survival and recovery of the cells. The prepared cell suspension was aliquoted into labeled 1mL cryovials, placed overnight in a -80°C freezer and then immediately transferred to a liquid nitrogen container for long term storage.

2.2.5. Millicell inserts

This work was carried by using a commercially available Millicell™ hanging cell culture inserts, to control the long-term proliferation and culture of the ARPE-19. Two types of Millicell inserts were used; Millicell-HA inserts had a mixed cellulose esters membrane with pore size of 0.45 µm and Millicell-PCF inserts which is hydrophilic PTFE membrane with pore size of 0.4 µm. Both inserts were used in a 24-well plate and had an effective growth surface area of 0.6 cm² and a thickness of 10.5 mm. All inserts were aseptically handled with sterile forceps throughout the work.

2.2.6. Treatment with laminin coat

The multi-layered extracellular matrix underlying the retina, Bruch's membrane (BrM's), contains several laminins that support the retinal pigment epithelial cells (RPE) to grow on. The presence of the basement membrane is essential for the polarization of RPE cells. It was shown by Aisenbrey and colleagues 2006 that RPE cells adhere robustly to all laminins of Bruch's membrane and that RPE cells synthesize these laminins. Therefore the filter supports were coated with $10\mu\text{g cm}^2$ purified mouse laminin (from Engelbreth-Holm-Swarm murine sarcoma/ Sigma). To coat filters, the laminin was thawed slowly in ice to prevent its transformation to gel then diluted 1:10 in PBS; coated filters were then incubated for two hours at 37°C co^2 incubator. Immediately before plating cells on coated filters the basolateral chamber of each cell culture well was rinsed several times with PBS.

2.2.7. Preparation of ARPE-19 cultures

Cell culture models of RPE can play an important role in understanding the native tissue. In this protocol ARPE-19 Passage between 7- 15 were used to set up the protocol. Cells were passaged and seeded onto coated Millicell-HA or Millicell-PCF filters of $0.40\text{-}0.45\mu\text{m}$ diameter (Millipore Millicell-HA Culture plate inserts, PIHA 01250 and Millipore Millicell-PCF Culture Plate Inserts, PIHP 012 50). Cells were seeded in laminin coated side (apical) of the filters at a density of $1.6 \times 10^5 \text{ cell/cm}^2$ in DMEM/Ham F12 supplemented with 2 mM glutamine and 100 IU/ml penicillin- streptomycin and 10% FBS then switched to serum free media after 48h. A corresponding amount of culture medium was added at a ratio of 400 μl : 600 μl (upper: lower) to the basal

compartment, levelling the height of the liquid to prevent hydrostatic pressure.

The medium was changed every 3-4 days **Figure 2.1**

Cells were cultured for at least a month to form differentiated monolayers, with the apical domain corresponding to the retina-facing side of the RPE monolayer and basal compartments side corresponding to the choroid-facing side of the RPE monolayer.

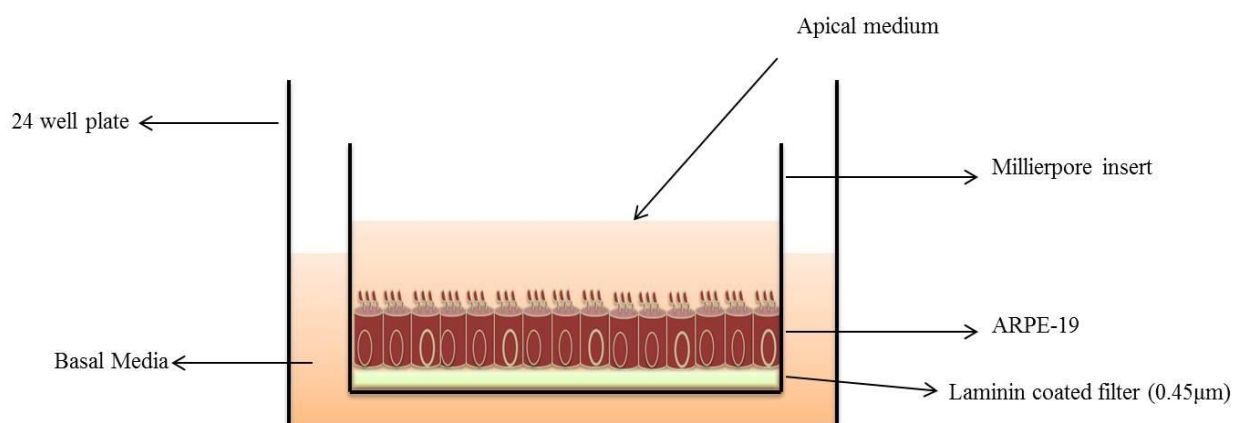


Figure 2. 1 A Schematic presentation of the typical ARPE-19 cell culture. Cells are cultured on laminin coated membrane. Media was replaced every 2-3 days at both apical (representing RPE facing the photoreceptors) and the basal (representing RPE facing choroid).

2.3. Analytical Procedure

2.3.1. Viable Cell Concentration

Trypan Blue is the most common stain used to distinguish between viable and dead cells. Only nonviable cells can absorb the dye and appear blue. 100 μ L of cell suspension sample was taken and diluted in 1:2 ratio in 0.5% Trypan Blue dye. The optimal concentration of cells for counting was found to be $5-10 \times 10^5$ cells/mL (50-100 cells per large square) after dilution

with the Trypan Blue solution. The stained cells were counted using a hemocytometer under the light microscope within 3 minutes upon mixing with Trypan Blue. After this time period the viable cells will begin to take up the dye and consequently the result of the test will be misleading. The viability of cells was also quantified by the ratio of viable cell to total cell density.

2.3.2. Nuclear Analysis and for cell culture viability

Analysis of nuclear viability was performed through fluorescent nuclear staining, ARPE-19 cell cultures were fixed and permeabilized with 0.1% Triton X-100, and labeled with 4', 6'-diamidino-2-phenylindole dihydrochloride hydrate (DAPI; Sigma-Aldrich) see **section 2.4.5.** . The percentage of nuclei was determined through the use of ImageJ software. Results were collected from a minimum of n=3 repeats.

2.3.2. Tight junction assessment

The percentage of continuous tight junction (TJ) presentation was calculated using the protocol provided (Ebrahim and Leach, 2014). Samples were labelled with ZO-1 antibody as described in **section 2.4.3.**, an average of random 10 images were taken for each sample of each condition. To ensure that all cell-cell junctional regions had an equal chance of being counted, a grid was placed on images and both continuous and discontinuous junctions were counted from every other square **Figure 2.2.** Results were collected from a minimum of n=3 repeats.

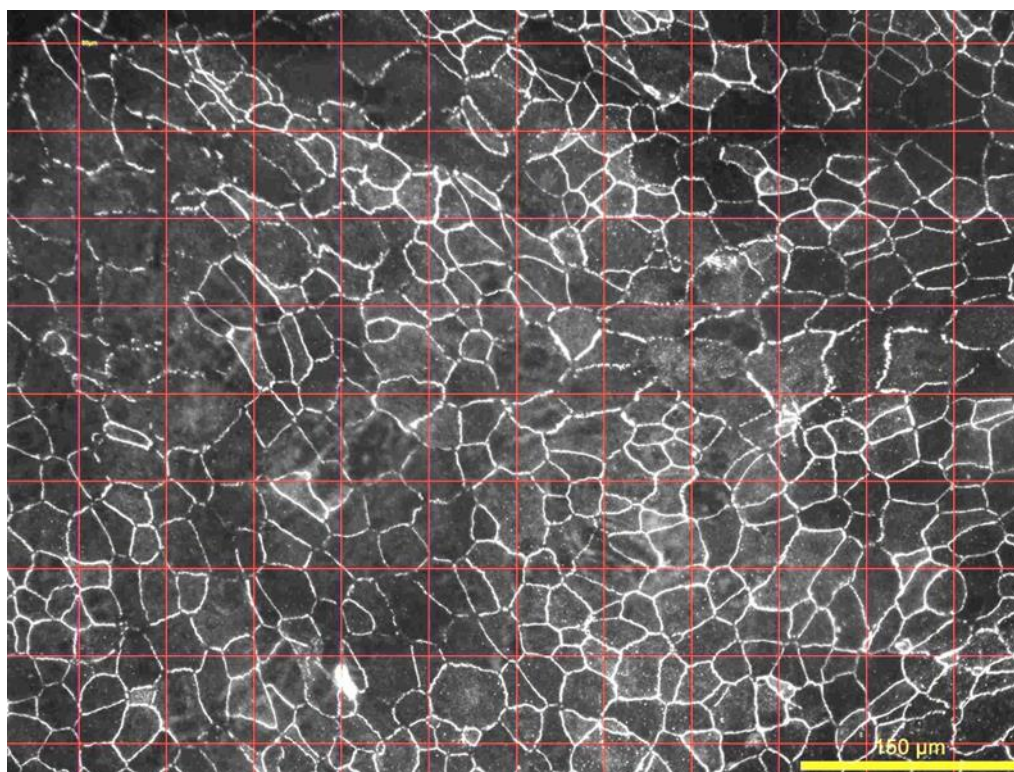


Figure 2. 2 The percentage of continuous tight junction assessment. ARPE-19 cell cultures were labelled with TJ protein ZO-1 to visualise TJ presentations. Through the use of Leica DMRB microscope, TIF images were taken from 10 random areas in each culture. A Fixed grid was used ensure that all cell–cell junctional regions had an equal chance of being counted at a fixed rate. Both continues and discontinues junctions were counted from every other square.

2.3.3. Preparation of Fatty Acids in Complex with BSA (PLO)

Complexes of BSA and fatty acids were prepared by a method adapted from van Harken, Dixon, and Heimberg (Heimberg et al., 1967, Van Harken et al., 1969). The 24% (w/v) bovine serum albumin (BSA) was prepared by gradually adding 6g of fatty-acid-free BSA (cat. No. A6003; Sigma) into 17.5ml 150mM NaCl in a beaker, over a slow period of time at room temperature with stirring. The pH was adjusted to 7.4 with 5M NaOH, and the volume made up to 25ml with 150mM NaCl. This solution was filtered through a 0.45μm filter and frozen in aliquots at –20°C. The fatty acid/BSA solution

was then prepared by a modification of the method of Van Harken and Dixon (1969). Briefly, 39.6 mg Sodium Oleic, 36mg palmitic or 40µl linolic acid was transferred to a glass beaker containing 0.5ml ethanol, and the ethanol was evaporated under a stream of nitrogen. Then 7 ml 24% (w/v) ice-cold BSA and 48µl of 5M NaOH were added. The solution heated gently with stirring on a hot plate until an emulsion is formed then the fatty acids were stored at -20°C until use.

Each new preparation of PLO mixture was tested prior to use in study. ARPE-19 cell cultures were incubated with different dilutions of PLO (0, 0.2, 1M) for 48h at 37°C in serum free culture medium. Lipid droplets were investigated by Nile red fluorescent dye **Figure 2.3**; microscopy was used to examine the localization of the accumulated lipids. Lipid quantification was carried by Image J (see **section 2.4**). Results were collected from a minimum of n=3 repeats.

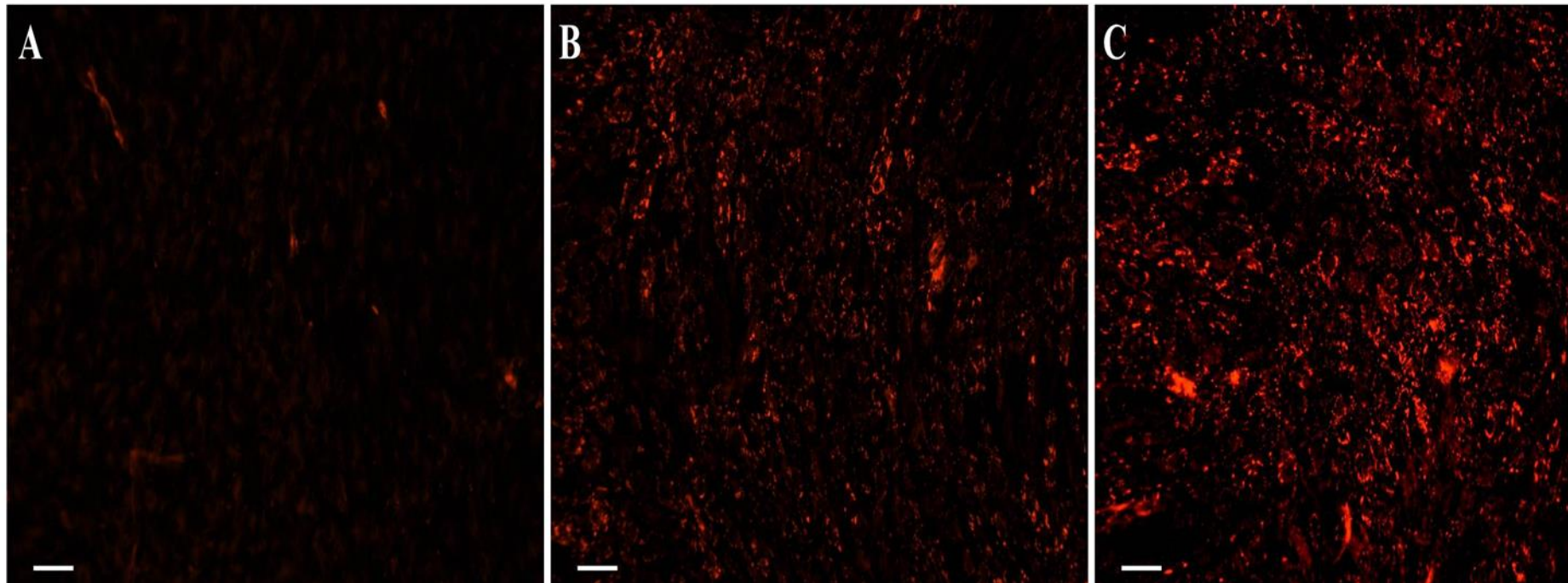


Figure 2. 3 Lipid loading with PLO mixture. ARPE-19 cells treated with (A) serum free media, (B) 0.20mM pol and (C) 1mM POL, to test the effectiveness of the newly prepped PLO mixture. ARPE-19 cells were grown in a laminin coated millicell-PCF filters for 48h then treated with different doses of PLO (0, 0.2 and 1mM). Cells were fixed and stained with Nile red (see section 2.4). An increase of accumulate lipids in a dose dependent manner is taken as a sign of effectiveness.

2.3.4. Trans-epithelial electric resistance (TEER) measurement

Trans-epithelial electric resistance (TEER) measurement by an EVOM voltohmmeter (World Precision Instrument) assessed the tightness of the ARPE monolayer. The instrument has a chopstick like electrodes (STX2) with the short electrode being inserted in the Millicell insert whereas the longer electrode is placed in the lower chamber **Figure 2.4**. Electrical resistance values of cell monolayers in the inserts TEER (Ωcm^2) was calculated by multiplying the resistance value (Ω) by the surface area of the cell monolayer. TEER measurements were taken within 3 mins of taken the culture out of the incubator for optimal readings. Results were collected from n=3 repeats.

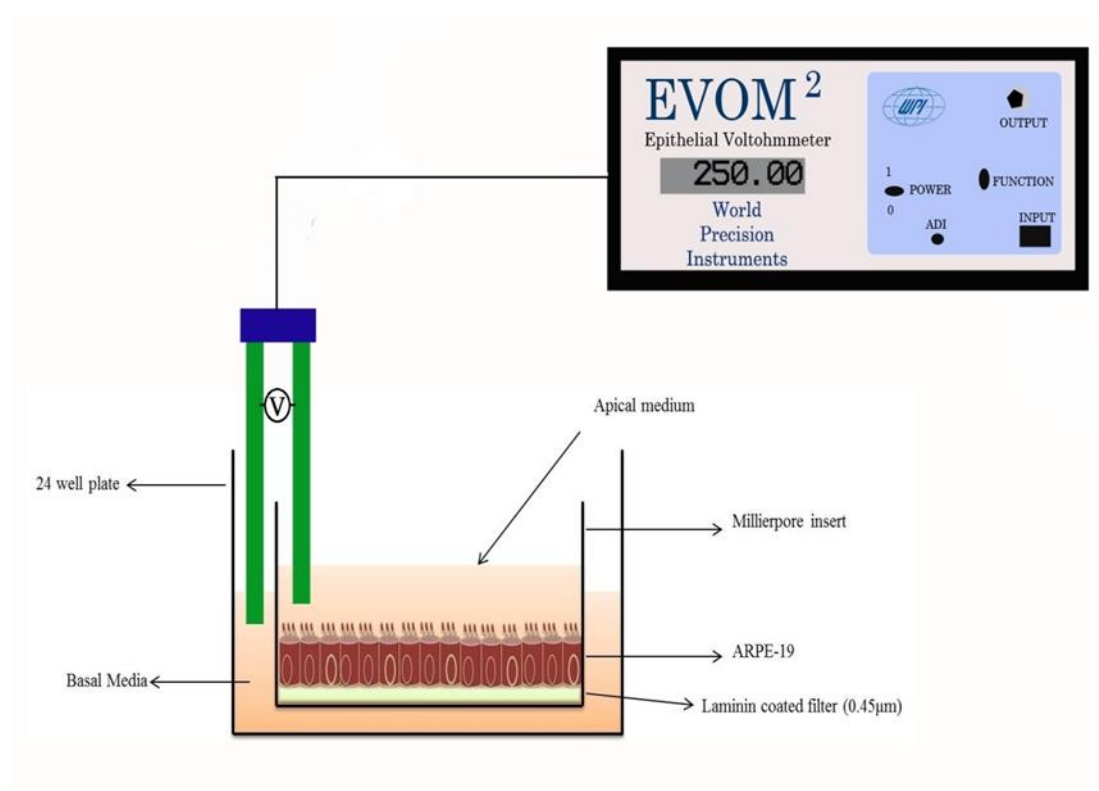


Figure 2. 4 Schematic illustration of in vitro trans-epithelial electrical resistance measurements (TEER). A monolayer of cells is formed on a porous membrane separating two compartments. TEER was measured using STX electrodes and an EVOM resistance meter. The instrument has a chopstick like electrodes (STX2) with the short electrode being inserted in the Millicore insert whereas the longer electrode is placed in the lower chamber. TEER value was calculated by multiplying the resistance value (Ω) by the surface area of the cell monolayer (0.6cm^2)

2.2.5. Preparation of Human Serum

Fresh human serum was obtained from blood samples taken in the absence of anticoagulant. Clotted blood (30 min, room temperature) was centrifuged at $1,500 \times g$ for 15 min, and the collected serum was aliquot and stored at -80°C . Human serum pool was used to activate the complement system in ARPE-19 cell culture. To assure that equal complement activation

was achieved in each test and to prevent human variation, a pool of was created from the collected sera.

2.2.6. Heat-Inactivation Procedure

To destroy the complement a heat-inactivation process was carried to the collected human sera. Chosen aliquot samples were placed in a 56°C water bath, allowing the water level to reach the top of the sample volume but not touching the rim of the cap of the tube. Samples were incubated for 1 hour to be heat-inactivated. Samples that were previously heat-inactivated were re incubated for 30-45.

2.4. Fluorescence imaging analysis

2.4.1. Fixation of cells

Cell cultures were washed 3 times with PBS and then fixed in 4% paraformaldehyde in PBS (PFA) for 20 minutes. The fixation reagent was removed by washing the cells four times with PBS. Cultures were stored in 0.4% paraformaldehyde pending sectioning and immunohistochemical analyses.

2.4.2. Sample preparation

2.4.2.1. Sample processing

Cell culture filters were imbedded in 5% Agros templates after fixation with 4% PFA and processed using an automated tissue processor (Leica TP1050, Leica Instruments GmbH, Nussloch, Germany). The Paraffin –

embedded samples were first sectioned into 17.5 μM sections mounted on amino silane (APS) coated glass slides using a SLEE CUT 4060 (Mainz) rotary microtome (Mainz) with a 4689 Accu-Edge Low-Profile Blade, before carrying out further processing. Sectioned samples were allowed to dry for 10 minutes, followed by 20 mins incubation at 40°C hot plate.

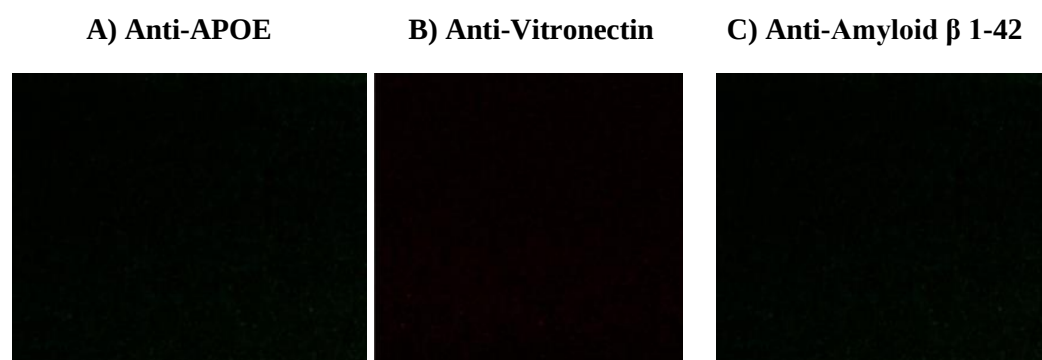
2.4.2.2. Dewaxing, rehydration and antigen retrieval

The slides were submerged in two changes of xylene (2 min) then rehydrated by immersion the slides in a serial ethanol baths starting at two changes of 100% then 90% ethanol (2 min) then 70% and finally 50% before they are washed in H₂O (2min) path twice. The slides were then allowed to sit on hot plate, and then covered in citrate buffer. The hotplate was then preheated to 60°C for 45 minutes. They were then treated with 3% (v/v) methyl hydrogen peroxide for 5 minutes to inactivate endogenous peroxidases.

2.4.3. Immunofluorescence

Immunofluorescence imaging was performed for 17.5 μm microtome sections or flat mounts of fixed ARPE-19 cell cultures. Sections were washed in PBS and blocked for 60 min in 10% goat serum. Sections were incubated with primary antibody overnight at 4°C, sections were washed in PBS three times for 5min, and incubated with secondary antibody for 60 min at room temperature. The sections were washed in PBS three times for 5 min then incubated with DAPI for 5 min followed a final three time washes and mounted. Negative controls omitting the primary antibodies were carried with a cell culture membrane without cells following exposure to cell culture media

(DMEM/HAM's F12 supplemented with 10% FBS for 48h then switched to serum free DMEM/Ham's F12 for 3 months) and were uniformly negative. A secondary antibody control was carried as well with negative results **Figure 2.5**. Images from sections were visualized and captured using through the use of Leica DMRB transmitted-light microscope or Leica DMIRB Inverted Microscope with appropriate filters. Data where taken as Tiff or Jpg and processed by using Volocity imaging software or Image J. Results were collected from a minimum of n=3 repeats.



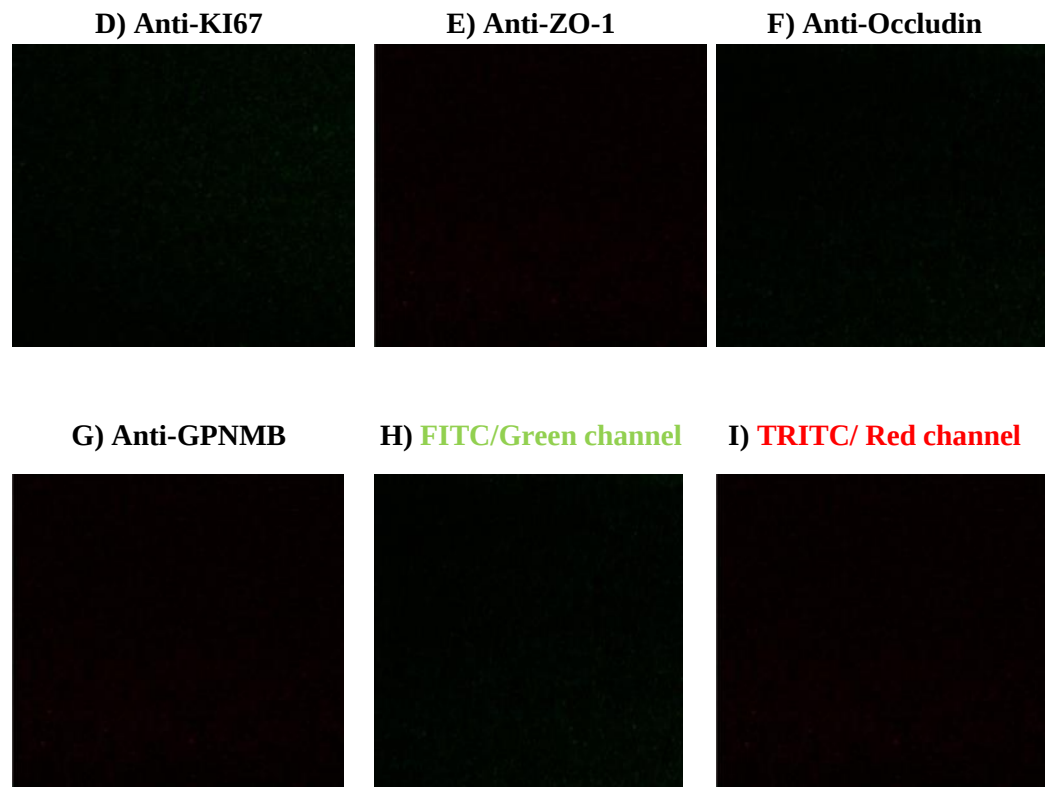


Figure 2. 5 Immunostaining of a millicell-HA cell culture membrane without cells following exposure to DMEM/HAM's F12 cell culture medium supplemented with 10% FBS for 48h then switching to serum free (DMEM/HAM's F12) for 3 months at 37 °C. (A) Anti-apolipoprotein E (APOE) ,(B) Anti- Vitronectin, (C) Anti- Amyloid β 1-42, (D) anti-KI67, (E)) anti- zona occuldens proteins 1 (ZO-1), (F) anti-Occludin and (G) anti-Glycoprotein NMB (GPNMB) A, B and C all shown negative for deposit-like immunoreactivity, indicating that the APOE and Amyloid β 1-42 immunoreactivity observed in association with sub-RPE deposits is not related to deposition of bovine APOE from the supplemented FBS in the medium, and that Amyloid β 1-42 immunoreactivity is not the result of complement activation by other components of the medium or the laminin coat. A secondary antibody control is shown in (H/FITC channel) and (I/TRITC channel).

Table 2. 1 List of primary antibodies used in this study

Antigen	Host species/type	source	Cat. No.	Dilution
ApoE	Rabbit/polyclonal	ABcam	Ab20874	1/1,000
ApoE	Mouse/monoclonal	ABcam	Ab1907	1/1,000
Amyloid β	Rabbit/polyclonal	ABcam	ab120301	1/500
Vitronectin	Mouse/polyclonal	ABcam	Ab13413	1/1,000
GPMB	Mouse/polyclonal	ABcam	Ab56584	1/500
ZO-1	Rabbit/ polyclonal	ABcam	Ab59720	1/1,000
Occludin	Mouse/Monoclonal	Life technologies	33-1500	1/200

2.4.4. Nile red Lipid staining

Nile red staining was used to visualise lipid droplets; cells were washed twice with Hank's Balanced Salt Solution (HBSS). A dilution of 30 μ M of Nile red in HBSS was added to the cells making sure that it covered the culture surface completely. Samples were incubated in the dark for fifteen minute, and then washed again with HBSS. Samples were then mounted with 80% glycerol plus DABCO (1, 4-diazabicyclo [2.2.2] octane) antifade and viewed using a Leica DMIRB Inverted Microscope with appropriate filter for Nile red with Excitation of 525 and Emission of 590 nm. Images were captured with Hamamatsu digital camera. Intracellular fat vacuoles, filled with neutral lipids such as cholesterol, lipoproteins and triglycerides will fluoresce green (EX 485nm/EM 590nm) while polar lipids, such as phospholipids, will fluoresce red. In this study a special filter for Nile red was used to ensure only

intracellular lipids are captured. Red color was added to the taken images as illustration only.

All Millicell-PCF were kept intact throughout the staining process to make sure that they were stained on the appropriate side. Once the staining was completed, the filter was removed by carefully cutting around its edge with a surgical scalpel and placed on a mounted cover slip cells facing down.

2.4.5. Filipin cholesterol staining

Filipin is a polyene macrolide antibiotic that is fluorescent and specifically binds to free non-esterified 3 β -hydroxy sterols, such as cholesterol, in a one-to-one molar stoichiometry, and that filipin fluorescence intensity is proportional to the amount of sterol bound (Drabikowski et al., 1973; Gatfield and Pieters, 2000). Treated cells were incubated with 0.05% filipin (30 min to 1 hour) at 37°C, then visualised using fluorescence microscopy with excitation (355 nm) and emission (420 nm) through a standard UV filter block. Results were collected from a minimum of n=3 repeats.

2.4.6. Nuclei staining

DAPI blue fluorescence staining was used to check the cell viability throughout this study. The visualisation of nuclei assessed counts of cell through the use of Image J. DAPI stains nuclei specifically, with little or no cytoplasmic labelling and it has a maximum excitation of 358 nm, and a maximum emission of 461 nm. Samples were equilibrated briefly with phosphate-buffered saline (PBS) then fixed with 4% paraformaldehyde (PFA)

for thirty minutes and then washed again with PBS. 300nM diluted DAPI stain was added to the samples, making certain that the cells were completely covered. Samples were incubated for five minutes then rinsed several times in PBS. Excess buffer was drained from the samples and they were mounted with 80% glycerol plus DABCO (1, 4-diazabicyclo [2.2.2] octane) antifade. Samples were viewed using Leica DMRB transmitted-light microscope or Leica DMIRB Inverted Microscope with appropriate filter (DAPI filter Excitation 350/50 – Emission 460/50). Images were captured with Hamamatsu digital camera. Results were collected from a minimum of n=3 repeats.

2.5 Analysis of Gene Expression at the mRNA level

2.5.1. Total RNA extraction

RNA was extracted from ARPE-19 cell cultures using Tri-reagent® (Ambion). A pool of two millicell filter inserts was homogenized in 1ml tri reagent using a Janke & Kunkel Ultra-Turrax T25 homogenizer. The homogenate was allowed to stand for 5 minutes at room temperature then 200µl of BCP (1-Bromo-3-chloropropane, Sigma) was added per ml of Tri-reagent used. The solution was vortexed and centrifuged at 10,000 rpm for 15 minutes at 4°C. RNA was precipitated from the upper aqueous phase by the addition of 250µl of 2M sodium acetate and 700µl 2- isopropanol per ml of solution. After mixing, the solution was chilled at -20°C overnight, followed by centrifugation at 10000g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed twice with 500µl 70% ethanol solution. The supernatant

was then removed, and the pellet air dried for 5 minutes and then re-suspended in 50 μ l RNase free water. Results were collected from a minimum of n=3 repeats.

2.5.2. DNase treatment of total RNA

Residual plasmid or genomic DNA was removed from RNA samples using DNase I (Invitrogen). Briefly, samples (up to 2 μ g) of total RNA were equilibrated with 10 x DNase buffer to which 1 U of DNase was added. Samples were then incubated for 15 minutes at room temperature and the enzyme was denatured by incubation at 65°C for 10 minutes. The RNA and purity and concentration were further measured on a nanodrop spectrophotometer (Nanodrop technologies, USA). The ratio of absorbances between 1.8-2.0 at both 260/280 and 260/230 was taken as an indication of acceptance of RNA purity.

For microarray studies, RNA quality was also measured by Agilent bioanalyzer 2100. Briefly, 1 μ l aliquots of each RNA sample were taken from the stock samples and diluted, if necessary, to give a concentration of less than 500ng/ μ l and then stored on ice. The stock sample was immediately returned to the - 80°C freezer. Prior to use, the Agilent 2100 bioanalyser electrode was cleaned with RNasezap by following the manufacturer's instructions. Next, preparation was made for the chip priming station. A new syringe was inserted into the station and 550 μ l of gel matrix was centrifuged through a spin filter for 10 minutes at 1500g. To a 65 μ l aliquot of filtered gel was added 1 μ l of well

mixed dye concentrate. After mixing, the gel dye mixture was centrifuged for 10 minutes at 13000g. A new RNA 6000 nanochip was inserted into the chip priming station and 9µl of the gel-dye mix was pipetted into well “G”. Next, the chip priming station was activated and the plunger was released exactly 30 seconds after activation. The plunger was then pulled back to the 1ml position. The chip priming station was then opened and 9µl of gel-dye mix was pipetted into all wells marked “G”. Next, 5µl of the Nano marker was pipetted into the ladder well and the 12 samples wells. Any unused wells had an extra 1µl of marker added so that the volume in all the wells was the same. Next, 1µl of heat denatured RNA ladder solution was pipetted into the ladder well.

All of the labelled test samples were then heat-denatured at 70°C for 2 minutes using a heat block and 1µl aliquots of each sample were pipetted into the relevant sample well. The chip was then vortex mixed for 1 minute in an IKA vortexer at 2400rpm. The chip was then loaded onto the Agilent 2100 Bioanalyser, the lid closed and the run started (takes 25 minutes). The Agilent 2100 expert software was used to analyse the electropherogram. First, the ladder data was viewed to assess the function of the chip. If this was satisfactory each individual sample was viewed to check for the presence of 2 distinct peaks (rRNA) and a normal distribution curve representing mRNA

Figure 2.6. Visual inspection of RNA integrity generates a ribosomal ratio and an RNA Integrity number (RIN). RIN is a comprehensive estimation of RNA integrity, as it takes the entire electrophoretic trace into account (Mueller & Schroeder, 2004). Samples were deemed acceptable for microarray studies if they had a RIN value more than 7.5

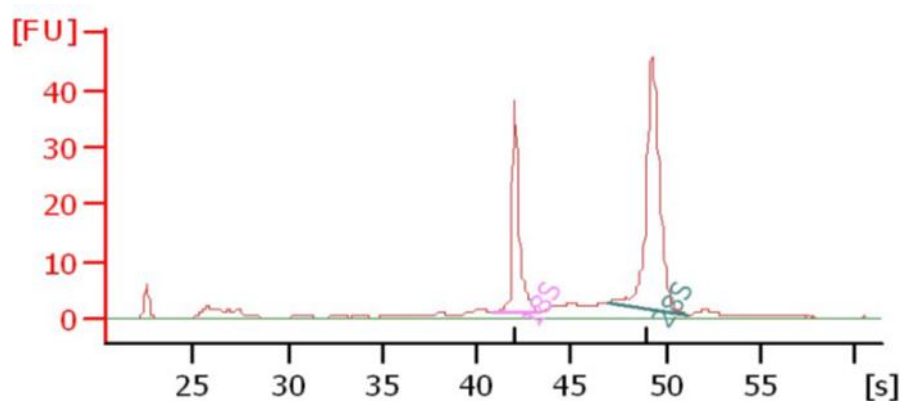


Figure 2. 6 Representative electrophoretogram demonstrating the total RNA quality and integrity extracted ARPE-19 cell culture. The 18s and 28s peaks are clearly visible at 40 and 50 seconds.

2.5.3. Single stranded cDNA synthesis

cDNA was produced by the reverse transcription of 500ng of total RNA. The following were added into 0.5 ml PCR tubes: the total RNA or mRNA, 1 μ L random primers (100ng), 1 μ L of dNTP's (10 mM) along with RNase-free water was added to give a total volume of 13 μ L. The tubes were briefly vortexed and centrifuged before being placed into a thermocycler (MWG-Biotech Primus 96 Plus) at 65°C for 5 minutes followed by a rapid chill on ice. Whilst on ice 4 μ L of 5 X First-strand buffer, 1 μ L of 0.1M DTT, 1 μ L of RNaseOUT (40u/ μ L) and 1 μ L of Superscript™ III RT (200u/ μ L) were mixed and then added to the primer:RNA mix. The contents of the tube were mixed and incubated at 25°C for 5 minutes, 50°C for 1 hour, 70°C for 15 minutes and then cooled to 4°C. cDNA was then stored at -20°C until RT-QPCR analysis.

2.5.4. Analysis of mRNA expression by TaqMan® real-time quantitative PCR

The mRNA expression levels of selected genes **Table 2.3** were quantified using the step one plus real –time PCR system (Applied Biosystems) based on Taqman standard curve method. The Taqman RT-PCR is a very selective and useful assay for the amplification of specific sequences encoding the gene of interest. It employs the use of a Probe where its ends are labelled with a fluorophore and a quencher respectively. During the RT-PCR reaction, the Probe identifies amplified targets and binds to them. The intensity of light emitted, which is due to the release of the probe, is measured in real time which reflects the expression levels of the gene in the sample being analysed. For a reaction with a total volume 25µL the following reagents were added to 5µl of cDNA;

Table 2. 2 Sample preparation for QRT-PCR reaction

TaqMan® Universal PCR Mastermix Applied Biosystems (4304437)	12.5µL
Forward primer (10 µM)	0.75µL
Reverse primer (10 µM)	0.75µL
Probe (dual labelled) (10 µM)	0.5µL
Water (molecular grade)	5.5µL

A negative/No Template Control (NTC) was also included by replacing 5µL of cDNA with 5µL RNase-free water. Thermo-cycling parameters were 50°C for 2 minutes, 95°C for 10 minutes (melting temperature) followed by 40 x (95°C for 15 seconds, 60°C for 1 minute) (annealing temperature). The

standard curve consisted of two-fold serial dilutions of cDNA reverse transcribed from a known quantity of RNA (NEAT). All standards, samples and negative controls were assayed in triplicate to ensure accurate results. The threshold cycle (Ct Value) is the point at which fluorescence rises to a point considered statistically significant above the baseline values. A standard curve was then constructed by plotting Ct values versus the logarithm of the serial cDNA dilutions. Any assay which did not have a standard curve with a slope between -3.0 and -3.6 (90-100% efficient) was rejected as were any results where the values for the triplicates had a range greater than 0.5 Ct **Figure 2.7**. Maintenance genes were used where appropriate. Results were collected from a minimum of n=3 biological repeats and a minimum of n=5 technical repeats.

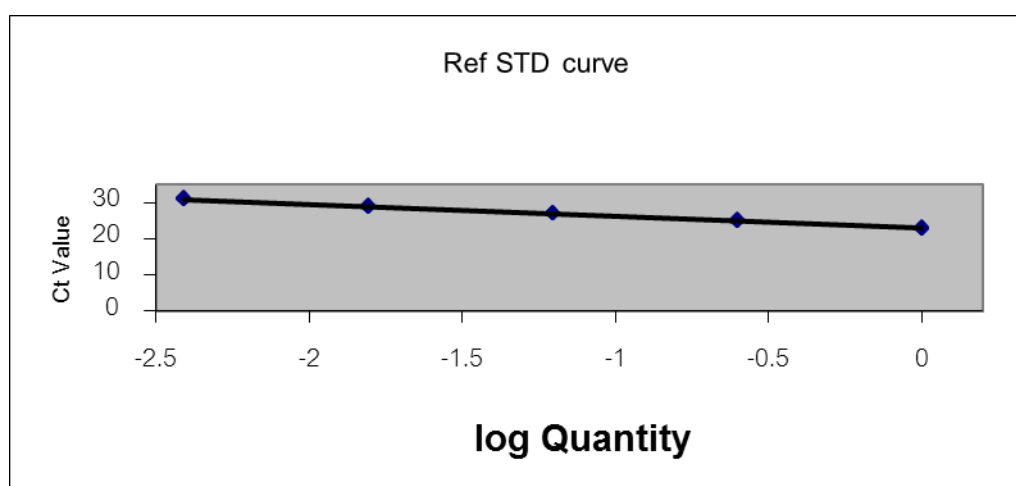


Figure 2. 7 TaqMan Real-time PCR standard curve for reference gene RNA polymerase II in ARPE-19 cell culture showing the slope value (-3. 31) and correlation co-efficient (r²) value (0.99)

**Table 2. 3 Taqman RT-PCR primer and probe sequences and sources**

Human Gene	Sequences (5' → 3')
GAPDH	Forward: CAA CAG CCT CAA GAT CAT CAG C Reverse: TGG CAT GGA CTG TGG TCA TGA G Probe: CCT GGC CAA GGT CAT CCA TGA CAA C
TBP	Forward: GGA GCT GTG ATG TGA AGT TTC CTA Reverse: CCA GGA AAT AAC TCT GGC TCA TAA C Probe: TAG AAG GCC TTG TGC TCA CCC ACC A
Human β -actin	Forward: CCT GGC ACC CAG CAC AAT Reverse: GCC GAT CCA CAC GGA GTA C T Probe: ATC AAG ATC ATT GCT CCT CCT GAG CGC
RBS18S	Forward: CGG CTA CCA CAT CCA AGG AA Reverse: GCT GGA ATT ACC GCG Probe: TGC TGG CAC CAG ACT TGC CCT C
RNA polymerase II / POLR2A	Forward: TGT GTT TCA TGT GGG CTT CCT Reverse: CCT TAA TCT TCG GGT TGT TAG AAT CT Probe: CGC TGT GTC TGC TTC TTT TGC TCC AA AC
Complement component 3	Forward: CCA CAG TTC TCT ATC GGA TCT TCA Reverse: GAC GCC AAG CTG GTT CTG A Probe: CAA CAT TGA GAA CCC GGA AGG CAT CC

2.5.5. Primer design and preparation

Primers are single stranded synthetic oligonucleotides that are used in molecular techniques including PCR and DNA sequencing. They are designed to have the reverse complement sequence of a region in a template or target DNA to which they anneal to in order to amplify a specific region of that template. Therefore all primers were designed according to a database sequence that was retrieved from NCBI using Primer Express 3 software **Table 2.3 and Table 2.4**. A sequence of around 300 to 700 nucleotides was targeted for amplification and therefore the primers were designed to amplify a template of such length. The manually designed primers were analysed using Integrated DNA Technologies online program to calculate their guanine-cytosine (GC) content, melting point, and their susceptibility to form hairpin structures. Their specificity was also examined by blasting them in NCBI online database search www.ncbi.nlm.nih.gov (nucleotide-nucleotide Blast). They were then sent to be synthesised by eurofins mwg/operon Genetic services.

Primers arrive in lyophilised form and get suspended by the provided buffer according to manufacturer's protocol (100pmol/ml). Out of the primers stock a 1/10 dilution is made to form 200ng/μl working solution of both forward, reverse primers and probe to carry on the PCR reaction.

Table 2. 4 Sequences of primers used in PCR

Human Gene	Sequences (5' → 3')	T _m °C	Prod. size bp	Gene bank accession NO.	Origin
APOE	Forward: CAGGTCACCCAGGAAGTCTGAG Reverse: CTTCTGCAGGTCATCGGCAT	59.96 60.04	317	>NM_000041.2	This work
MTP	Forward: AGTGTGTACCTGCAGACGTG Reverse: ACGTTGGAGGAAATGCGGTA	59.97 59.68	328	>NM_000253.2	This work
ApoB	Forward: TTGTACAACTGGTCCGCCTC Reverse: CAAGTTGGTCGTTGCACTGG	59.97 59.97	885	>NM_000384.2	This work
RPE65	Forward: ATGTGGGCCAGGACTCTTTG Reverse: TGGTCTCTGTGCAAGCGTAG	60.11 59.74	341	>NM_000329.2	This work
RLBP1 (CRALBP)	Forward: GACAAGTATGGCCGAGTGGT Reverse: ACGGCCTTGCCATCATACTT	59.75 59.74	449	>NM_000326.4	This work
Otx2	Forward: AGAGGACGACGTTCACTCGG Reverse: GCTGGGCTCCAGATAGACAC	61.58 59.89	343	>NM_001270523.1	This work
HNF4a	Forward: CATGGACATGGCCGACTACA Reverse: GTACTTGGCCCACTCAACGA	59.82 59.97	586	>NM_000457.4	This work
COUP-TF I	Forward: GGTAAGTAGCAGCTGGCGAGA Reverse: CCACAGGCGTCTGACGTGAA	61.89 62.69	993	>NM_005654.4	This work
COUP-TF II	Forward: AAGCTCCCTGAAACCTTGTCT Reverse: AGGTACGAGTGGCAGTTGAG	59.22 59.40	422	>NM_001145155.1	This work
VEGF	Forward: CATACCCCATGCCAGTTGT Reverse: ACTCTAGCTGCCTCGCTTTG	60.03 60.11	494	>NM_001114232.1	This work

2.5.6. Affymetrix Microarray

Whole-genome GeneChip® Human Transcriptome Array 2.0 was used. The RNA was isolated from ARPE-19 cells grown on laminin coated Millicell filters after being subjected to different treatments. The labelling and hybridization steps were performed at the Nottingham Arabidopsis Stock Centre (NASC). Results were collected from n=3 repeats.

2.5.7 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis® (IPA) software (www.ingenuity.com) was used for functional annotation of the differentially expressed gene lists produced by genespring GX 13. The generated data set from GX 13 was uploaded into IPA to compute functional analysis of probesets. Each identifier was mapped to its corresponding object in the Ingenuity Pathways Knowledge Base (IPKB). A cut-off of 1.5 fold was set to identify genes whose expression was significantly differentially regulated. These network eligible molecules were overlaid onto a global molecular network developed from information contained in the IPKB. Networks of network eligible molecules were then algorithmically generated based on their connectivity. IPA analysis was performed using standard settings to examine significance against biological functions and disease and canonical pathways (using Fischer Exact tests and a p value of <0.05). The interaction networks produced indicate literature-curated interactions with other molecules. Each network was given a score, calculated by IPA according to the number of Network Eligible Molecules in

the network, the network's size, the total number of network eligible molecules analysed and the total number of molecules in IPA's knowledge base that could be included in networks (www.ingenuity.com). The network score is based on the hypergeometric distribution and is calculated using a right-tailed Fisher's Exact Test, with the score being the negative log of the p-value. This score is used to rank the networks.

2.6. Protein Expression Experiments

2.6.1. Reagents

10% Ammonium persulfate (APS)

0.1g of Ammonium persulfate was dissolved in 1ml of distilled water immediately prior to use

4x Separation Buffer (pH 8.8)

1.5 M Tris.Cl

0.4%SDS

4x Stacking Buffer (pH 6.8)

0.5 M Tris.Cl

0.4%SDS

SDS-PAGE Separating Gel (12% for 2 gels)

30% Acrylamide/bisacrylamide: 8 ml

4x separation buffer: 5ml

Distilled water: 6.8ml

10% APS: 200 μ L

TEMED: 20 μ L

SDS-PAGE Stacking gel (10ml)

30% Acrylamide/bisacrylamide: 1 ml

4x stacking buffer: 2.5ml

Distilled water: 6.5ml

10% APS: 120 μ L

TEMED: 12 μ L

6x Protein sample loading buffer (50ml)

1 M Tris (pH 6.8) :4.5 ml (90mM)

6% (v/v) beta-mercaptoethanol

4% (w/v) SDS:2g

Bromophenol blue (0.01% w/v)

7M deionized urea (dissolved in water and deionized by incubation with

Amberlite Monobed resin for 30 min with gentle stirring)

SDS-PAGE running buffer (500ml 10x)

1.92 M Glycine: 72g

0.25 M Tris base: 15g

10% (w/v) SDS: 5g

Transfer buffer (500ml 10x)

1.92 M Glycine: 72g

0.25 M Tris base: 15g

Tris-Buffered Saline (10xTBS PH 7.4)

Tris Base: 15g

NaCl: 4.4g

KCL: 1.0g

pH adjusted to 7.4 and made up to 500 mL with distilled H₂O

TBS-T

10xTBS 1:10

0.1% (v/v) TweenTM20

Blocking buffer

3% fish gelatine in TBS-T

Stripping buffer (pH 2.0)

0.25 M glycine

2% SDS

Ponceau Red

0.1% (w/v) Ponceau S (3-hydroxy-4[2-sulpho-4-(4-sulphophenylazo)-phenylazo]-

2, 7-naphthalenedisulphoric acid) in 5% (v/v) acetic acid.

2.6.2. Protein extraction and quantification

Proteins were precipitated by adding isopropanol to the organic bottom layer following the RNA extraction with trizol method, and centrifuging at 5000rpm for 10 minutes at 40°C. After removal of supernatant, the pellet was washed with 0.3M guanidine HCl/ 95% ethanol and then stored in 2 ml 70% ethanol (-20°C). The pellets were dissolved in SDS (Sodium Dodecyl Sulfate)-Urea sample buffer. For complete dissolving of the proteins, 3x 10 second sonication was performed.

Proteins in RIPA buffer (Radio Immuno Precipitation Assay) [150mM NaCl 25mM Tris-HCl pH 7.6, Triton X-100, 1% Sodium deoxycholate 0.1% SDS, 1mM Na₃VO₄, 10mM NaF, and stock 25 × Complete Protease inhibitor (Roche)], were dissolved by sonicating 3 times for 10 seconds and rotating the homogenate for 45 minutes on a daisy wheel in cold room followed by centrifugation at 15000g for 10 minutes. The supernatant was then separated from the pellet and assayed for total protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Sample loading buffer was used before heating and loading of the sample in the polyacrylamide gels for electrophoresis.

2.6.3. Western blotting gel electrophoresis

Three volumes of cell lysates (50 µg of protein/ sample) were mixed with one volume of 4x protein sample buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 8% β -mercaptoethanol, bromophenol blue, and 40% v/v glycerol) and boiled for 5 minutes. The mixture was spun briefly to bring down condensate and then loaded onto to 10% or 12%, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. 5 µl of protein markers (SeeBlue[®] Plus2 Prestained Standard (1x); Invitrogen) were also loaded onto each gel. The gel was run at 150 V for 1 hour until the bromophenol blue dye reached the end of the gel. The gel was then transferred on to nitrocellulose paper soaked in transfer buffer; a western blotting sandwich was prepared in a plastic cassette with secure clamps, the gel and membrane were sandwiched between 2 pieces of soaked Whatman No. 3 filter paper and then between two sponges. The nitrocellulose-gel filter paper sandwich then was placed with an ice-pack in a Mini Trans-Blot Cell Tank (BioRad) containing Transfer Buffer and run at 105v for 1 hour. Ponceau Red stain solution, 0.1 % (w/v) in 5% acetic acid (Sigma) was used to stain the membrane in order to verify the successful of transfer. The stain was washed with distilled water and the membrane then blocked in 5% (w/v) Marvel skimmed milk solution (Cadbury, London, UK) in 1X TBST for 1 hour at RT.

2.6.4. Immunodetection following western blotting

Blots were incubated with the primary antibody see Table 2.1 in TBST, 5% skimmed milk overnight at 4°C with gentle shaking. Blots were washed three times in TBST buffer for 10 minutes and then incubated with IR-Dye[®]

conjugated secondary antibody (*LI-COR*[®] Biosciences, 1:10000 dilution in 3% (w/v) skimmed milk) as appropriate. The membrane was then washed three times in TBST buffer for 5 minutes. Analysis of blots was performed using a *LI-COR*[®] ODYSSEY infrared imaging system (*LI-COR* Biosciences, Lincoln, NE/USA). Results were collected from a minimum of n=3 repeats.

2.7. ELISA

ELISA was performed collected supernatant using VEGF ELISA kits (Human VEGF ELISA, Invitrogen/KHG0111), according to the manufacturer's instructions. Cell cultures were grown as described before, supernatant was collected at several time points (7, 30 and 90 days). All samples were kept at – 80°C till analysis. Results were collected from a minimum of n=3 repeats.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Results were collected from an average of n=3 repeats. Values were expressed as mean $\pm$ S.E.M. Statistical comparisons of different groups were made by one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-hoc test Unpaired two tailed t-test was also used to compare between two variable. A P value <0.05 was taken as the level of significance.

3

USE OF ARPE-19 CELL LINE AS AN IN-VITRO MODEL FOR AGE RELATED MACULAR DEGENERATION

3.1. Introduction

Age related macular degeneration (ARMD) is a retinal degenerative disease that leads to loss of central vision; it affects 5–10% of the population over the age of 60 (Iranmanesh et al., 2007, Slakter and Stur, 2005). The onset of ARMD is a complex process and its pathophysiology is poorly understood. The disease affects the retinal pigment epithelium (RPE) monolayer. Damage to the RPE cells, especially in the macular region, has devastating effects on vision. Although the pathophysiology of ARMD is far from simple, lipid rich

lesions that involve RPE and Bruch's membrane (BrM) are the most prominent clinical and histopathological features of ARMD. These lesions have several names regarding to their form and site of occurrence (see **chapter 1 section 1.3.1.**), yet they all occur between the RPE and the BrM or within the BrM. The accumulation of lipid rich lesions is considered one of the main early characteristics of the dry form of ARMD (early ARMD). These deposits are widely accepted as contributors to the aetiology of ARMD. Their composition and the mechanism of their formation are not understood but histochemical and immunocytochemical studies have shown that they contain a variety of lipids, polysaccharides, and both early and late stage complement proteins C3, C5, C5b-9(Hageman et al., 2001).

Lipid deposit formation in the RPE (Imanishi et al. 2004) and "lipidization of the RPE" is a recognised feature of aging in the RPE. The histological features are individual RPE cells with loss of pigment and intracellular accumulation of lipid (el Baba et al. 1986;Green and Harlan, Jr. 1999;Stempien-Otero et al. 1999). The macaque monkey also gets an ARMD-like syndrome with drusen formation and RPE degeneration. Here too, lipid loading of RPE cells overlying lipid rich lesions has also been reported (Anderson et al. 2006;Feeney-Burns et al. 1981)and lipoidal RPE degeneration (Fine 1981;Fine and Kwapien 1978). Pauleikhoff et al also highlighted that there is a progressive accumulation of lipid in Bruch's membrane with relation to age(Pauleikhoff et al., 1990). They suggested that this accumulation of lipids is an obstruction to the passage of fluid and materials through the RPE monolayer. If the BrM becomes more hydrophobic, because of this lipid

accumulation, fluid passage could be impaired. Consequently, any change in Bruch's membrane may affect the efficient transport of the nutrients from choriocapillaris to the retina. Furthermore the fluid collected between the RPE and the BrM may cause the RPE to detach (Bressler et al., 1988, Fine and Kwapien, 1978). This evidence gives rise to a lipo toxic model of ARMD caused by progressive lipid accumulation.

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor which is known to be a major stimulator for ocular neovascularisation (Dahrouj et al., 2014). In AMRD an increase in VEGF is positively correlated with the progression of the disease from dry form to neovascular (wet) form, as it was shown in **Chapter 1** (Leung et al., 1989). In the work carried by Rudolf et al in 2005, VEGF expression was detected in the outer retinal layers of LDL receptor deficient mice and VEGF expression appeared to correlate with the amount of lipid particles present in BrM (Rudolf et al., 2004, Rudolf et al., 2005), further supporting a role for lipo-toxicity in ARMD pathology.

During the past decade, cross-sectional, case-control, and cohort studies have demonstrated an increase in body weight (especially abdominal fat) as a factor associated with the formation of drusen and other sub-RPE deposits (Abdelsalam et al., 1999, Del Priore et al., 2002). For example, a cohort study in a hospital-based retinal practice found higher Body Mass Index increased the risk for progression to the advanced forms of ARMD (Seddon et al., 2003). Abdominal obesity was also found to increase the risk of ARMD pathology, as a higher waist circumference, as well as waist to hip ratio(WHR), were

associated with a two-fold increase in ARMD progression in both lesion size and progression to neovascularisation (Evans, 2001, Dali-Youcef et al., 2012).

The toxic consequences of lipid overload will depend on the magnitude and duration of the imbalance between lipid efflux and lipid influx in a given tissue, therefore appropriate homeostasis is maintained by protective mechanisms triggered by the tissue itself or cells present in the surrounding environment e.g. macrophages. In addition to the pro- and anti-inflammatory effect of macrophages, they also express the cholesterol transporter ABCA1. Sene et al demonstrated that depletion of ABCA1 specifically in macrophages results in increased cholesterol retention in macrophages which significantly increased neovascularization in an animal model for neovascular ARMD (Sene et al., 2013)

There is evidence that the RPE can produce lipoprotein particles suggesting the presence of self-protective mechanisms to avoid lipo-toxicity (Li, Presley, Zhang, Dashti, Chung, Medeiros, Guidry, & Curcio 2005b; Malek, Li, Guidry, Medeiros, & Curcio 2003). These observations lead to a lipo-toxic model for ARMD development and form the basis of the development of an in vitro model of the disease.



3.2. The aim of this chapter

This chapter describes the development of an *in-vitro* model for ARMD based on RPE lipid loading. Neutral lipids are ubiquitously found in nature and they serve as energy storage compounds. The idea of this model is that, excess intracellular lipid is potentially toxic and that the protective cellular response is to export those excess lipids as lipoprotein particles. It is hypothesised that these particles can precipitate and form extracellular sub-RPE deposits, or drusen. In addition, the introduction of chronic lipid loading will lead to lipotoxicity, which will lead to an increase in vascular endothelial growth factor (VEGF) expression, inflammation and cell death.



3.3. Study design

In this study we used two different designs; the first was conducted to study the effect of growing ARPE-19 cells on millicell inserts long term in serum free DMEM/Ham F12 media. The second design utilized this culture model as a base to create a model of lipid toxicity that can capture aspects of ARMD pathology. ARPE-19 cell cultures were grown and cultured as described in **Chapter 2 (section 2.2.8)**. After 30 days of culture in serum free media ARPE-19 cell cultures were treated with serum free medium supplemented with 0.2mM PLO (see **Chapter 2, section 2.3.3**). Cultures were incubated with the PLO mixture for another 60 days (90 days in total) to ensure lipid loading of cultures.

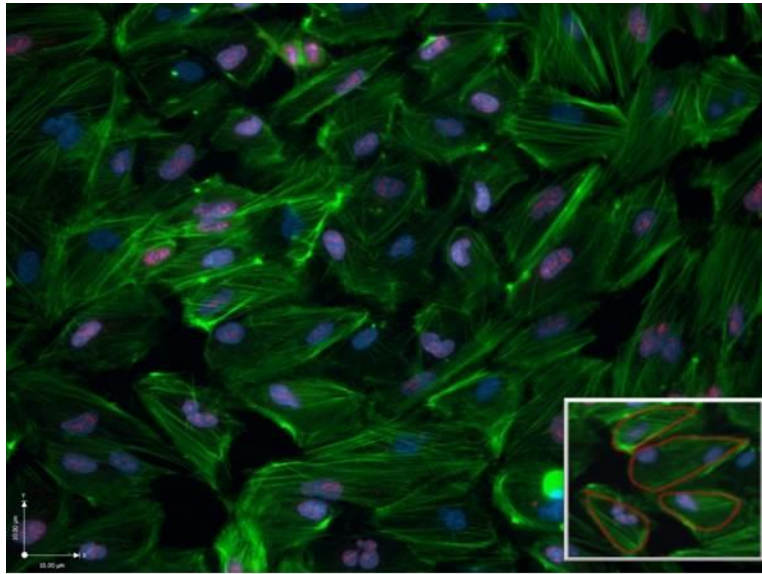
3.4. Results

3.4.1. Characterization of an aged ARPE-19 cell culture model

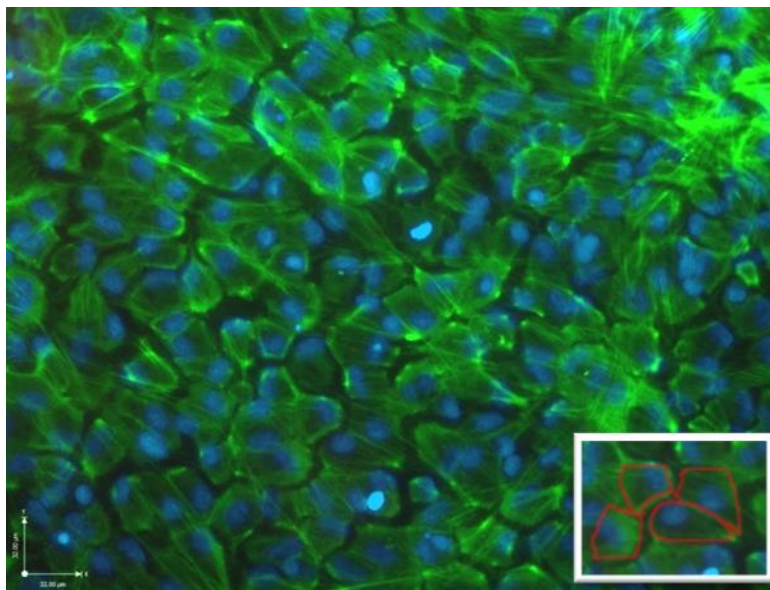
3.4.1.1. Morphology

ARPE19 cells were grown on laminin coated inserts for 90 days under serum free conditions and the cell morphology was monitored. The actin cytoskeletal structure of the ARPE19 monolayers was visualised with phalloidin staining. As shown in **Figure 3.1**, RPE cell cultures that are left confluent for twenty-four hours presented with tear-shape morphology; all cells expressed Ki67 in their nuclei indicating proliferative activity **Figure 3.1A/** red. Initially, significant spaces were noted between the cells (yellow arrow in **Figure 3.1B**). When the cells were switched to serum free DMEM/Ham's F12, the cell morphology changed to cobblestone type and the gaps disappeared **Figure 3.1C**. No proliferation was seen after 30 days in culture cell **Figure 3.1B /** red and this was maintained until the endpoint of culture incubation period (90 days) **Figure.1C/red**.

A)



B)



C)

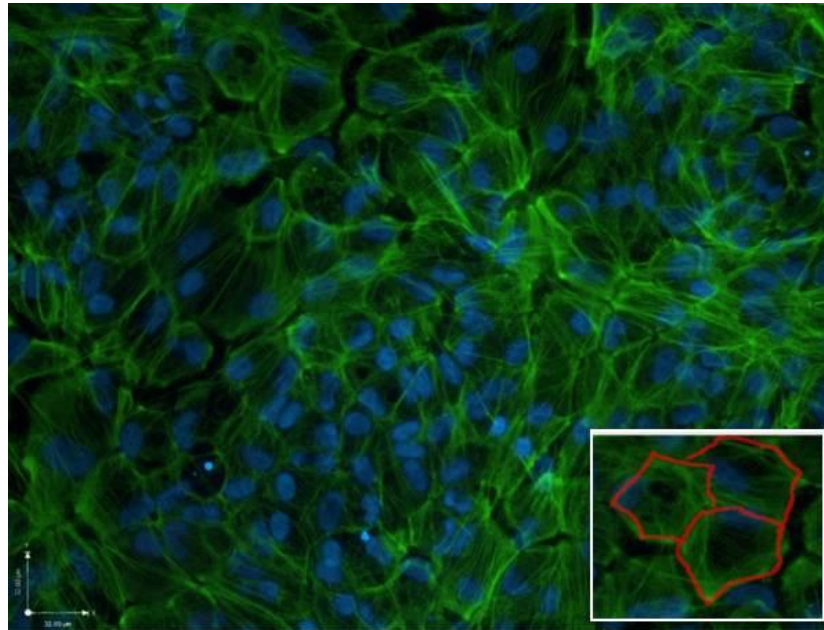


Figure 3. 1 RPE cell morphology in long term serum free culture. ARPE-19 cells were grown on laminin coated Millicell-PCF inserts. (A) ARPE-19 cells after 24h in culture, (B) ARPE-19 cells grown in serum free media for 30 days , (C) ARPE-19 cells grown in serum free media for 90 days. The morphology was visualised with phalloidin (green) and the proliferation level was monitored with the expression of KI67 protein (red) ARPE-19 cells changed from proliferating tear-shape cells to a non-proliferating cobblestone monolayer after 30 days in serum free culture which was maintained till day 90 (A-C). Scale bar 10 μ M in A, 32 μ M in B and C. n=3.

3.4.1.2. Measurement of RPE-gene expression markers

The mRNA was isolated from ARPE-19 cultures and the expression of known specific markers for differentiated RPE cells; RPE65, OTX2 and CRALBP were all detected by RT-PCR **Figure 3.2.**

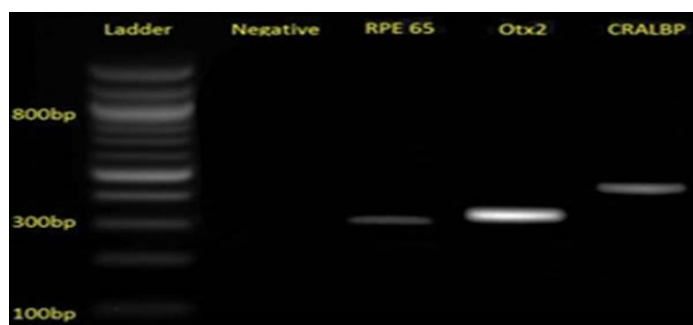


Figure 3. 2 The expression of RPE-specific Genes determined by RT-PCR showing expression of RPE markers RPE65, OTx2 and CRALBP. Negative lanes were loaded with samples containing water in place of cDNA in the PCR reaction.

3.4.1.3. Tight junction protein expression

The expression of two tight junction (TJ) proteins (Zo-1 and Occludin) was investigated by Immunocytochemistry (ICC) and western blot (WB). 90 days serum free cultures showed positive expression of both Zo-1 **Figure 3.3AII**/red and Occludin **Figure 3.3III**/Green. TJ protein expression was validated with WB analysis **Figure 3.4B**. Protein expression of ZO-1 showed a single band (~250 kDa) **Figure 3.4BI** when detected with a mouse monoclonal antibody, while a Rabbit polyclonal antibody raised against a C-terminal peptide of occludin showed the expected multiple bands that represent potential post-translational states (Ghassemifar et al., 2002), which are a weak band at ~53 kDa, some 7 kDa below the predominant band at 65 kDa **Figure 3.3BII**. HepG2 cell lysates were used as a positive control for both ZO-1 and Occludin expression in western blot analysis (Schmitt et al., 2004).

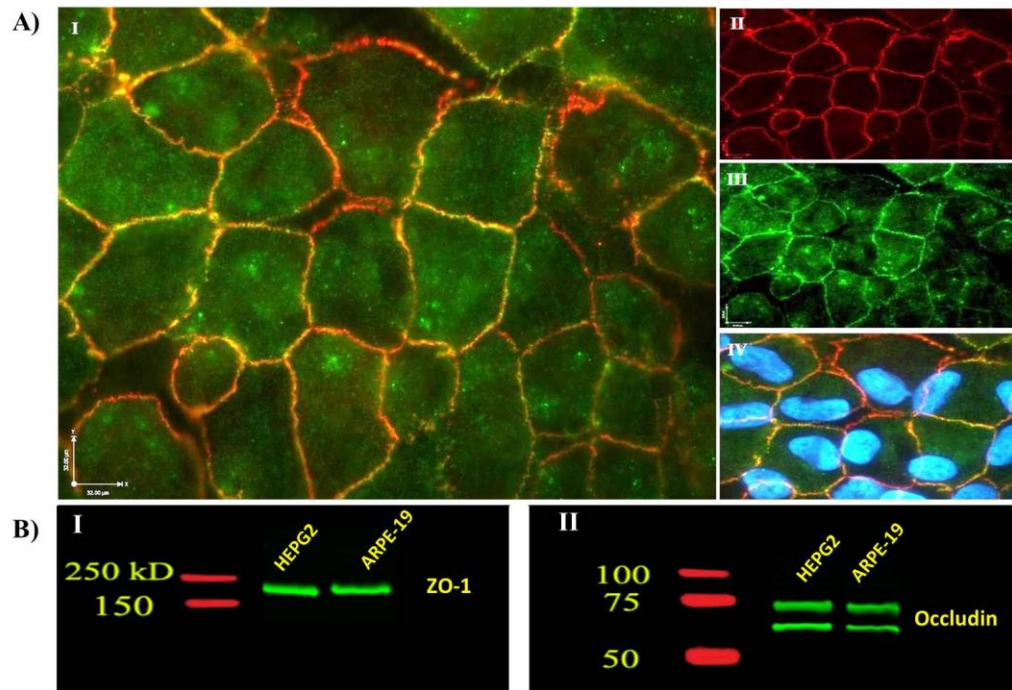


Figure 3. 3 Immunocytochemical characterization of the ARPE-19 monolayers maintained in serum free media for 90 days. Fluorescence microscopic images showing the expression of ZO-1 (red)/Occludin (green) the nucleus was visualised with DAPI (blue) (A). Scale bar 32μm. (B) western blot analysis showing the expression of both TJ markers ZO-1 (BI) and Occludin (BII), HEPG2 cell lysates were used as a positive control (Schmitt et al., 2004).

3.4.1.4. Cell culture Trans-epithelial Electrical Resistance TEER measurements

Trans-epithelial electrical resistance (TEER) measurements were recorded throughout the 90 days of culture **Figure 3.4**. The TEER readings increased rapidly during the first week of culture and plateaued by four weeks, with a mean level of $77 \pm 2.3 \Omega \times \text{cm}^2$.

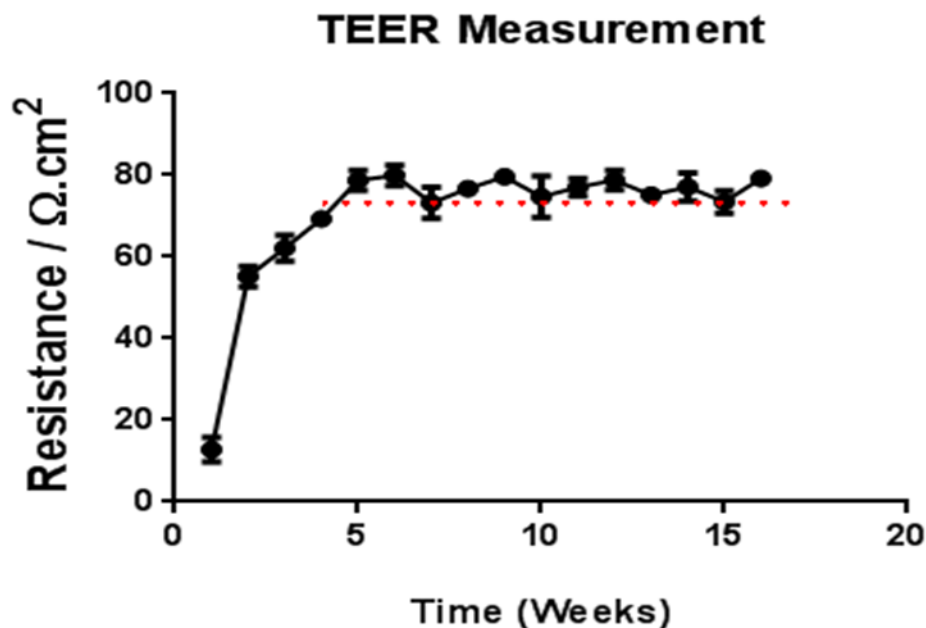


Figure 3. 4 TEER measurement of the ARPE-19 monolayer over time. TEER measurements were recorded once a week throughout the 90 days using the protocol mentioned in Chapter 2, section 2.3.5 . ARPE-19 cell culture permeability reached a maximum reading within 3-4 weeks main of $77 \pm 2.3\Omega \times \text{cm}^2$. Each data point is presented as an average reading of 3 wells. Readings were taken from the same wells throughout the experiment.

3.4.1.5. VEGF-A expression in media

Asymmetric secretion of vascular endothelial growth factor (VEGF) by retinal pigment epithelial (RPE) cells in-vivo is critical in maintaining the health of the retina and choroid. Elevation of VEGF expression is involved in the development and progression of the dry form of ARMD to the wet neovascular form of ARMD(Kannan et al., 2006), hence we investigated VEGF expression in our model of aged RPE monolayers. RT-PCR showed that ARPE-19 cell express the VEGF-A gene (product length 494bp) **Figure 3.5A**. The levels of VEGF-A in the media were measured using ELISA. Cell culture supernatants were collected at four time points (7, 30, 60 and 90 days) from

both the apical and basal compartments of each well (n = 3) **Figure 3.5B**. No significant difference was seen in VEGF-A secretion between the apical and the basal surface, yet a positive correlation between VEGF-A secretion was seen over time ($r^2 = 0.9990$, $P < 0.0010$). Data are represented as mean $\pm$ SEM. Statistical analysis was done using unpaired student t-test and Pearson r for the correlation.

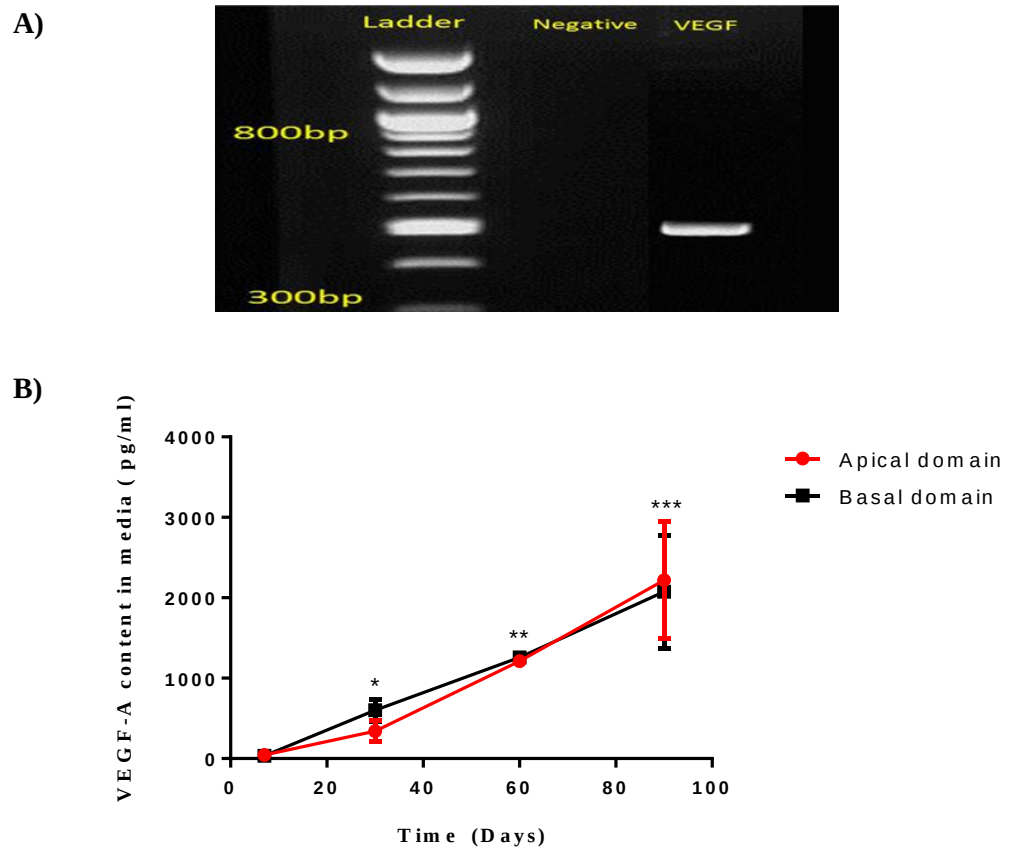
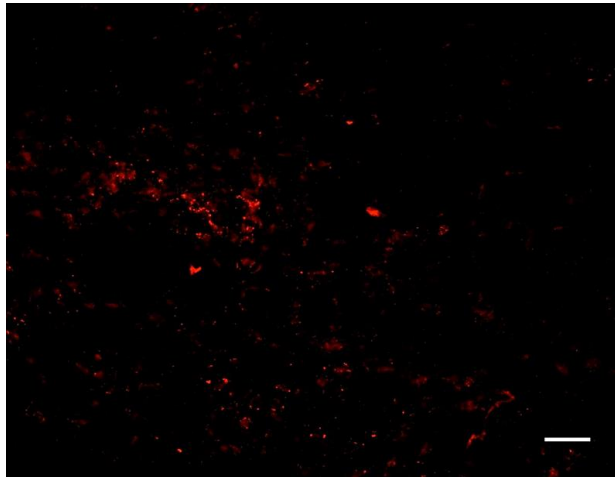


Figure 3. 5 VEGF-A expression at the mRNA level and in terms of secreted protein. (A) RT-PCR showing gene expression of VEGF-A gene in ARPE-19 cells grown for 90 days in serum free media. (Negative) lanes were loaded with samples containing water in place of cDNA. (B) The VEGF-A concentration in the media from the ARPE-19 cells on the different time points was measured with an ELISA. Data are represented as mean $\pm$ S.E.M. Statistical analysis between apical and basal domain was done using unpaired student t-test. Time (Days) Vs expression of VEGF-A correlation analysis was conducted via Pearson $r^2 = 0.9990$, $P < 0.0010$ (Ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). N=3

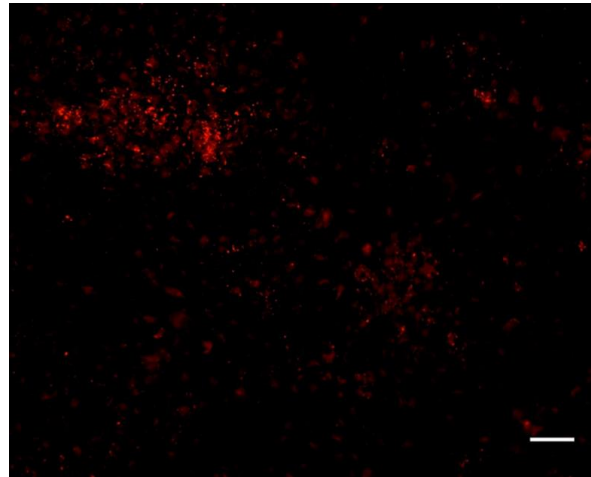
3.4.1.6. Intracellular lipid accumulation in long term cultures

A key morphological hallmark of retinal aging, is the accumulation of intracellular natural lipids in the RPE cells (Curcio et al., 2009). Growing ARPE-19 cells long term in serum free media showed evidence of an increase in lipid accumulation in a time dependent manner **Figure 3.6A** and **B**. ARPE-19 were stained with Nile red, a lipophilic stain used to localize and quantitate lipids, particularly neutral lipid droplets within cells. The corrected total cell fluorescence (CTCF) of the Nile red stain was calculated using the method of (Burgess et al., 2010, McCloy et al., 2014, Gavet and Pines, 2010, Potapova et al., 2011). The formula = Integrated Density - (Area of selected cell X Mean fluorescence of background readings) was used to calculate the CTCF through the use of Image J software **Figure 3.6B**.

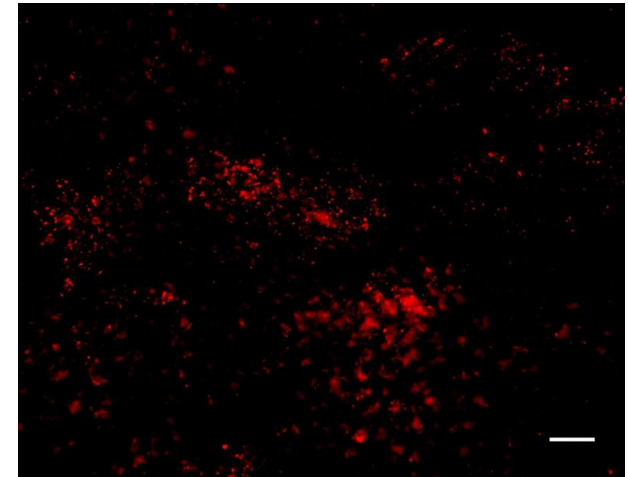
A) 7 days



B) 30 days



C) 90 days



D)

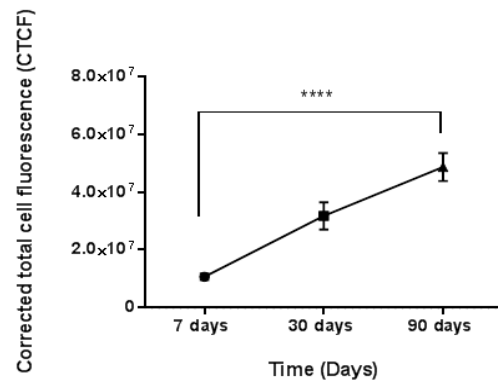


Figure 3. 6 Intracellular lipid accumulation in ARPE-19 cell cultures. Long term growth of ARPE-19 cell in serum free media showed evidence of an increase in natural lipid accumulation when stained with Nile-Red in a time dependent manner (A-C). Scale bar 10µm. Higher number of Nile Red-positive lipid particles were seen in ARPE-19 cultures at day 90 (c) when compared with early stage day 7 (A). (D) Using the ICC images we calculated the CTCF through the use of Image J software (10 pictures per sample). Data are represented as mean ± SEM. Statistical analysis was done using one way ANOVA. (ns: no significance, * P < 0.05, ** P<0.01, *** P< 0.001 and **** P < 0.0001). N=5.

3.4.1.7. *The formation of Sub-RPE deposits*

Another Hallmark of natural aging is the accumulation of Sub-RPE deposits between the BrM's membrane and the RPE monolayer, we investigated the presence of sub-RPE deposit using proteins reported in sub-RPE deposits isolated from healthy aged individual (APOE and Vitronectin) as well as Amyloid Beta ($A\beta$ 1-42) a protein only found in drusen taken from ARMD patients (Ding et al., 2011, Wang et al., 2009, Löffler et al., 1995). ARPE-19 cells cultured on membranes were sectioned into 17.5 μ m samples. The sections underwent immuno-histochemical staining for the presence to Apolipoprotein E (ApoE). A double staining of APOE and Transmembrane glycoprotein NMB (GPNMP), (an abundant RPE surface protein (Alcazar et al., 2009)) showed globular extracellular deposits present on the support material underneath the ARPE-19 cells **Figure 3.7 A**. To assess the distribution pattern of these sub-deposits flat mounted images were obtained using a DMIRB fluorescence inverted microscope. The de-convoluted z-stack images were tilted on the Z-axis to form a flat mounted image using velocity imaging software. Immunofluorescence results showed APOE deposits to be typically spheroid; sometimes tubular in shape with an uneven distribution, with areas of high aggregation **Figure 3.7A III**/blue arrows and others with no deposits **Figure 3.7AIII**/blue asterisk. No deposits were seen in cells cultured for 7 days **Figure 3.7 B**. This irregular distribution of sub-RPE material resembles the pattern of deposits observed in aged individual.

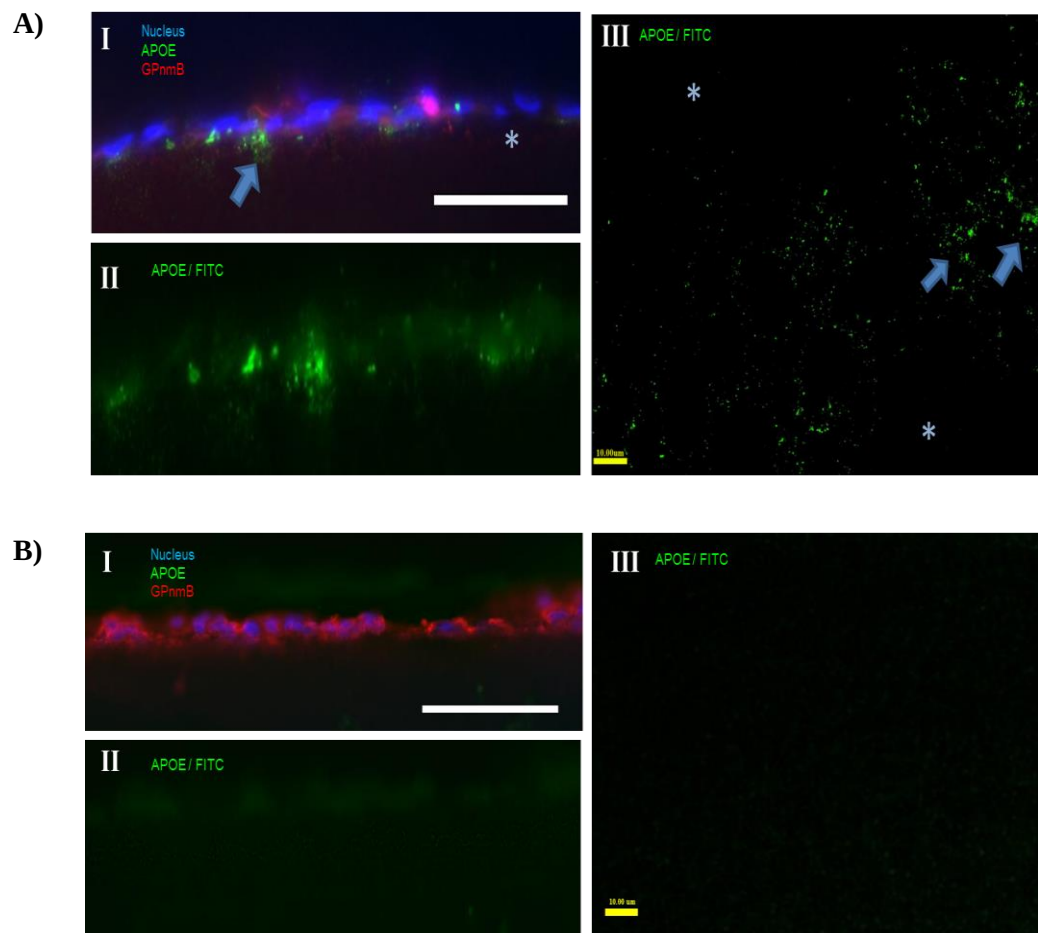


Figure 3.7 Sub-RPE deposits accumulated in aged ARPE-19 cell culture. (A I and II) side sections showing the accumulation of sporadic deposits underneath the RPE monolayer that are immunoreactive for APOE antibody (green). GPNMP is a transmembrane glycoprotein used to identify the borders of RPE cells (red). DAPI was used to show the nucleus. (AIII) De-convoluted z-stack images were tilted on the Z-axis to form a flat mounted image with Volocity software; a random scatter for these deposits with areas of high density (arrows) and other with low to none (asterisk) are seen. Scale bar, 50µm in A I -II, 35 µm in B I -II and 10 µm in AIII - BIII.

Cultures were also examined for two other proteins; vitronectin and Amyloid β , found in drusen **Figure 3.8**. A low level of vitronectin was seen **Figure 3.8 A**/blue arrow, but no A β 1-42 was observed **Figure 3.8 B**.

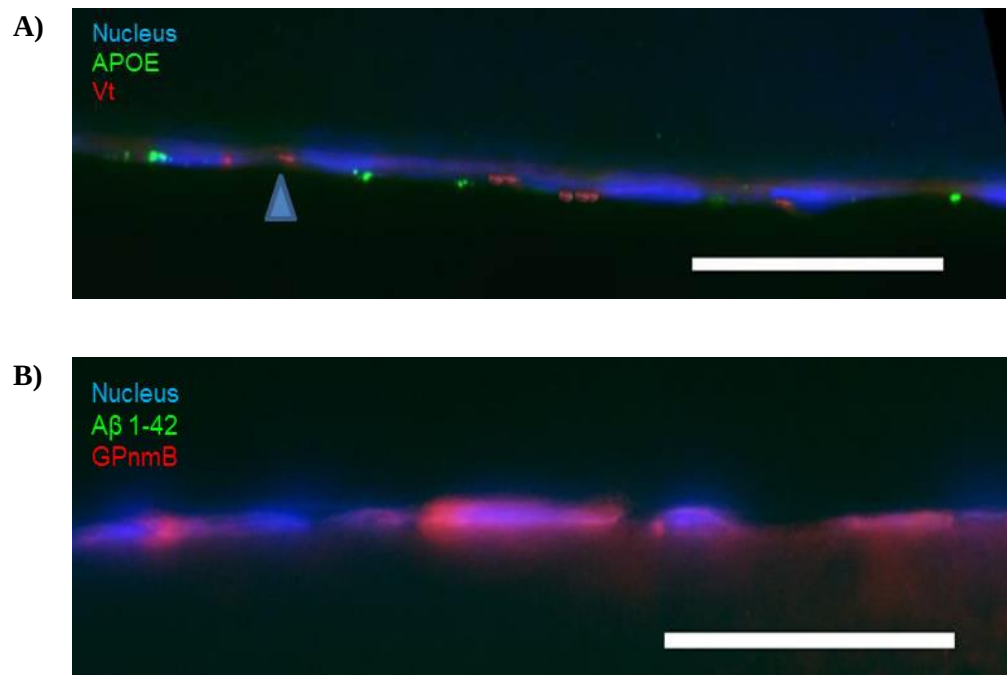


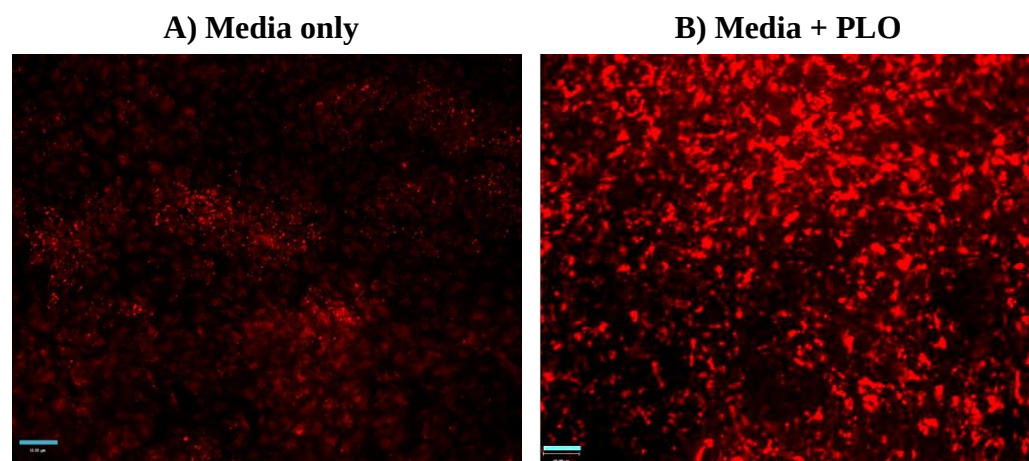
Figure 3. 8 Immunofluorescence detection of drusen-related protein expression. The immunoreactivity of two drusen related proteins were tested. ARPE-19 cell cultures showed low expression for Vitronectin (A). No immunoreactivity was seen for Aβ 1-42 antibody (B). DAPI was used to show the nucleus. (Scale bars, 50μm).

Together these results conclude that by 30 days we had achieved a stable monolayer of RPE cells with TJ formation and reasonable trans-monolayer resistance suitable for experimentation. The 30 days serum free ARPE19 cell cultures can be considered a model of normal aged RPE monolayer. This model of aged RPE captured some of the features of normal aging which include low levels of VEGF expression, low levels of drusen formation and no evidence for amyloid deposition.

3.4.2. in-vitro model for RPE lipid toxicity

3.4.2.1. *The effect of chronic PLO treatment on lipid accumulation in ARPE 19 Cells*

The effect of chronic 0.2mM PLO treatment was investigated by fluorescence analysis of Nile red staining using an appropriate filter as mentioned in **Chapter 2 Figure 3.9**. Lipid treated cultures showed an increase in their intracellular lipid accumulation compared to lipid free cultures **Figure 3.9A** and **3.9B** respectively. The total CTCF of lipid droplets was calculated using the method used by (Burgess et al., 2010, McCloy et al., 2014) **Figure 3.9C**.



C)

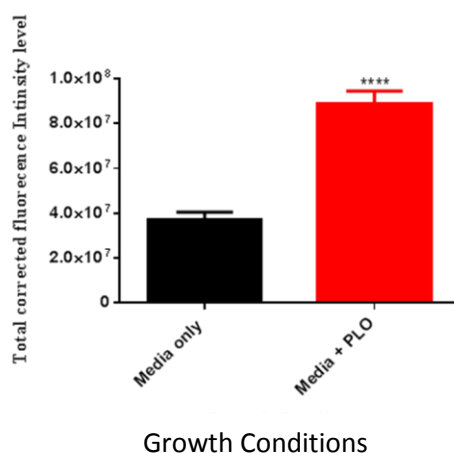


Figure 3. 9 The effect of chronic 0.2mM PLO treatment on lipid accumulation. 0.2mM PLO intervention increased the intracellular level of lipids in ARPE-19 cell culture.(A) 0.2mM treated ARPE-19 cultures. (B) Serum free ARPE-19 cultures. Nile Red-positive lipid droplets were visualized with lecia inverted DMIRB microscope. Scale bar 10µm. (C) Total CTCF analysis, the total CTCF was calculated using the ICC images through the use of Image J software (10 pictures per sample). Statistical analysis was done using unpaired t test. P<0.05 . (ns: no significance, * P < 0.05, *** P < 0.0001). N=4.

3.4.2.2. The effect of chronic lipid loading on monolayer integrity.

The outer Blood Retinal Barrier (oBRB) integrity is maintained by a series of tight junction (TJ) proteins, responsible for regulating RPE cell-to-cell contact and limiting the intercellular space (see **Chapter 1 section 1.3.2**). Destruction of TJ proteins in the RPE monolayer was shown to contribute to the pathophysiology of ARMD (Rizzolo et al., 2011, Erickson et al., 2007).

We investigated the effect of chronic lipid loading on the ARPE-19 cell culture barrier function by recording weekly (TEER) measurements throughout the culture period (90 days). In contrast to lipid free cultures, which maintained a consistent TEER rating beyond 60 days, the lipid-supplemented culture showed a gradual decrease in TEER after 60 days, reaching a significant low value of $54.0 \pm 4.2 \Omega \times \text{cm}^2$ ($P < 0.0062$) **Figure 3.10**.

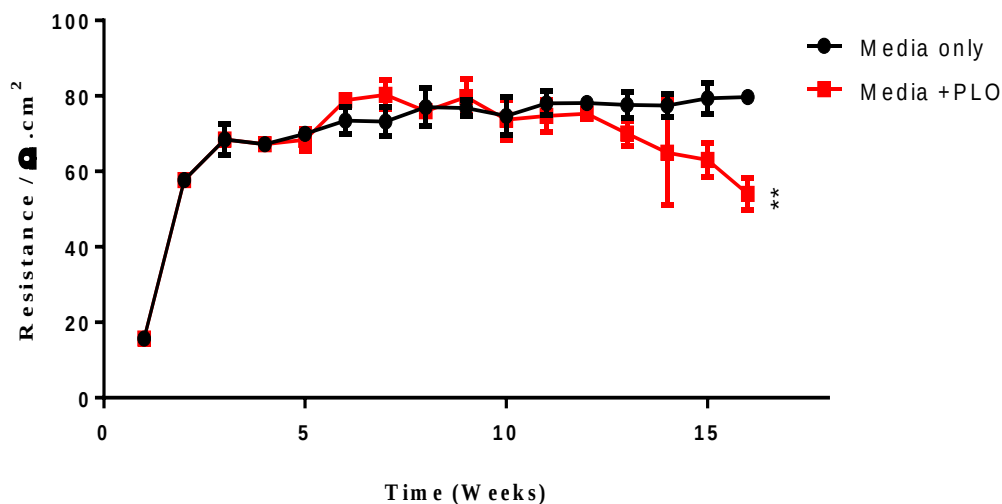
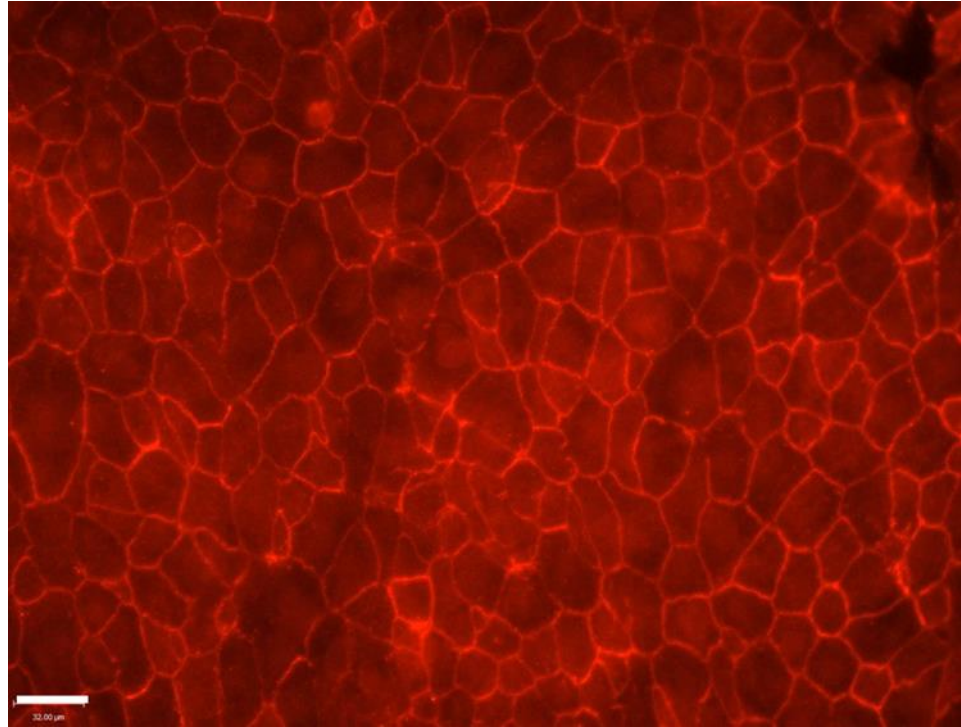


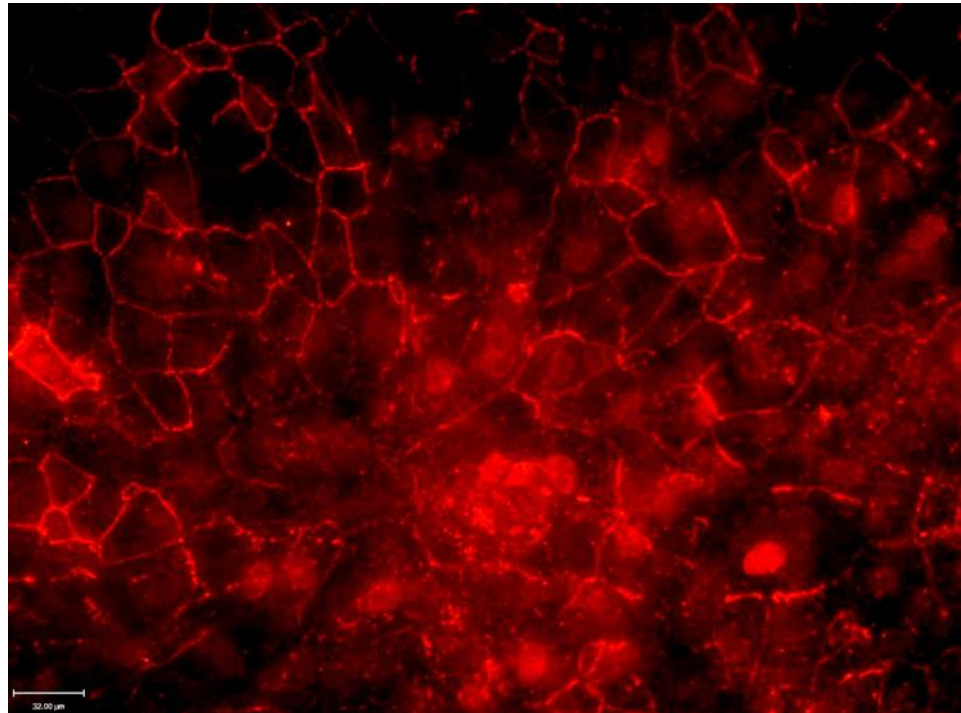
Figure 3. 10 Effect of chronic lipid loading on TEER value. TEER measurements were recorded once a week throughout the 90 days for both the lipid loaded cultures (Red) and a control culture using serum free media only (Black). By week 13 a sharp fall in TEER is seen reaching a low $54.0 \pm 4.2 \Omega \times \text{cm}^2$. Reading were analysed with prism using unpaired t test. with $P < 0.0062$. Data presented as mean $\pm$ SEM (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$), $N=4$.

TJ structure in RPE cells was observed after 60 days of 0.2mM PLO treatment. Through the use of immune-fluorescence analysis for the ZO-1, cultures grown in serum free media only and those that were exposed for PLO treatment for the same period were compared **Figure 3.11A** and **B** respectively. An average of 10 images were taken for each sample in each condition ($n=4$) and the percentage of continuous TJ presentation was calculated using the protocol provided (Ebrahim and Leach, 2014) (See **chapter 2 section 2.3.3.**). 60 days lipid loading intervention caused an apparent decrease in the percentage of continuous TJ compared to cultures grown in serum free media only **Figure.11C.**

A)



B)



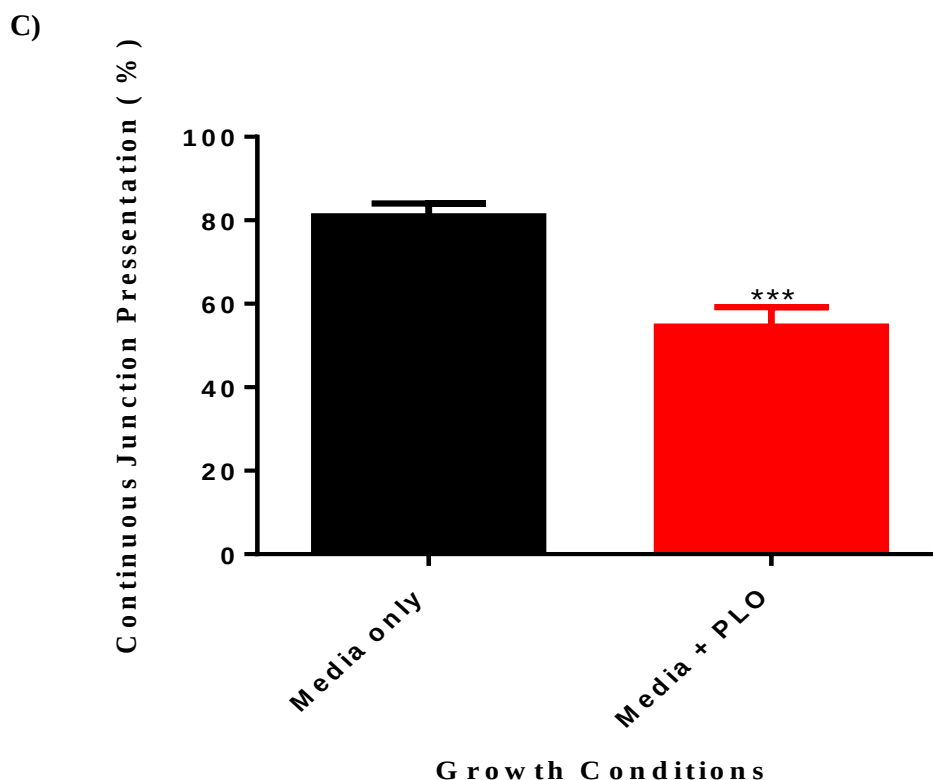


Figure 3. 11 Effect of chronic lipid loading on the TJ integrity. (A) 0.2mM treated ARPE-19 cultures. (B) Serum free ARPE-19 cultures, scale bar 32µm. (C) Through the use of fluorescent microscopy an average of 10 images were taken for each sample of each condition. The percentage of continuous TJ presentation was calculated using the protocol provided by (Ebrahim and Leach, 2014). Data presented in $\pm$ S.E.M, n=4. Statistical analysis was done using unpaired t test $P < 0.0002$. (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$).

3.4.2.3. VEGF-A secretion into Media

Total VEGF-A release into media was quantified through the use of ELISA. This was done using the protocol mentioned in Section 3.4.1.6. Cell culture supernatants were collected at four time points (7, 30, 60 and 90 days) from both the apical and basal domains of the same insert (n = 3). From previous results (see **Section 3.4.1.6**), it was

concluded that there is no significant change between apical and basal expression of VEGF-A in media, therefore media collected from both apical and basal domain were mixed to capture total VEGF-A levels in media. **Figure 3.12** represents a comparison of VRGF levels after 60 days intervention of either 0.2mM PLO or lipid free media. A significant increase in VEGF-A secretion level was recorded after lipid loading ($P < 0.0001$) compared to lipid free media cultures.

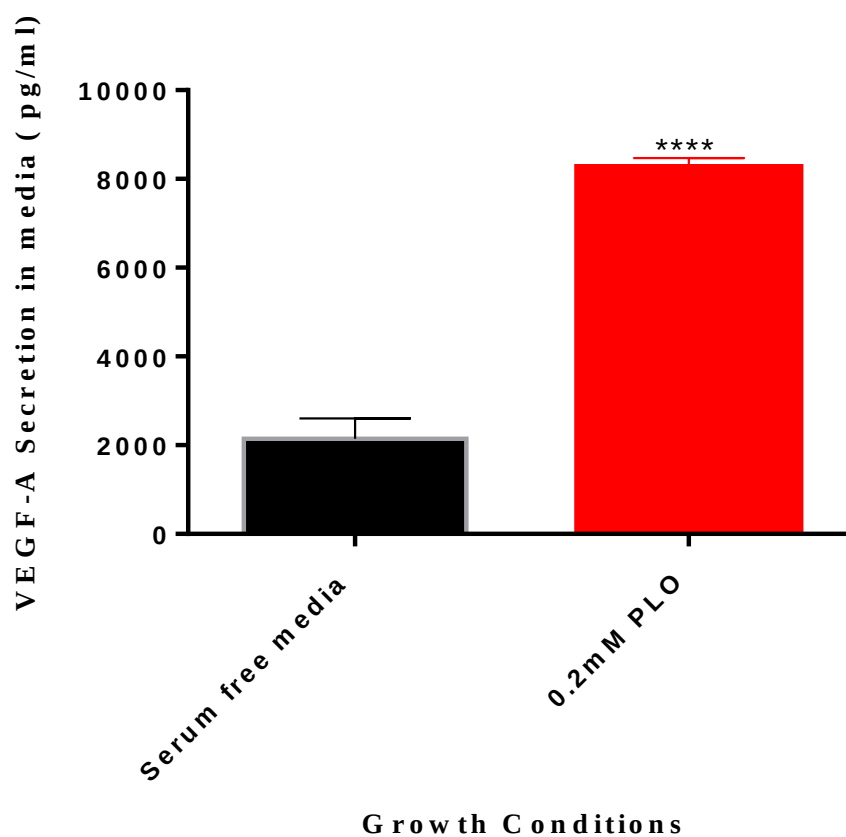


Figure 3. 12 VEGF-A secretion in media after 60 days intervention of either serum free or lipid loaded cultures. The VEGF-A concentration in the media from the ARPE-19 cells cultures was measured with an ELISA. A significant increase in VEGF-A expression was seen after lipid loading intervention ($P < 0.0001$). Data are represented as mean $\pm$ SEM. Statistical analysis was done using unpaired t test $P < 0.0001$. (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$). $N=3$.

3.4.2.4 Sub-RPE deposit formation

The immuno-reactivity of two major constituents found in drusen was investigated (APOE and A β 1-42). Side section analysis showed that lipid loading of ARPE-19 cell culture led to a greater accumulation of sub-RPE deposits. These deposits were positive for both APOE and A β 1-42 **Figure 3.13 AII and BII/Green**. To have a better visualization of the pattern of deposits across the culture membrane, a flat mount images were created **Figure 3.14A**. The total CTCF was quantified through the use of Image J software. Sub-RPE deposits positive for APOE increased significantly after 60 days of 0.2mM PLO intervention **Figure 3.14BI**. Interestingly A β 1-42 deposits were only visible after PLO treatment **Figure 3.14BII**

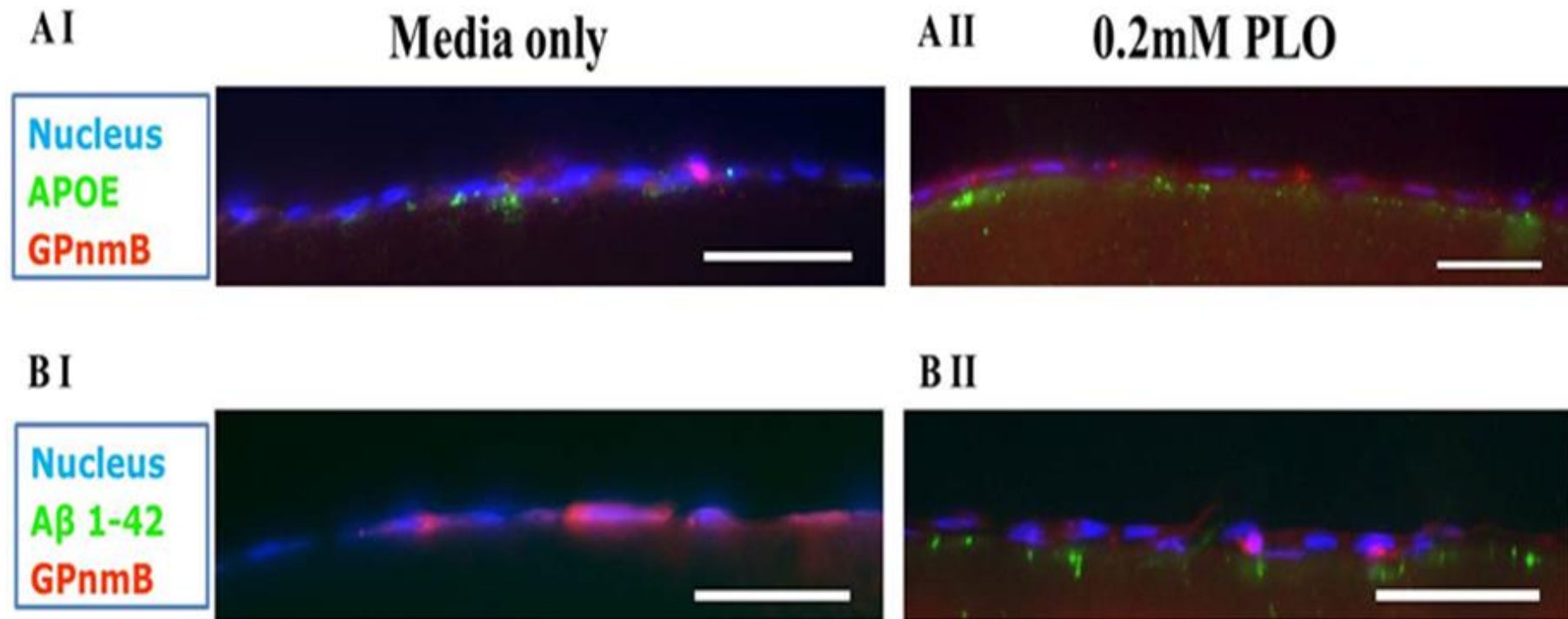
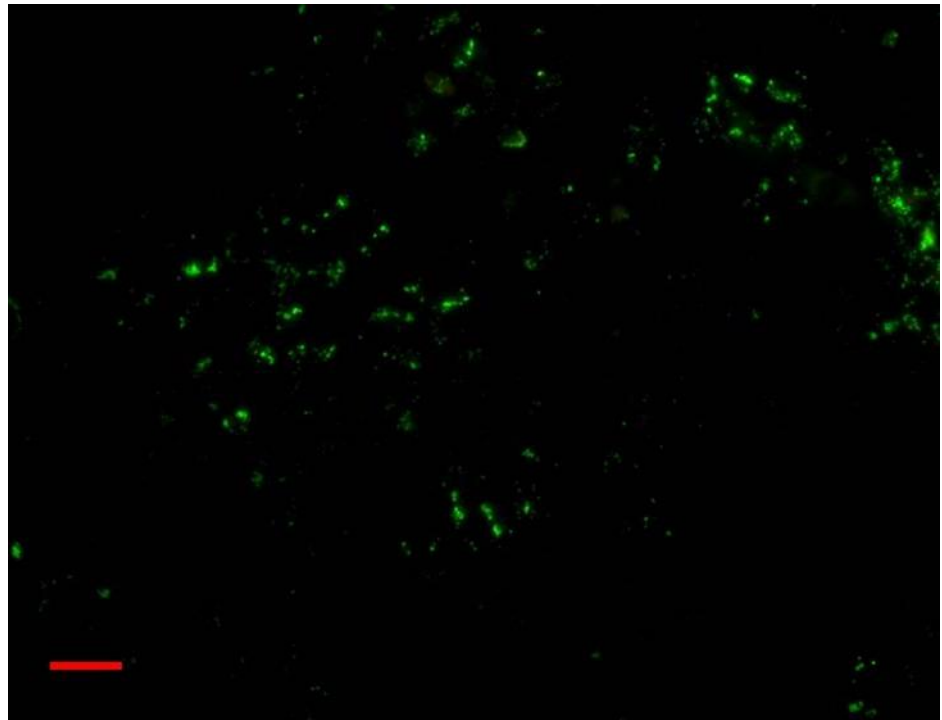
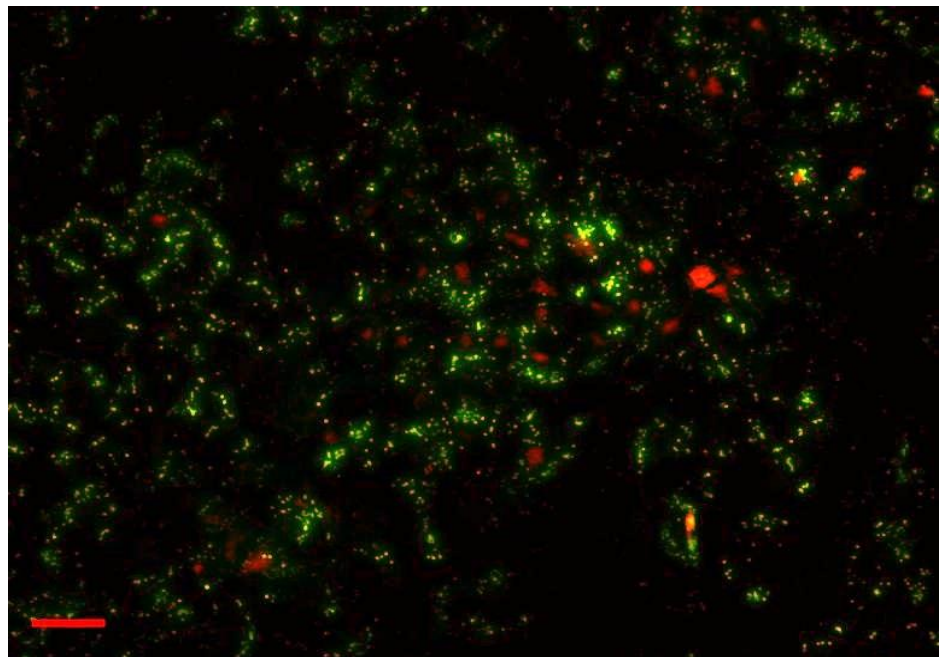


Figure 3. 13 Sub-RPE deposit formation. Samples were embedded in paraffin wax and side sectioned into 17.5μm ribbons using a microtome. The immune-reactivity of two drusen related proteins (APOE and Aβ 1-42) were tested. Lipid loaded ARPE-19 cell cultures showed an increase of APOE expression compared to cultures grown in serum free media only (AI and AII). Interestingly the chronic lipid loading intervention induced positive immune-reactivity of Aβ 1-42 protein, which was completely absent in lipid free media cultures (BI and BII). DAPI was used to stain the nucleus (Blue at A and B). (Scale bars, 50μ.

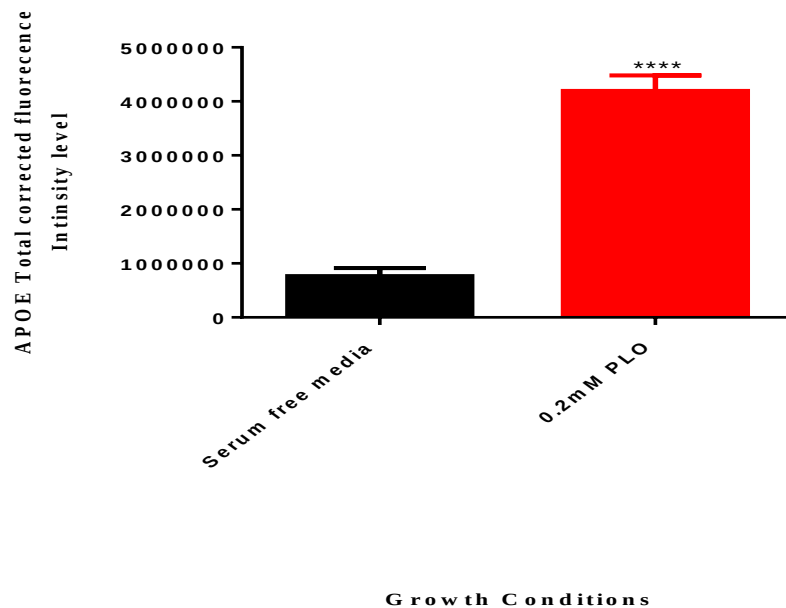
A)I



A)II



B) I



B) II

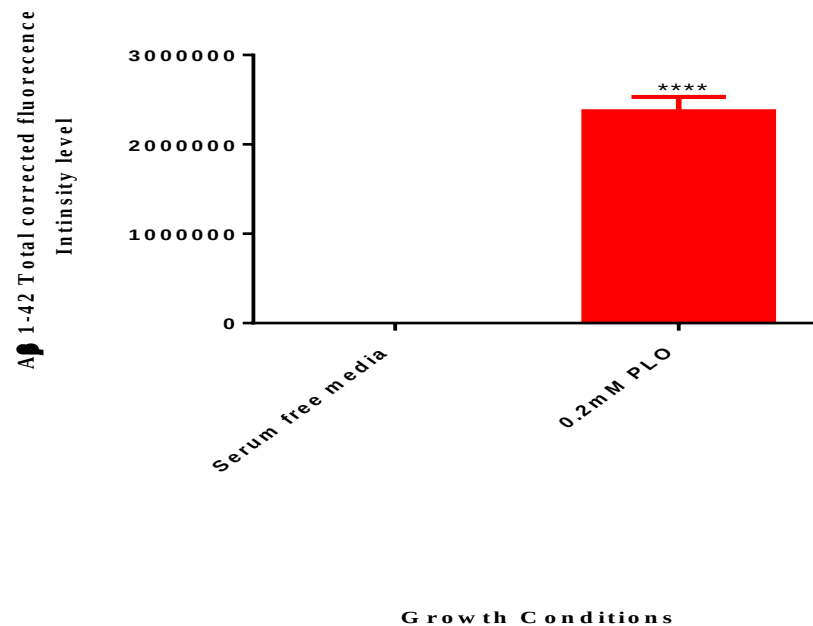


Figure 3. 14 the effect of lipid loading on sup-RPE deposit formations. (A) De-convoluted z-stack images were tilted on the Z-axis to form a flat mounted image with velocity software. An increase in APOE deposits (Green in AI and AII) and the expression of Aβ 1-42 (Red in AI and AII) was seen after 60 days of chronic lipid loading when compared to cultures grown in serum free media only. Scale bar 10μm. through the aid of Image J software deposits were counted using an average of 10 florescence images taken of each sample in each condition. Data are represented as mean ± SEM. Statistical analysis was done using unpaired t test. (ns: no significance, * P < 0.05, ** P<0.01, *** P< 0.001 and **** P < 0.0001).n=4.

Treating the ARPE-19 cell cultures with 0.2mM PLO for 60 days captured many of the features seen in ARMD: - the increase of intracellular lipids, loss of monolayer integrity and barrier function, increased VEGF-A secretion and, increased sub-RPE deposit formation and most importantly presence of amyloid β in subcellular deposits.

3.5. Discussion

The retinal pigment epithelium (RPE) cells play an important role in age related macular degeneration (ARMD). RPE cells irregularities such as accumulation of intracellular lipids (Tian et al., 2005, Curcio et al., 2001) and formation of sub-RPE lipid deposits “drusen” (Ambati et al., 2003) are recognised as the first clinical signs of a cascade of events leading to the dry from of ARMD. Because of the complexities of ARMD and the unique features of the human eye, animal models, although useful in some respects, fall short in capturing all aspects of ARMD (Zeiss, 2010). Cell-culture models are important tools used to study the physiology and pathology of cells and tissues. During the last decade much progress has been made in the establishment of cell culture models of the retinal pigment epithelium, there has been an increased interest in RPE function and its role in this disease that lead to the creation of many in vitro models. Johnson et al introduced a cell culture model that can mimic facets of drusen formation and complement activation in vivo (Johnson et al., 2011). This model was developed from RPE cells isolated from human fetal eyes. Such a model present a great potential for dry from of ARMD studies, yet the use of fetal eye is somewhat problematic due to stringent ethical issues and the limitation of the of the tissue supply. Other research groups investigated an alternative to primary RPE, using human cell lines. Hamilton et al introduced a 3D tri-layer model resembling RPE, Bruch’s membrane and choroid, using a human retinal pigment epithelial cell line (ARPE-19) and human umbilical vein-derived endothelial cells

(HUVECs), in a co-culture on amniotic membrane. This work demonstrated that ARPE-19, a human derived cell line can produce a monolayer with suitable TJ expression under controlled growth conditions. The potential use of filter grown ARPE-19 was also shown to be sufficient in drug delivery studies, as active transporters in the ARPE-19 membrane was shown to be qualitatively similar, with isolated RPE-choroid tissue (Shen et al., 2003) and their nuclear receptor (NR) expression was almost similar to primary isolated RPE cells, where 32 members out of the family of 48 were shown to be expressed in ARPE-19 compared to 36 out of 48 in primary RPE (Dwyer et al., 2011).

The use of cell line based culture models can be useful for studying dry ARMD mechanisms, yet caution should be applied when trying to relate them to age related retinal pathology, as they recapitulate phenotypes associated with the young and does not provide a model for age-related changes (Cristofalo, 1976, Cristofalo, 1997). In the present study, we introduced an RPE cell culture model that captured some of the characteristics of aged RPE in-vivo using long term serum free culture of ARPE-19 cell line. We then used the established aged RPE cultures and introduced them to lipid toxicity, a process that captured an aspect of ARMD pathology. Growing the ARPE-19 cells on laminin coated filters for 30 days in serum free cultures displayed the classic quiescent cobblestone morphology (Strauss, 2005) seen in vivo. The monolayer integrity was presented in these cultures through the expression of tight junction proteins (ZO-1 and Occludin) and the maintenance of steady TEER value. In vivo TJ are an essential component of the RPE monolayers that

form a barrier between neighbouring tissues. This presence of TJ in the RPE insure strong formation of the out blood retinal barrier (oBRB) that is responsible for the specialized transport of selected nutrients and metabolic products between the choroid and the retina. Therefore recreating similar a monolayer with integrity in vitro similar to that seen in vivo, will allow the ARPE-19 cells to behave as closely as possible to RPE cells in vivo

A prominent age-related change that occurs in human RPE monolayer is the accumulation of neutral lipid. In the in-vitro cell culture model described here it was shown that by growing the ARPE-19 cell long term under serum free conditions, an accumulation of intracellular lipids in a time dependent manner occurs. Intracellular lipid accumulation was reported as one of the age related changes seen in vivo along with lipid rich sup-RPE deposits build-up. Findings by Pauleikhoff et al and Marshall et al independently, suggested a strong correlation between age, intracellular lipid accumulation and sub-RPE deposition (D et al., 1990, Sheraidah et al., 1993, Pauleikhoff et al., 1990). Immunofluorescence analysis showed evidence of the production of extracellular sub-RPE deposits which accumulate within the pores of the supporting material over several weeks in culture, which were not found in 7 days cultures. These deposits were sporadic with a similar pattern as that seen in sub-RPE deposits in vivo. Immuno-reactivity analysis indicated that they contain major protein constituents of human drusen, APOE and Vitronectin; yet there was no immuno-reactivity for A β 1-42 observed in the sub-RPE deposits - a possible key contributor associated with the development of

ARMD (Löffler et al., 1995, Johnson et al., 2002, Anderson et al., 2004, Bruban et al., 2009, Wang et al., 2009).

From these results; it was concluded that by growing the ARPE-19 cells on a laminin coated Millicell filter for 30 days under serum free conditions, a stable healthy monolayer of RPE cells with TJ formation and reasonable trans-monolayer resistance suitable for experimentation is achieved. The 30 days serum free ARPE19 cell cultures model can be considered a model of normal aged RPE monolayer.

It is hypothesised that lipid accumulation is a key process in the pathogenesis of ARMD and so we preceded to lipid load the cells. The addition of 0.2mM PLO, as free fatty acids bound to albumin (a mixture of palmitic oleic and linoleic acids to reflect the FFA composition in human blood) to the serum free media caused a significant ($P<0.0001$) increase of intracellular lipids level within the ARPE-19 cells. This increase of intracellular lipids caused a disturbance to the monolayer integrity; this effect is seen as the TEER values start to drop below the control (serum free cultures) beyond 60 days of intervention. With the increase of intracellular lipid accumulation tight junctions start to be disrupted also. This disruption probably explains the fall in TEER value.

Vascular endothelial growth factor (VEGF) derived from the RPE has been also implicated in the physiological regulation of the choroidal vasculature, and overexpression of VEGF in this epithelium has been considered an important factor in the pathogenesis of choroidal

neovascularization in age-related macular degeneration (Vargis et al., 2014, Nozaki et al., 2006). A significant increase in VEGF-A secretion was seen in the spent media of lipid loaded cultures when compared to those grown in serum free media (control). The interaction of VEGF with its tyrosine kinase receptors (VEGF-R2) is a strong modulator of hyperpermeability as well as the delocalization of ZO-1 and ZO-2 from the cell borders (Fischer et al., 2004) which may explain the disruption of TJ's. Abnormal VEGF secretion by the RPE is believed to promote the progression of the choroidal neovascularization associated with the advanced form of age-related macular degeneration (ARMD) (Wang et al., 2010a). However, little is known about the molecular regulation of VEGF in the RPE. VEGF is a potent modulator of barrier function in both the retinal endothelia and the RPE and is required for choroidal vasculogenesis. It is the principal cytokine responsible for neovascularization in the mature eye (Geisen et al., 2006). In ARMD an abnormal increase in VEGF expression as well as TJ damage is seen and which drives the pathology of the dry form of ARMD to progress into the wet form of ARMD and the lipid loaded model described here model was able to demonstrate and increase in VEGF secretion (Rudolf et al., 2005, Geisen et al., 2006, Schmitt et al., 2004).

In addition to the intracellular lipid accumulation, the level of sub-RPE deposits within the lipid loaded cultures were significantly greater; these sub-RPE deposits also contain some of the most abundant protein constituents of human drusen. Immuno-reactivity assessment showed positive expression of APOE, a known drusen-associated protein that is synthesized and secreted by

the RPE (Wang et al., 2010b, Malek et al., 2005) and A β 1-42 is a cytotoxic protein that is also known to be a major component of Alzheimer's disease (AD) plaques. The presence of A β aggregation was reported in drusen isolated from ARMD donor eyes (Dentchev et al., 2003, Isas et al., 2010, Luibl et al., 2006) and was found to be association with RPE abnormalities, suggest that A β may play a role in the early phases of disease pathogenesis (Anderson et al., 2004, Dentchev et al., 2003, Johnson et al., 2002). However, whether the A β presence is a cause or result of RPE and photoreceptor degeneration is still unclear. The most likely source of A β in drusen is the overlying RPE cells, which are known to deposit material into drusen. Like most other cell types, RPE cells express amyloid precursor protein (APP), a membrane glycoprotein cleaved by proteases to produce the A β peptide (Johnson et al., 2002). The biological functions of A β are unclear, but both A β multimers and fibrils were shown to be neurotoxic (Selkoe, 2002). None the less, the quantity of A β present in drusen may depend upon alterations in protease activity producing A β and upon its clearance rate. PLO treated ARPE-19 cell cultures showed a positive deposition of A β 1-42, suggesting a possible a defect in A β clearance mechanisms. Together these findings strongly support the suggested idea of lipo-toxicity as contributing factor in ARMD pathology.



3.6. Conclusions

This chapter introduce a cell culture model that captures many of the features of ARMD. This model was generated from culturing ARPE19 cells to form a monolayer which was then allowed to differentiate under serum free culture conditions and then stressed by lipid loading to produce a model of lipo-toxicity. This lipo-toxicity model successfully captured several of the features of ARMD including an increase in intracellular lipids, an increase in VEGF expression, the loss of TJ, the development of sub-RPE lesions and deposition of A β 1-24. This model formed the basis of the subsequent studies in this thesis.

4

THE EFFECT OF COMPLEMENT ACTIVATION IN AN *IN VITRO* MODEL OF ARMD

4.1. Introduction

In recent years there has been increasing evidence of both genetic and molecular anomalies which have suggested that inflammation and complement cascade dysfunction play a critical role in the development and the progression of ARMD and are believed to be an initiator for its pathology (Ambati et al., 2013). However, the basis for these defects in the immune system remains unclear. Inflammation can be defined as a localized physical condition in which part of the body becomes reddened, swollen, hot, and often painful, especially as a reaction to injury or infection. The role of the complement

system in ARMD was first proposed about two decades ago following immuno-histochemical detection of complement components in specimens of sub-retinal membrane taken from patients with the wet form of ARMD (Hageman and Mullins, 1999, Gehrs *et al.*, 2010). This hypothesis was reinforced in 2005 with the discovery of a clear association between mutations in the complement factor H, a circulating regulator of the alternative complement activation pathway and ARMD (Klein *et al.*, 2005, Clark *et al.*, 2010). Since then, similar genetic associations have been reported in other complement factors (B, C2, C3, and C5), but factor H remains, the factor with the greatest impact on ARMD risk (Hughes *et al.*, 2011, Yu *et al.*, 2013)(see **Chapter 1 section 1.3.2.4.**). It is important to point out that that even though genetic polymorphisms associated with ARMD are clearly defined, their presence does not always cause the disease. Altered blood levels of complement proteins have been reported in patients with ARMD (Haas *et al.*, 2011, Sivaprasad *et al.*, 2007, Reynolds *et al.*, 2009). Though the evidence linking complement dysregulation to ARMD risk are strong, the mechanisms by which they act are still not fully understood.

There is a recent classification of ARMD that describes the disease not as a classical inflammatory disease, but a product of prolonged para-inflammatory response. Para-inflammation is the term that describes the low grade chronic/subclinical immune response caused by endogenous inducers which adapts tissue to harmful environments and helps maintain adequate functionality (Xu *et al.*, 2009, Chen *et al.*, 2012, Chen *et al.*, 2011).

The RPE cells structure and their support for the photoreceptor place them under a relentless stress of high levels of noxious substances which makes them highly dependent on the process of para-inflammation to preserve their homeostasis and health. Although the physiological purposes of para-inflammation is to act as an adaptive response of the injured tissue, prolonged inflammation and activation of immune response at one site can cause chronic damage. In the case of ARMD the accumulation of intracellular lipids in the RPE monolayer was seen to increase oxidized lipid accumulation as Drusen in sub-RPE form (Ebrahimi et al., 2013). Immuno-histochemical analysis of Drusen identified the presence of complement components C3, C5 and C5b-9. This observation highlights the potential involvement of the complement system in ARMD pathology.

There is a known correlation and relation between lipids and their metabolism with the function of complement cascade. Lipodystrophy was found to coincide with hypo-complementemia, prior to first descriptions of familial C3 deficiency presenting with partial lipodystrophy (Patrick et al., 2009). This interplay between lipid metabolism and inflammation driven by the complement activation is seen in other disorders that share ‘Drusen’-like deposits in their clinical presentation including atherosclerosis and Alzheimer Disease (AD) (Klein et al., 2013, Johnson et al., 2002). This may suggest a possible similar pathology in the mechanism of their deposit formation, as well as emphasizing the importance of this interplay between lipid metabolism and the activation of the complement cascade.

In the previous chapter, a lipid loading- driven model of ARMD was presented, this model captured certain aspects of ARMD (see **Chapter 3**), which have been shown to be exhibited in association with epidemiological factors such as dietary fat and caloric intake that have been identified as risk factors for ARMD. This raises the question of how do complement activation and lipid loading interact with each other and is the role played by complement a damaging or protective role.

4.2. The aim of this chapter

One of the challenges that complicate the understanding of ARMD pathology is the interaction of constituents from both local and systemic origin (Scholl et al., 2008). None the less many of the components of drusen are produced by RPE, retina, or both (Mullins et al., 2000) which makes it harder to identifying the trigger(s) for complement deposition. This chapter will explore the cross-talk between lipids and inflammatory responses by studying the effect of 10% human serum (vol/vol) in the presence and absence of lipid loading using the ARMD model in **Chapter 3**.

4.3. Study design

We generated our tissue culture based model of ARMD as follows: ARPE-19 cells were required to grow on a millicell filter support for 30 days in serum free medium prior to treatment or the introduction of any intervention. This period of time allowed TJ formation and stabilization of TEER (See **Chapter 3**). Once the cultures were stable and their TEER was $< 65\Omega \times \text{cm}^2$, cells were treated with either 0.2mM PLO, 10% human serum (HS) or the two

together for another 60 days (90 days total). Serum free media was used as a control (cultures grown in serum free media for 90 days). This model was used to investigate the accumulation of intracellular lipids and the RPE monolayer barrier formation (TJ expression and TEER value). VEGF expression, an; important component of ARMD pathology, was measured. The level of VEGF in media was monitored throughout the experiment by ELISA. We also investigated the effect of these interventions in the formation of sub-RPE deposits and for their immuno-reactivity for APOE and A β proteins.

4.4. Results

4.4.1. Evaluating the use of human serum

4.4.1.1. Cell culture viability

In this project the activation of the complement system was accomplished through the addition of 10% human sera (See **Chapter 2 section 2.2.6**) to serum free DMEM/Ham F12 media in a volume/volume manner. It was previously recommended by Johnson et al., 2011 that 10% HS be used to achieve complement activation in a cell culture model similar to that used here (Johnson et al., 2011); therefore we evaluated the tolerance of our system to chronic use of different range of human serum addition in media to evaluate cell culture viability.

ARPE-19 cell cultures were cultured in serum free media for 30 days then introduced to serum free DMEM/Ham F12 media supplemented with several concentrations of HS (5, 10, 25 and 50%; vol/vol) for another 60 days (90 days in total), replacing the media with freshly supplemented media every 3-4 days. The average cell count was calculated to assess the cell culture viability through counting the cell nuclei using DAPI fluorescence and ImageJ software **Figure 4.1**. Results were converted to percentage relative to the untreated control cells (serum free cultures). No significant effect on cell viability was seen after the addition of up to 10% HS in media (vol/vol), although 50% (vol/vol) was toxic, the use of 25% HS seemed to increase cell proliferation by ~20% an unwanted effect that will disturb the cell culture monolayer.

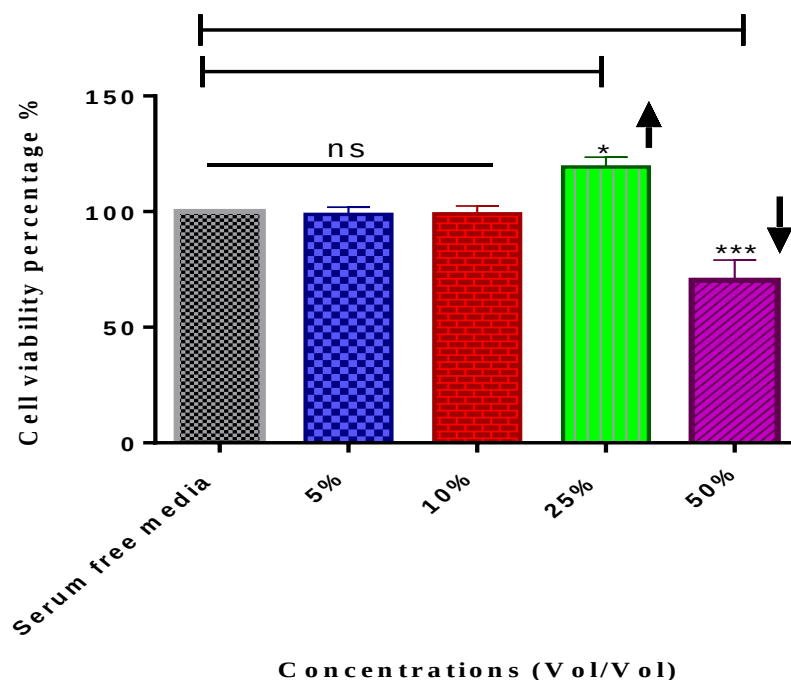


Figure 4. 1 The assessment of chronic human sera treatment on cell culture viability. ARPE-19 cell cultures were initiated in serum free media for 30 days then introduced to serum free DMEM/ham F12 media supplemented with several concentrations of human sera (5, 10, 25 and 50%; vol/vol). Cells were cultured for another 60 days (fresh supplemented media was added every 3-4 days) giving a total cell culturing period of 90 days. Average cell count was calculated to assess the proliferation levels. Cell nuclei were counted using DAPI fluorescence and ImageJ software. Reading were analysed with prism using one way ANOVA (dunnett multiple comparisons test). Data presented as mean $\pm$ SEM (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$), $N=3$.

4.4.1.2. The activation of the complement system in ARPE-19 cell cultures

C3 is an acute phase protein, and its plasma concentrations increases at the early steps of acute inflammation. A number of studies have examined complement gene expression in inflamed tissue and found that the level of C3 gene expression broadly correlates with the severity of tissue infection and inflammation (Pratt et al., 2000, Sasaki et al., 1997, Passwell et al., 1988, Passwell et al., 1990). In this study it was established that the chronic use of

10% human serum does not affect the ARPE-19 cell culture viability, therefore the influence of chronic 10% human serum addition on C3 gene expression was investigated. ARPE-19 cell cultures were initiated in serum free media for 30 days then introduced to serum free DMEM/ham F12 media supplemented with 10% HS or 10% heat-inactivated HS. Heat-inactivation was achieved through heating the samples in water for 30 min at 56°C to destroy complement. After 60 days of culture in media supplement with human serum (changing media every 3-4 days), RNA was isolated and gene expression was investigated through the use of Taqman. The mRNA relative levels were normalized to the mean value used in the $\Delta\Delta C_t$ method in comparison to RNA polymerase II expression (reference gene). Taqman results showed that C3 gene expression was present in unstimulated ARPE-19 cultures and was increased by stimulation with active 10% HS **Figure 4.2**. The increase in C3 mRNA expression of approximately 70% is indicative of a low level activation of complement gene expression which has been observed in ARMD.

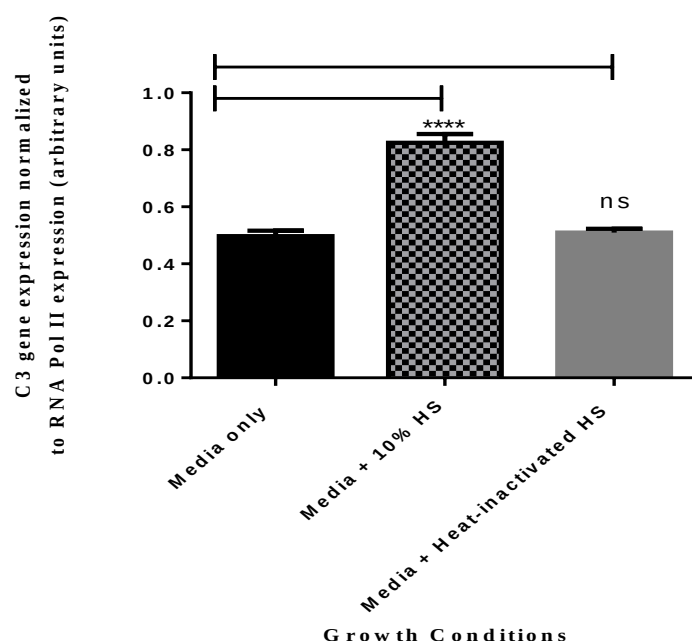


Figure 4. 2 The assessment of 10% HS treatment on C3 gene expression. ARPE-19 cell cultures were supplemented with either 10% human sera (HS) or 10% heat-inactivated human sera (vol/vol) for 60 days (fresh supplemented media added every 3-4 days). RNA was isolated from culture and C3 gene expression was detected by Taqman. The mRNA relative levels were normalized to RNA polymerase II expression. The addition of 10% HS significantly increased the level of C3 gene expression, this increase was not seen when the human sera were depleted of complement by heat-inactivation. Data presented as a mean S.E.M. statistical analysis was done using one way ANOVA with tukey post-test (ns: no significance, * $P < 0.05$, *** $P < 0.0001$).

4.4.2. Effect of chronic 10% human serum (HS) on lipid accumulation

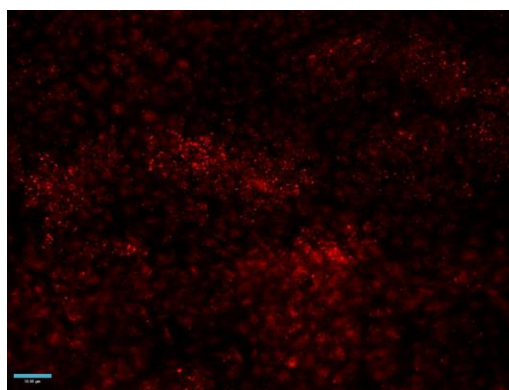
Complement C3 is a pivotal component of three cascades of complement activation (see **chapter 1, section 1.3.3.**) and secretion of C3 by the RPE was shown to increase during acute inflammation (Volanakis, 1995, Sacks et al., 1993). We showed that the C3 gene is expressed in non-stimulated ARPE-19 cell cultures and that its expression increased by approximately 70% after stimulation with 10% HS. To further understand the role of complement in lipid accumulation we cultured ARPE-19 cells in media supplemented with 0.2mM PLO with, and without, 10% HS. After 60 days of treatment, cultures

were fixed with 4% PFA and stained with Nile red fluorescent stain to visualize intracellular lipid droplet formation.

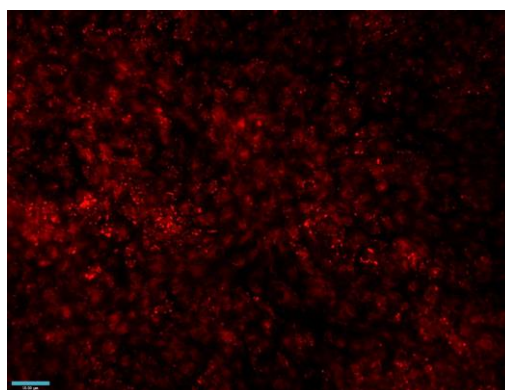
4.4.2.1. Microscopic visualization of Nile red fluorescent staining

It was previously demonstrated that treating ARPE19 cultures with 0.2mM PLO resulted in intracellular lipid accumulation (**Chapter 3, section 3.4.2.**). Fluorescence microscopic analysis of Nile red stain showed that the addition of 10% HS **Figure 4.3A** had no significant effect on the intracellular accumulation of lipids compared to control (serum free cultures) **Figure 4.3B**. As expected the treatment of 0.2mM PLO increased the accumulation of intracellular lipid accumulation **Figure 4.3C** yet, this effect of PLO treatment was reversed in the presence of complement **Figure 4.3D**.

A) Media Only



B) Media + 10% HS



C) Media + PLO

D) PLO + 10%HS

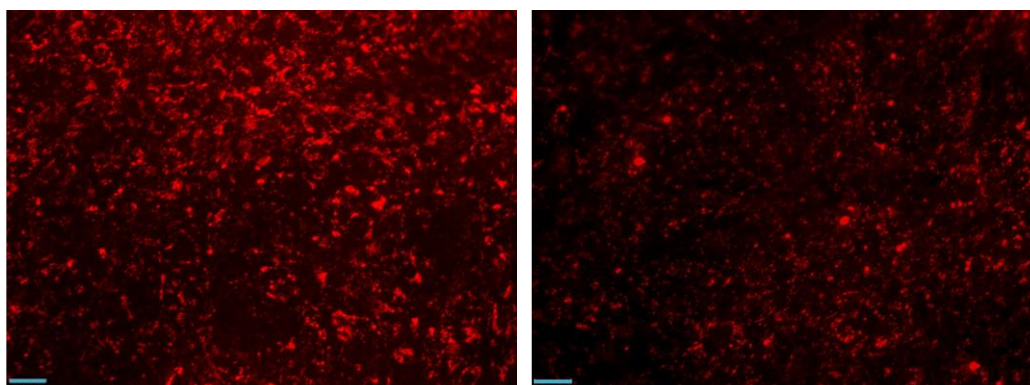


Figure 4. 3 The effect of chronic 10% HS treatment on lipid accumulation. Fluorescence analysis of Nile red staining was used to visualize the intracellular lipid accumulation. ARPE-19 cells grown on Millicell-PFC inserts were cultured in serum free DMEM Ham F12 for 30 days then induced to different interventions for a further 60 days; A) 10% HS (Vol/Vol), B) Serum free media (control), (C) 0.2mM PLO and D) dual interventions of 10% HS and 0.2mM PLO. Scale bar 50µm, N= 5.

To further verify the results, a quantitative corrected total cell fluorescence (CTCF) analysis was calculated using the method used by (Burgess et al., 2010) **Figure 4.4**. A reversal of lipid accumulation was observed in complement activated cultures. Together these results indicate a process of increase lipid metabolism or lipid transfer.

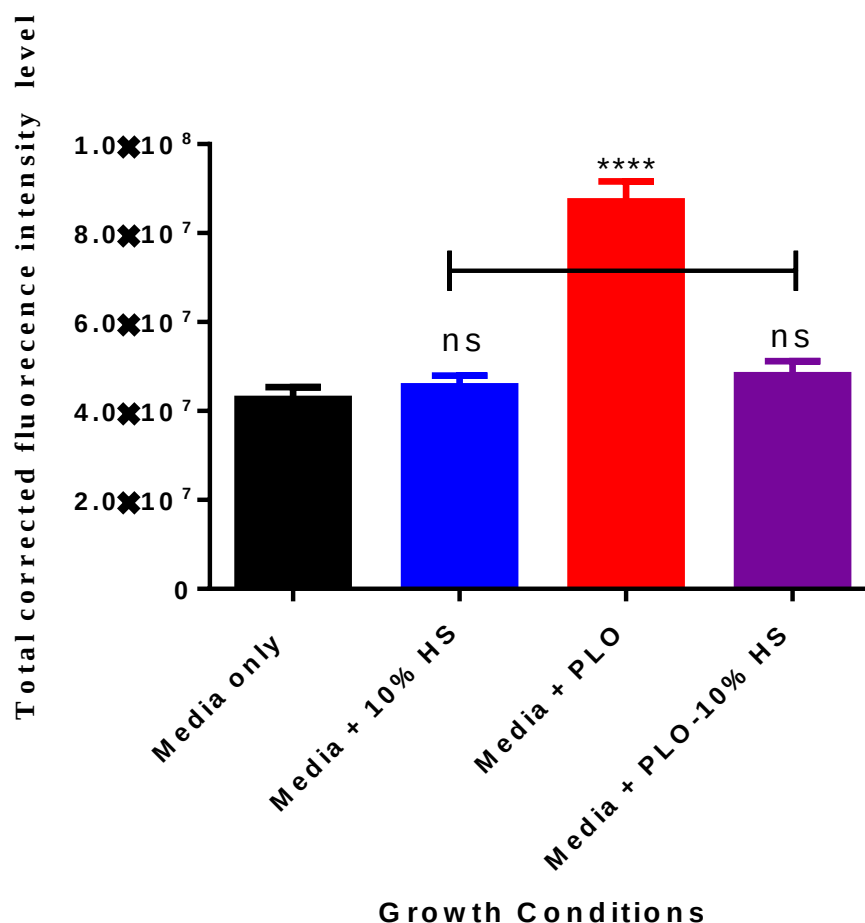


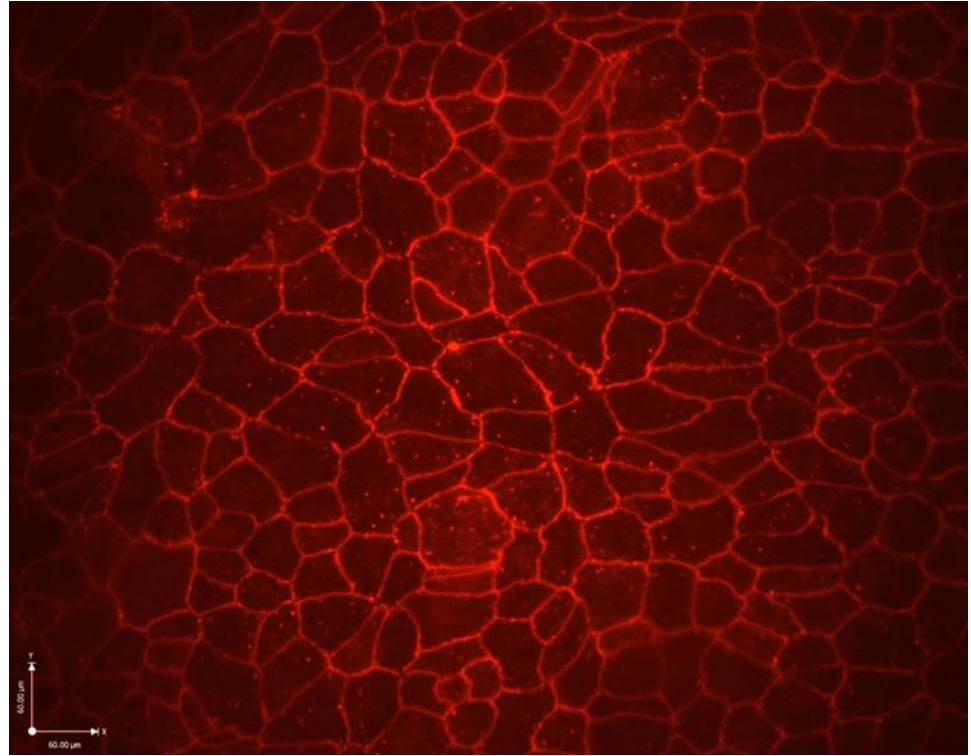
Figure 4. 4 The effect of chronic 10% HS treatment on lipid accumulation. 10% HS was used to activate the complement system in ARPE-19 cell cultures in the presence and absence of 0.2mM PLO. Cells were fixed with 4% PFA then stained with Nile red stain to visualise lipid accumulation. Total CTCF was calculated using the ICC images through the use of Image J software (10 pictures per sample). Reading were analysed with prism using one way ANOVA (Tukey's multiple comparisons test). Data presented as mean $\pm$ SEM (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$), $N=5$.

4.4.3. Effect of 10% HS treatment on cell culture monolayer integrity

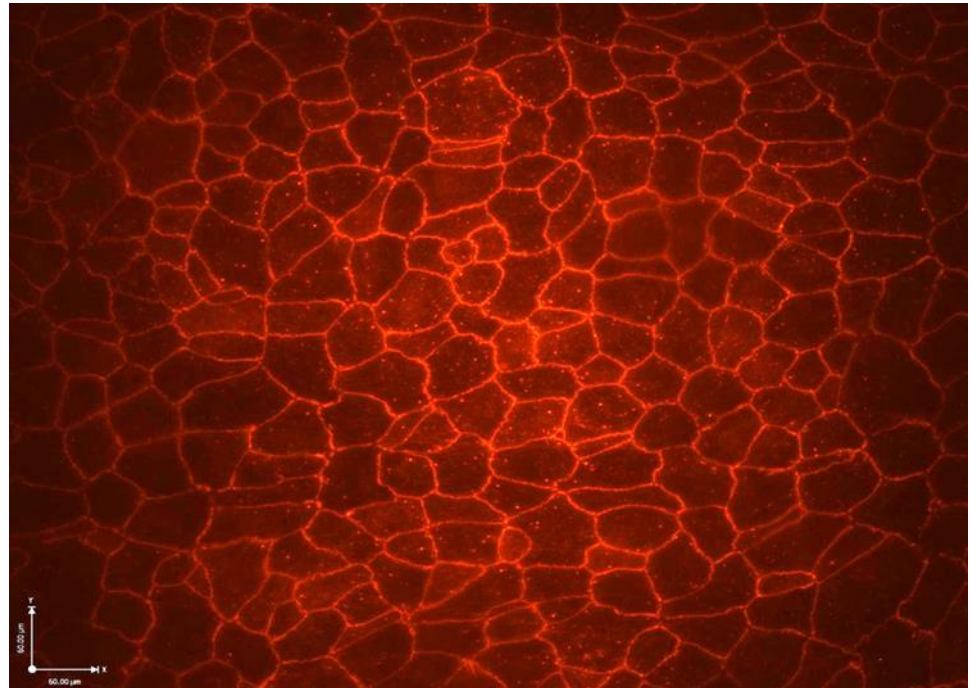
4.4.3.1. Tight junction formation

It was shown, in **chapter 3** that there was a correlation with intracellular lipid loading and reduced barrier function with impaired TJ and monolayer integrity of ARPE-19 cell cultures (see **Chapter 3 Figure 3.11 and 3.12**). To understand how the presence of an active complement cascade might affect the RPE barrier function, the effect of 10% human serum in the presence and absence of lipid accumulation was investigated through the use of ZO-1 immuno-reactivity analysis. Fixed cultures were visualized for their TJ formation by staining with ZO-1 antibody **Figure 4.5**. In comparison to cultures grown in serum free media (control) **Figure 5.4A**, treatment with 10% HS showed no significant effect on ZO-1 TJ marker by itself **Figure 4.5B**. As expected the chronic treatment of 0.2mM PLO reduced ZO-1 TJ staining **Figure 4.5C**, yet the combination of 10% HS and 0.2mM PLO showed a maintenance of ZO-1 protein expression levels **Figure 4.5D**.

A)



B)



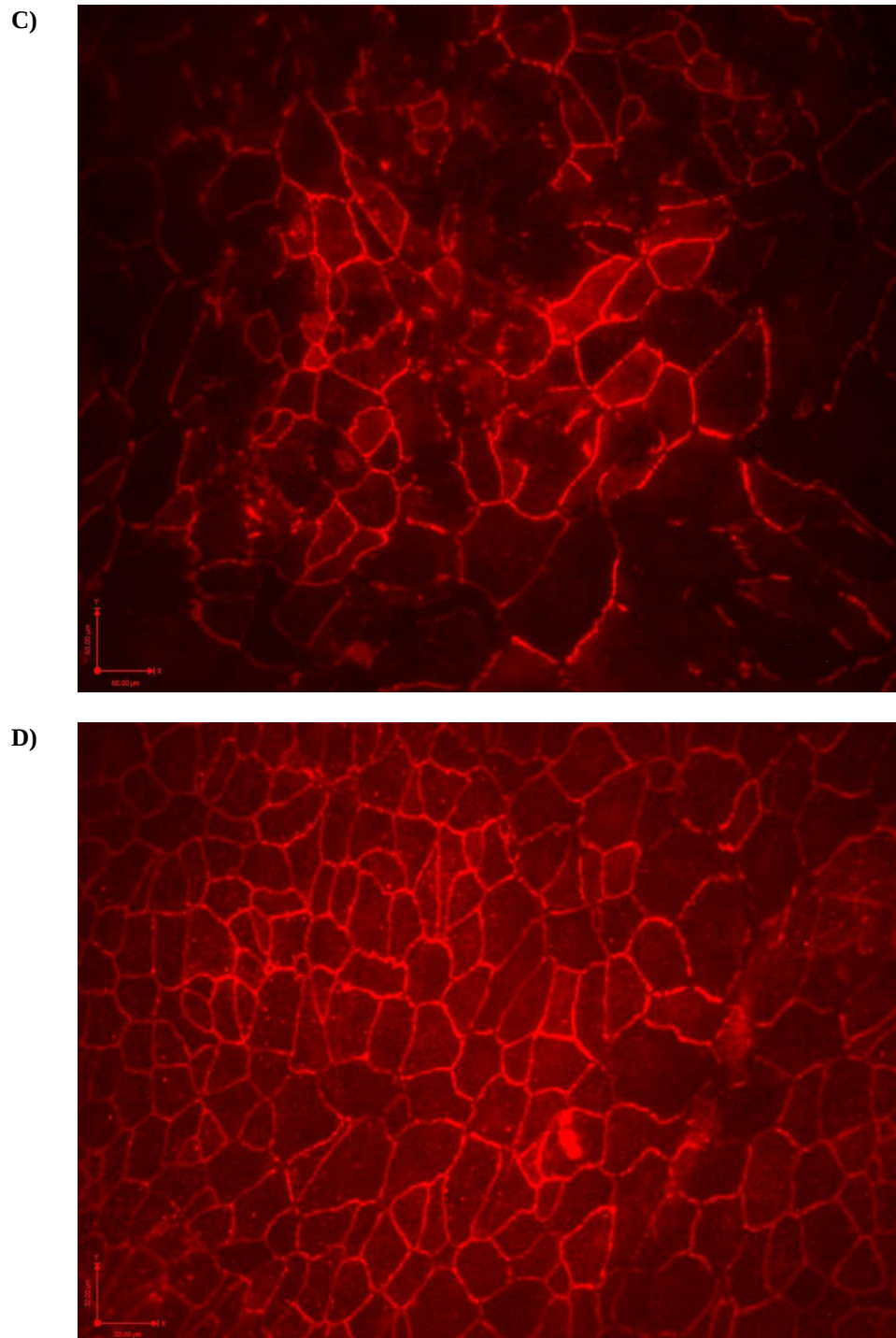


Figure 4. 5 Effect of 10% HS with and without lipid loading on tight junction formation. ARPE-19 cells grown on Millicell-PFC inserts were cultured in serum free DMEM Ham F12 for 30 days then induced to different interventions for a further 60 days; (A) Serum free media only (control), (B) 10% human serum (C) 0.2mM PLO and (D) dual interventions of 10% human sera and 0.2mM PLO. Cells were fixed with 4% PFA and stained with anti ZO-1 antibody (known TJ marker). Scale bar 60µm. n=4 for A and D and n=8 for B and C.

The percentage of continuous TJ was further assisted as described previously in **chapter 3. Figure 4.6.**

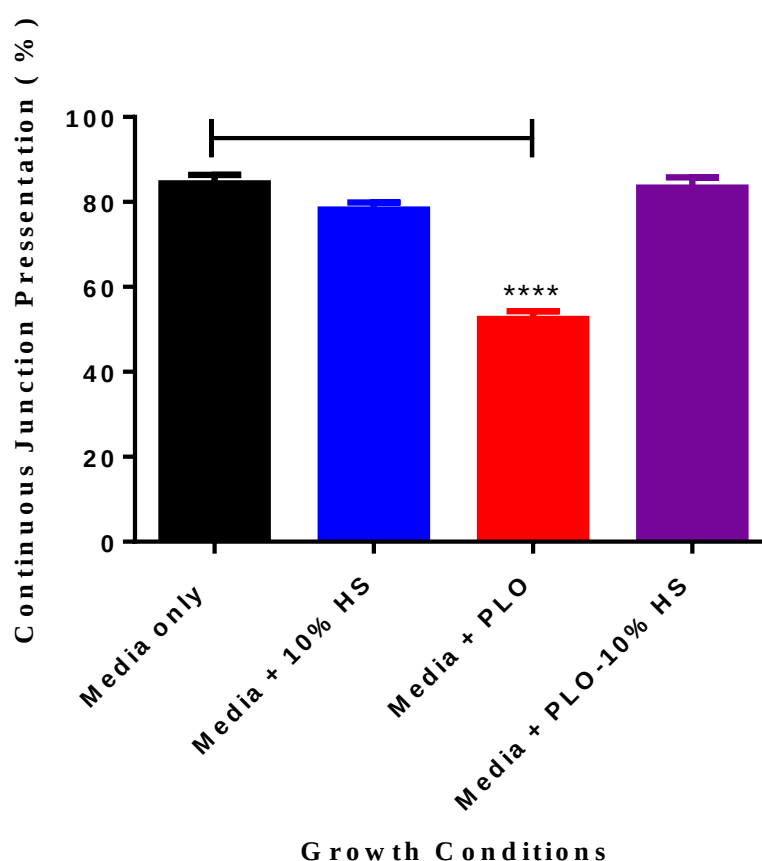


Figure 4. 6 Effect of 10% HS with and without lipid loading on the continuous TJ. Through the use of fluorescent microscopy an average of 10 images were taken for each sample (n=4) and the percentage of continuous TJ presentation was calculated using the protocol provided by (Ebrahim and Leach, 2014). Reading were analysed with prism using one way ANOVA (dunnett multiple comparisons test). Data presented as mean $\pm$ SEM (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$), N=4.

4.4.3.2. TEER measurements

The TEER value was calculated as before using readings obtained from three cultures for each intervention on a weekly basis **Figure 4.7**. Lipid loaded cultures showed a similar pattern in their TEER value expression as seen before in **Chapter 3 section 3.2.3.2**. At week 13 the TEER values start to gradually decrease reaching a low of $54.0 \pm 4.2 \Omega \times \text{cm}^2$. The effect of 10% HS alone or in conjunction with lipid loading showed a TEER level similar to that observed in control cultures (serum free media) that was maintained throughout the experiment. These results support the data obtained from ZO-1 TJ immunostaining, providing stronger evidence of the protective role in terms of reducing intra-cellular lipid and maintaining tight junction integrity, played by the addition of 10% HS treatment in the ARPE-19 cell cultures.

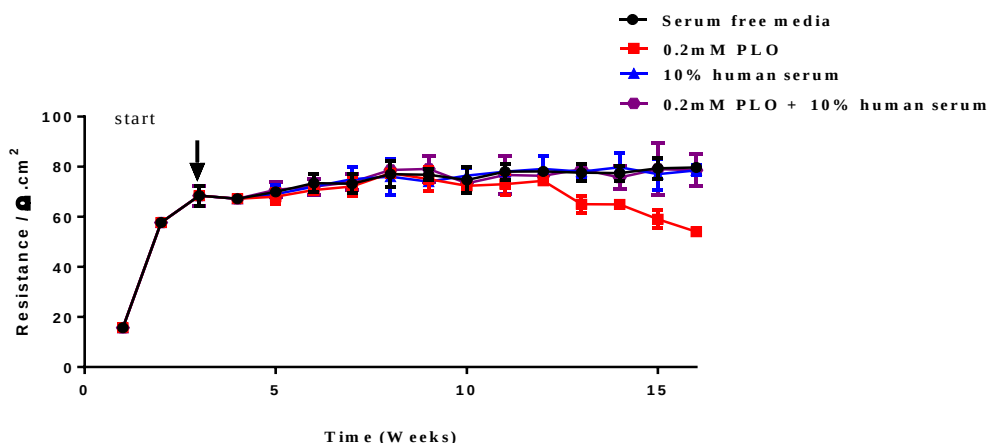


Figure 4. 7 Effect of 10% HS treatment on TEER value. TEER measurements were recorded once a week throughout the 90 days for all interventions. All cultures were maintained in serum free for 30 days before the induction of chronic interventions (start) of serum free media (Black), 10% HS (Blue), 0.2 mM PLO (Red) and dual treatment of 0.2mM PLO + 10% HS (Purple). The increase of in complement activity showed a protective characteristic to the TEER value. (N=3).

4.4.4 VEGF-A secretion in media

It was shown previously that lipid loading caused a significant increase in VEGF-A secretion in the media that may be involved in the reduction of tight junction formation (See **chapter 3, section 3.4.2.3**). The effect of 10% HS on VEGF-A secretion into media was investigated using ELISA, as described in **Chapter 3**. 60 days treatment with 10% HS was shown to significantly increase VEGF-A secretion in the media in the presence and absence of lipid loading compared to serum free media **Figure 4.8**, yet the activation of the complement system under lipid loaded conditions showed a non-significantly lower secretion of VEGF-A into media compared to cultures that were treated with 0.2mM PLO alone.

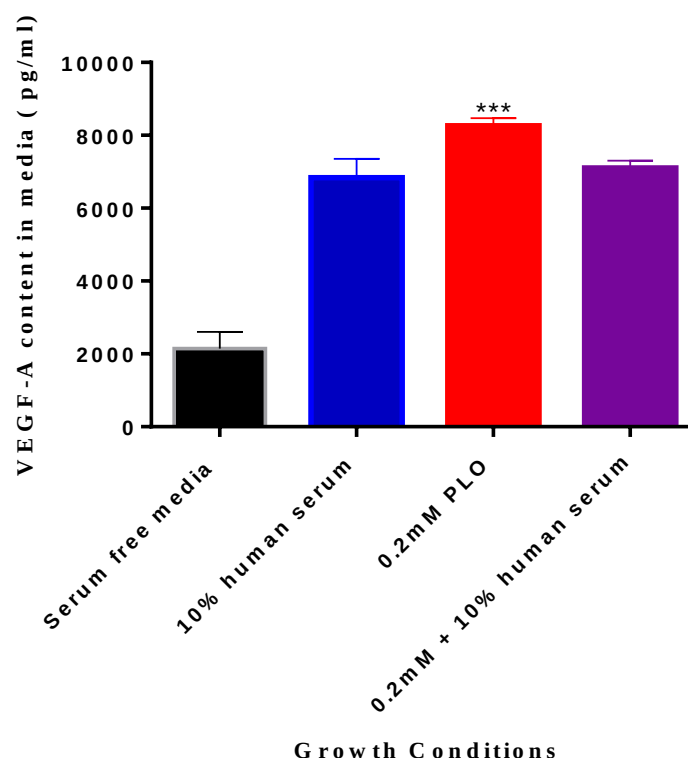
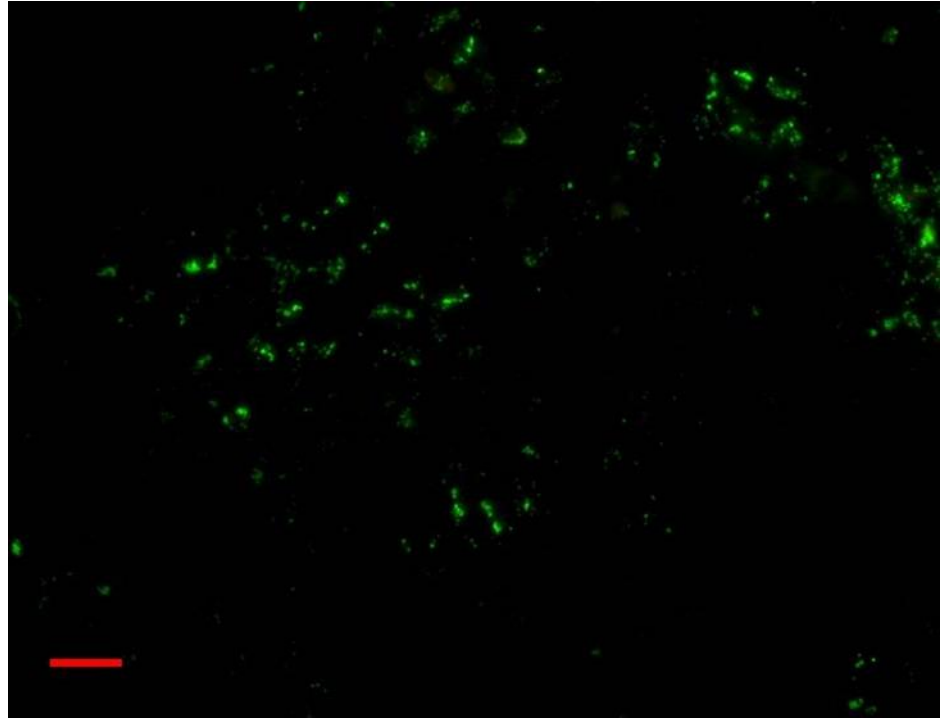


Figure 4.8 The effect of 10% HS on VEGF-A secretion in media. VEGF-A concentration in the media from the ARPE-19 cells cultures were measured with an ELISA. Data are presented as mean $\pm$ SEM Reading were analysed with prism using one way ANOVA (Tukey's multiple comparisons test). Data presented as mean $\pm$ SEM (ns: no significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$), $N=3$.

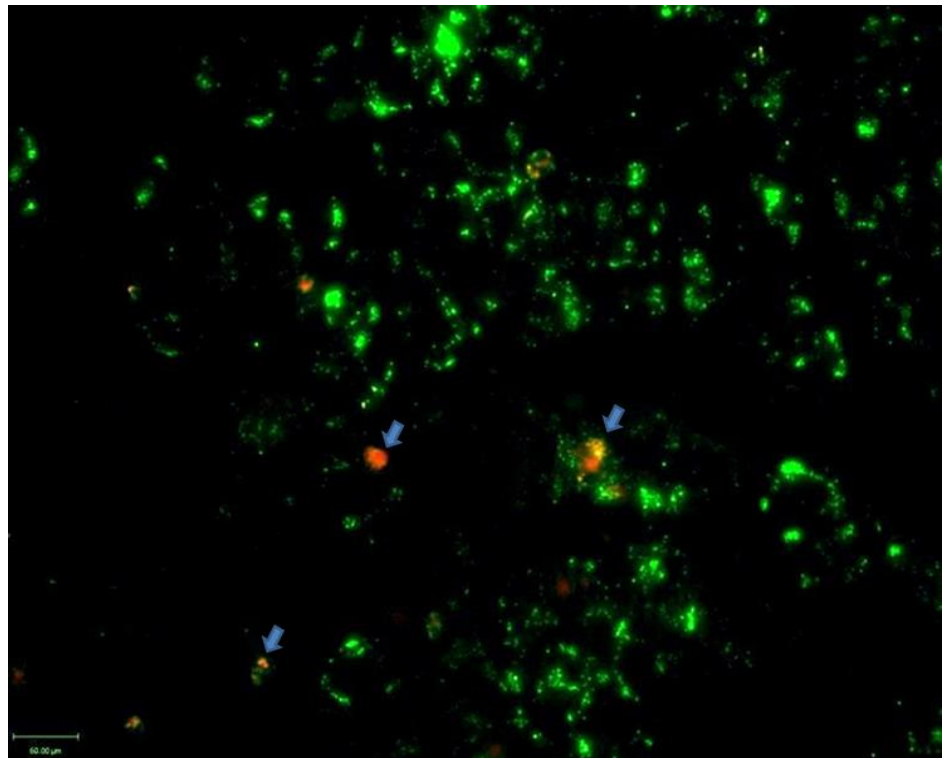
4.4.5. Sub-RPE deposit formation

The immuno-reactivity for drusen related proteins APOE and A β 1-42 were investigated through fluorescent microscopy **Figure 4.9**. Flat mounted images showed an increase in both APOE and A β 1-42 deposits under both interventions (0.2mM PLO and 10% HS) when compared to control cultures. Cultures treated with 10% HS showed co-localization of APOE and A β 1-42 in deposits **Figure 4.8 B** and **4.D** /arrow. The same effect was seen in side section images **Figure 4.10**.

A)



B)



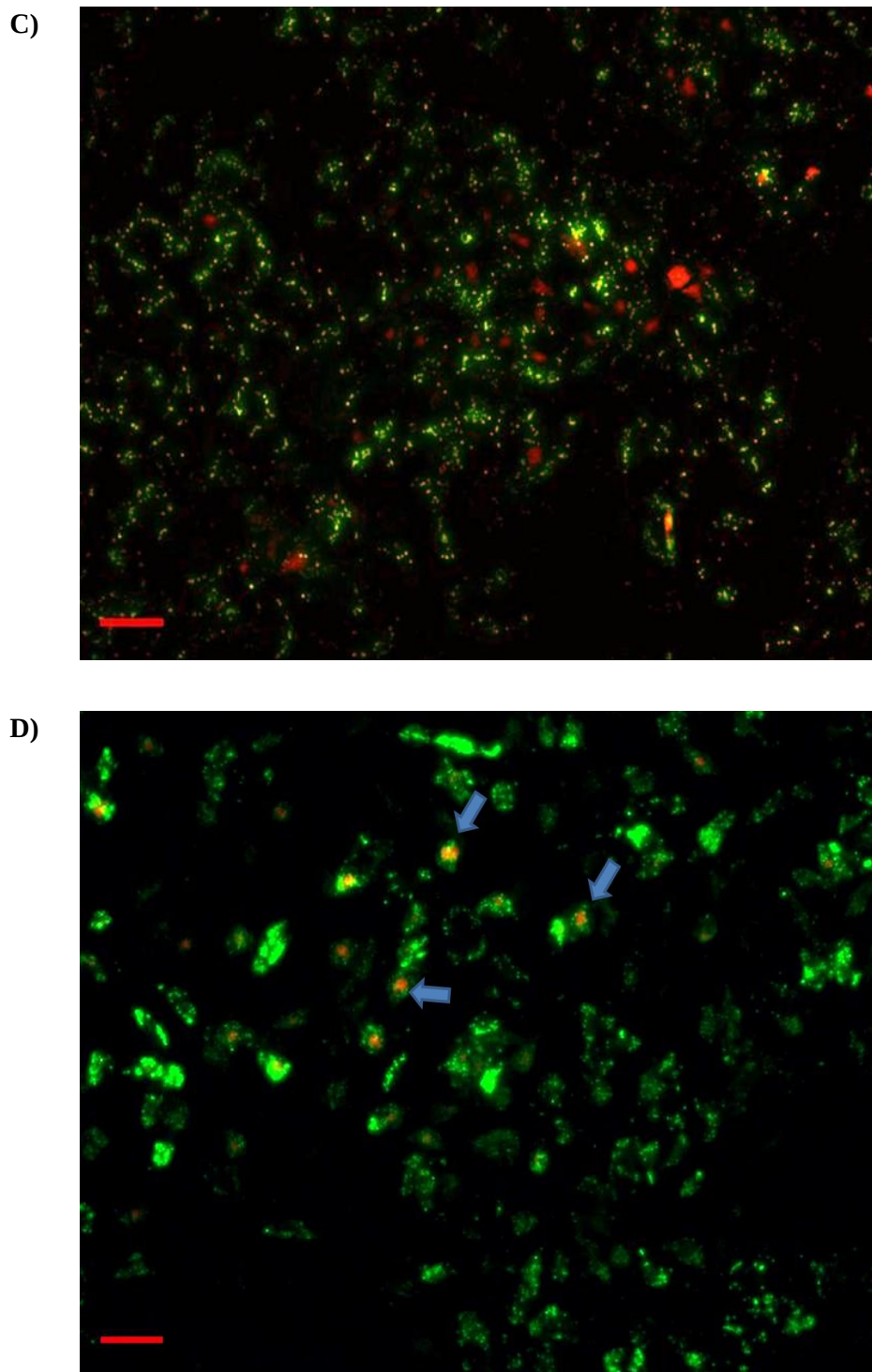


Figure 4. 9 The effect of 10% HS treatment on sub-RPE deposits formation. De-convoluted z-stack images were tilted on the Z-axis to form a flat mounted image with velocity software. (A) Serum free media only (control), (B) 10% human serum, (C) 0.2mM PLO and (D) 0.2mM PLO + 10% human serum. Co-localization of APOE/ A β 1-42 complexes (Blue arrows) was seen in complement activated cultures. Scale bar 10 μ M, N=4.

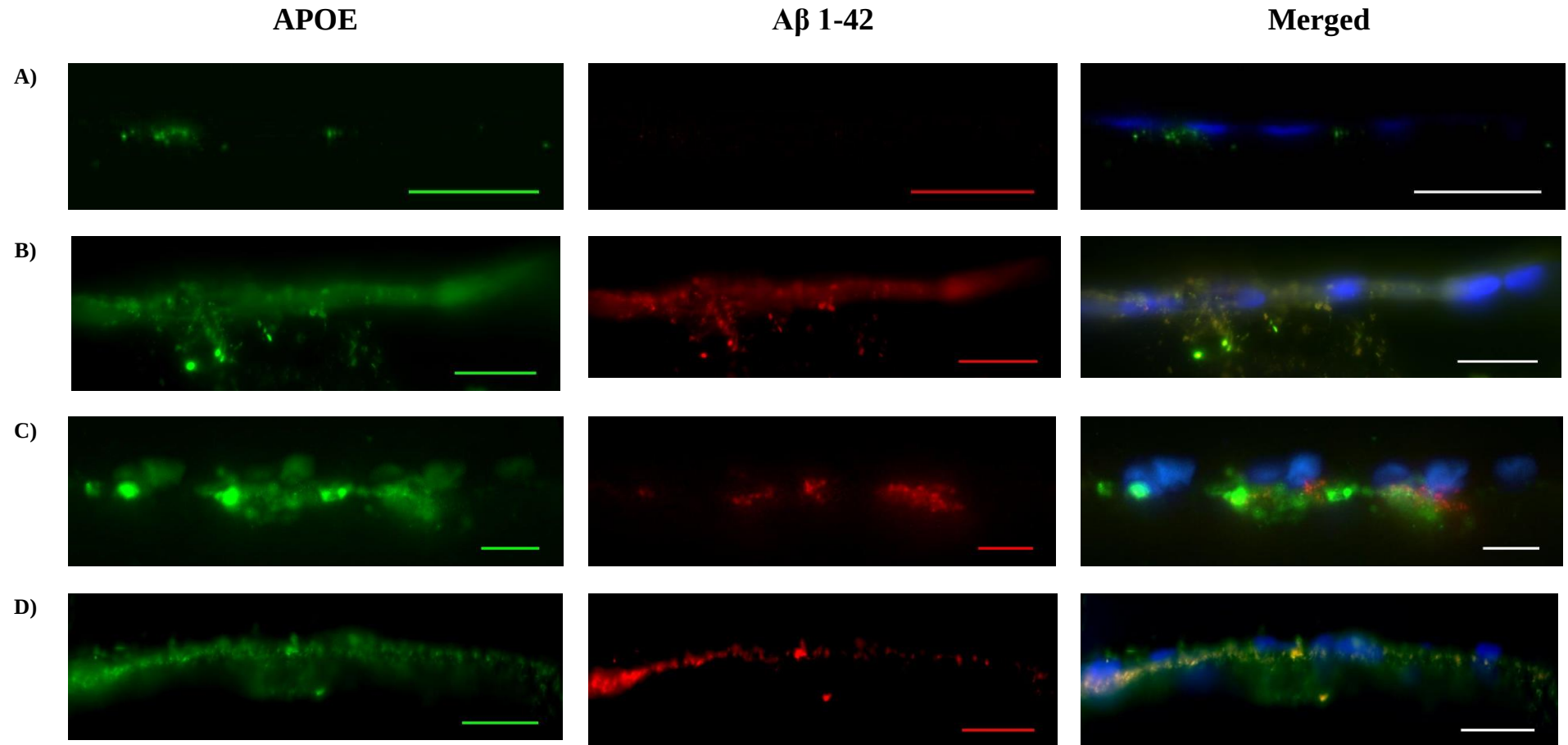


Figure 4. 10 Sub-RPE deposit formations in 10% HS treated cultures. Samples were embedded in paraffin wax and side sectioned into 17.5 μ m ribbons using a microtome. The immune-reactivity of two drusen related proteins (APOE and A β 1-42) were tested.(A) Serum free media only (control), (B) 10% human serum, (C) 0.2mM PLO, (D) 0.2mM PLO + 10% human serum. APOE deposits (green) showed strong co-localization (yellow) with A β in complement activated cultures. Scale bar 50 μ m, N=5.

To further analyse the immunofluorescence results, the number of deposits were quantified by calculating the total corrected cell fluorescence using the method used by (Burgess et al., 2010). An average of 10 randomly selected images were taken for each sample in each intervention (n=4 per intervention). The accumulation of ApoE **Figure 4.11A** and A β 1-42 **Figure 4.10B** in sub-RPE positive deposits increased significantly after the addition of 10% HS in the presence and absence of PLO treatment compared to control (serum free media) . PLO + 10% HS treated cultures showed a significantly lower APOE levels in their deposits, yet higher levels of A β 1-42 (P < 0.05).

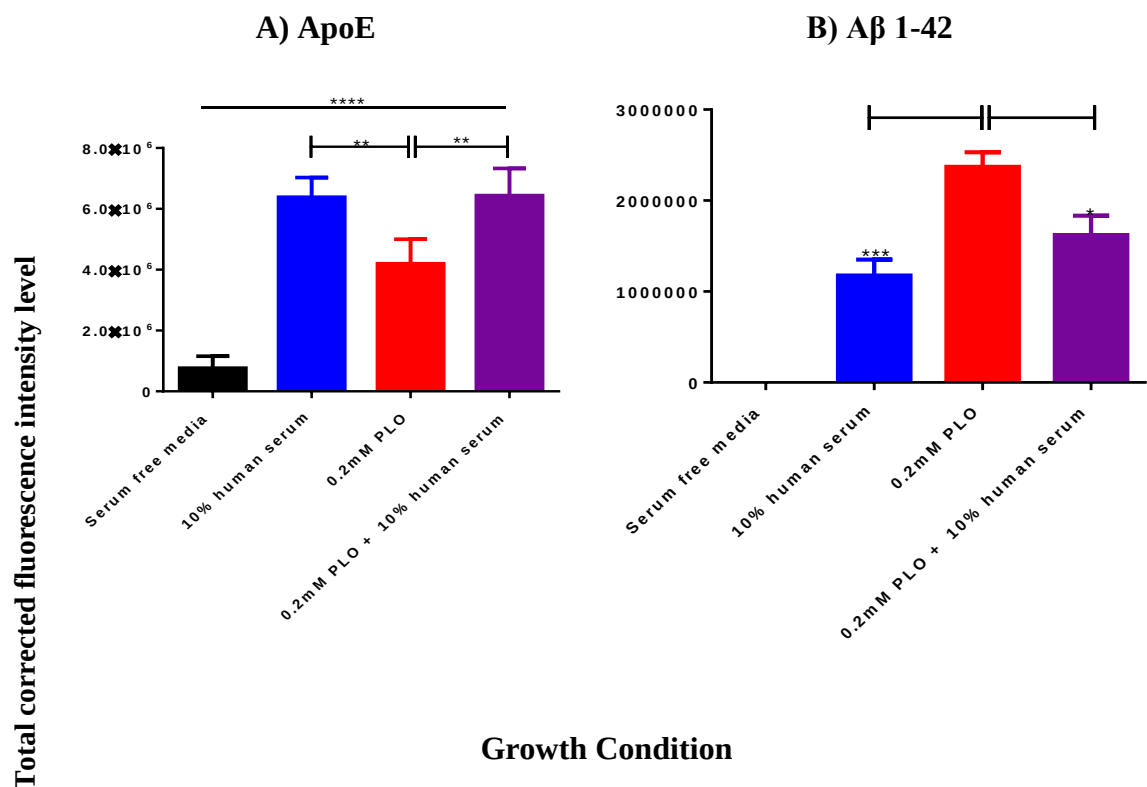


Figure 4. 11 The effect of 10% HS treatment on sub-RPE deposits formations. De-convoluted z-stack images were tilted on the Z-axis to form a flat mounted image with velocity software. Total CTCF analysis was calculated using the ICC images through the use of Image J software (10 pictures per sample). Reading were analysed with prism using one way ANOVA (Tukey's multiple comparisons test). Data presented as mean $\pm$ SEM (ns: no significance, * P < 0.05, ** P<0.01, *** P< 0.001 and **** P < 0.0001).n=4

4.5. Discussion

In this chapter we have examined the role of the 10% human serum treatment as a source of complement component C3, in an attempted to identify its association in ARMD pathology using our cell culture model of lipid toxicity.

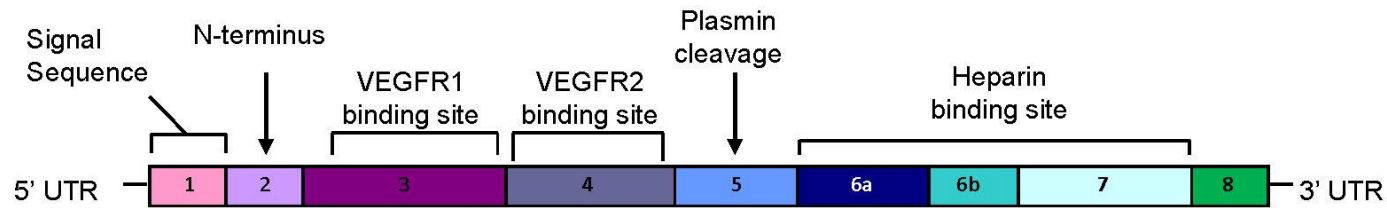
Long term (60 days) 10% human serum (HS) intervention showed no effect on ARPE-19 cell culture viability and monolayer integrity unlike the effect seen with chronic lipid loading. Lipid loaded cultures that were treated with 10% HS, had reduced intracellular lipid accumulation and did not exhibit the reduction in TJ and TEER observed in the PLO treated cells. The level of VEGF secretion has been previously shown correlate with TJ expression and barrier function (Ghassemifar et al., 2006). In the previous chapter both high secretion of VEGF-A in media and loss of TJ integrity were seen after lipid loading. The addition of 10% HS also showed an increase in VEGF-A secretion, yet with no alteration to the TJ suggesting a possible different VEGF-A isoform production between the two interventions.

The vascular endothelial growth factor (VEGF) family is comprised of a number of closely related ligands, that exists in a number of different isoforms which differ in the number of amino acids contained in the mature secreted protein and, most importantly, in their solubility and heparin-binding properties. In addition to generating VEGF-A isoforms that differ in length and domain composition, the pre-mRNA splicing machinery also gives rise to proximal and distal splice forms: proximal splice forms are pro-angiogenic

whereas distal splice forms are anti-angiogenic **Figure 4.12** (Holmes and Zachary, 2005, Robinson and Stringer, 2001). In the human eye, VEGF₁₂₁ and VEGF₁₆₅ are the major isoforms expressed, while high-affinity receptors for VEGF (VEGFR 1 and VEGFR 2) are expressed by the RPE cells and choriocapillaris (Campa and Harding, 2011, Saint-Geniez et al., 2006). VEGFR 2 appears to be the major signal transducer of VEGF-A, whereas VEGFR1 is considered as a natural decoy by regulating VEGF-A isoforms ability to bind VEGFR 2. In contrast to VEGFR 1, VEGF-A isoforms binding to VEGFR 2 leads to a robust autophosphorylation and the induction of various signalling cascades including, Mitogen-activated protein kinases (MAPK), Phospholipase C (PLC), Protein kinase B (PKB), also known as “Akt”, and Proto-oncogene tyrosine-protein kinase Src, all of which are critical for endothelial cell proliferation, migration, and survival (Olsson et al., 2006). VEGFR 2 activity is not only regulated by the availability of VEGF-A but also by the presence of the co-receptors neuropilins (NP-1), the binding of VEGF-A to NP-1 mediates formation of complexes between it and VEGFR 2, potentiating the effects of VEGF-A. Even though both VEGFR 1 and VEGFR 2 bind to all isoforms of VEGF-A, NP-1 binds specifically to VEGF₁₆₅ via the exon-7-encoded domain of VEGF, which VEGF₁₂₁ lacks suggesting that individual VEGF isoforms serve specific functions. This observation was further supported through the use of engineered mice expressing single VEGF isoform (Shima et al., 1996, Ng et al., 2001, Saint-Geniez et al., 2009). None the less, the use of whole human serum can provide other components beside complements components. Several studies dealing with measurement of circulating VEGF in healthy



induvial serum and plasma were conducted through the past decade, showing a range of Serum-VEGF-A <500 ng/l; Plasma-VEGF-A <80 ng/l (Larsson et al., 2002), therefore the use of human serum can provide a source of false positive increase in cultures that were treated with 10% HS. All in all in this study, the limitation of the ELISA kit as it detected total VEGF-A in media regardless to the type of isoforms and the absence of background reading for the serum used can both prevent a proper analysis, therefore the role of VEGF-A secretion in media in this cell culture model require further investigation for an appropriate analysis.



Pro-angiogenic
(a isoforms)

Anti-angiogenic
(b isoforms)

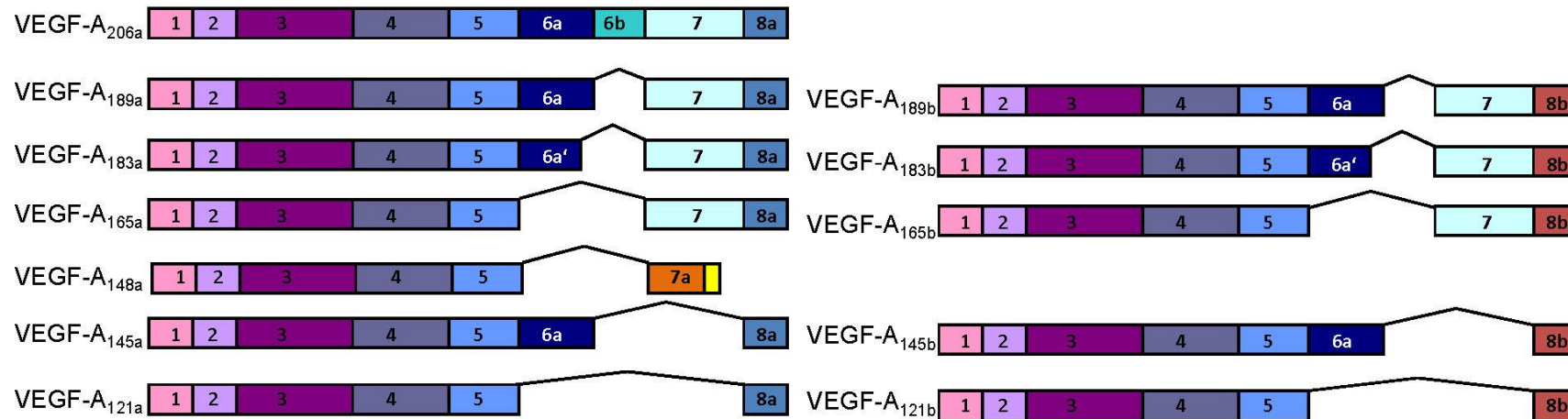


Figure 4. 12 Vascular endothelial growth factor (VEGF) exists in multiple isoforms that differ in their solubility and heparin binding characteristics

Regardless of the high VEGF-A secretion in the media, the intact TJ integrity and general monolayer health support the hypothesis presented in this chapter, that complement activation has, initially a protective rather than damaging role in terms of protecting RPE cells from potentially toxic lipid accumulation and loss of junctional integrity.

The complement system play a central role in the innate response, besides its obvious involvement in eliminating microbes, complement participates in such diverse processes as immune surveillance, homeostasis, and mediation of inflammatory responses (Ricklin et al., 2010, Kawa et al., 2014, Machalińska et al., 2012). Although acute or chronic inflammation is a common component of many clinical disorders, the underlying processes can be highly distinct. In recent years, the complement system has been associated with a growing number of immunological and inflammatory conditions that include degenerative diseases such as ARMD, cancer, and transplant rejection (Ambati et al., 2013). It becomes evident that excessive activation or insufficient control of complement activation on host cells can cause an immune imbalance that may fuel a vicious cycle between complement, inflammatory cells leading to tissue damage a process known as “para-inflammation” (Xu et al., 2009). In the case of age-related and degenerative diseases such as ARMD or AD, it is suggested that the slow steady accumulation of debris can act as a promoter for inflammation and considered an initiator of the pathology, while the suitability of the complement system is considered a key determinant in how fast a symptomatic threshold is reached “secondary response to primary initiator” (Allende-Vigo, 2010, Buschini et al.,

2011, Emanuela et al., 2012). In the case of complement component genetic polymorphisms such as those seen in small number of ARMD patients, the dysregulation of the complement system is found to contribute to the insufficient clearance of complement component as well as the dysregulation of the inflammatory responses triggered by the primary instigator (Clark et al., 2010, Fritsche et al., 2010, Gold et al., 2006, Klein et al., 2005). This chronic vicious cycle of para-inflammation eventually leads to tissue damage and vision lost.

Even though complement inhibition at various levels has shown promise in models of ARMD, the translation into a clinical application has proven challenging. (Yehoshua et al., 2014) investigated the effect of Eculizumab (Soliris, Alexion Pharmaceuticals, Cheshire, Conn.), a C5 antibody that binds to C5 and prevents downstream activation and formation of MAC. Their results involving 30 eyes of 30 patients at six months showed that systemic treatment with eculizumab did not decrease drusen volume; in addition it did not decrease the growth rate of geographic atrophy (GA), in fact their data showed a non-significant increase in GA. They argued that part of the reason is the duration of the study and the need for a higher drug dose and inadequate penetration. The data presented in this chapter the role of the complement system as a secondary protective response that might trigger para-inflammation in the long run.

The increased activity of the complement system showed no effect in reducing the volume\number of sup-RPE deposit in ARPE-19 cell culture model; in fact a significant increase was seen in both ApoE and A β 1-42

deposits under both interventions compared control samples (Serum free media). None the less a higher rate of co-localization of APOE/A β complex was seen in complement activated cultures

Therefore this work supports a model where the primary pathology is lipid loading of cells proposing potential therapeutic target. The activation of the complement in this model triggered a protective response in the ARPE cells, the cells compensate for the lipid loading in two ways:-

- In exports excess lipid in the form of lipoprotein particles that can precipitate out as deposits – drusen, which may be, at least initially, a protective mechanism.
- Excess apoE lipoprotein particles act as a chaperone for toxic Amyloid β deposits.

ApoE is a plasma lipoprotein involved in the transport and metabolism of lipids; it was also found to directly bind A β (Hyman and Terry, 1994, Weisgraber, 1994). ApoE/A β interaction is thought to play a critical role in A β deposition. Binding studies with lipoprotein-associated apoE have demonstrated that APOE isoforms; E2 and E3 (see **chapter 1 section 1.4.3.**) form more stable complexes with a higher affinity to A β which are believed to aid in its clearance, unlike APOE4 a known risk factor of ARMD(Yang et al., 1997, Martinelli et al., 2009). In addition, rat apoE, which is similar to human APOE4 in binding poorly with A β , whereas rabbit apoE have a higher affinity of binding, similar to human APOE3 that allows the formation of stable complexes with A β (LaDu et al., 1997). Other studies showed that the interactions with A β depend on the lipidation state of the apoE-containing

lipoproteins. For example, purified apoE4 binds A β with a higher affinity than apoE3 (LaDu et al., 1995, Hu et al., 2015). Together, these findings along with the results reported in this chapter suggest that apoE may contribute to ARMD pathology by its poor binding to A β and thereby reducing its uptake and clearance from the cell. However, how the complement system influences ApoE/A β complex formation and secretion remains unclear.

4.6 Conclusions

In this chapter it has been demonstrated that treatment with 10% HS exerted a 70% increase in C3 component gene expression, but this effect was not seen in cultures treated with 10% inactivated HS cultures. This protective/preventive role seen by 10% HS treatment suggests that the presence of fluid-phase complement components C3 might be important to maintain normal RPE monolayer. Therefore it is hypothesized that the complement system plays a secondary protective response role to a primary destructive initiator “lipid loading”. The complement activation suggested of the 10% HS treatment, was found to be protective against lipid accumulation and loss of barrier function in RPE cells. This treatment also led to an increase in sub-RPE lipid rich deposits.

It is interpreted that these deposits are formed from lipoprotein particles which would be expected to be cleared by passing across Bruch’s membrane to the choroidal circulation to be cleared outside the BRB. The interplay between complement system and lipid metabolism and transport remain elusive, therefore the next chapter explores the gene expression differences between cultures subjected to chronic lipid loading, chronic complement activation and both by micro-array analysis.

5

MICROARRAY STUDY OF LIPOTOXICITY MODEL SYSTEM OF ARMD PATHOLOGY

5.1. Introduction

Initial studies on retinal lipid metabolism focused on membrane assembly and turnover in photoreceptors, it is only recently has attention has moved towards lipid accumulation as the causative factor in the toxic insult to RPE and ensuing retinal dysfunction (Fliesler and Bretillon, 2010). The origin of excess FFAs in the human eye is unknown, recent work suggests that the lipoprotein rich lesions underneath the RPE are distinct from plasma lipoproteins and have been found to contain un-esterified (UC) and esterified cholesterol (EC) (Haimovici et al., 2001). The RPE cells both *in vivo* and *vitro* were shown to express the mRNA for apoB100, apoA-I, apoE, microsomal triglyceride transfer protein, and other genes involved in lipoprotein synthesis

(Mullins et al., 2000, Li et al., 2005, Wu et al., 2010). These observations have raised the possibility that the RPE assembles and secretes a novel lipoprotein particle, and that the lipoproteins which accumulate underneath the RPE may be generated locally. Lipoproteins act as transporters, transporting FFAs and cholesterol in and out of tissues. In the RPE the local generation of lipoproteins is believed to prevent the toxic accumulation of lipid.

5.2. Aim of this chapter

The previous chapter demonstrated that complement activation reduces the effects of lipid loading; this effect may be mediated by secretion of lipid as lipoprotein like particles which accumulate beneath the RPE cells in culture. However the molecular mechanisms and pathways underlying the changes observed with lipid loading and the subsequent decrease in intracellular lipid and increased lipid secretion observed with complement treatment are unclear. In order to uncover which pathways may be involved whole transcriptome microarray analysis was carried out to assess global changes in gene expression in the ARPE-19 cells.

5.3. Experiment design and methods

5.3.1. Sample collection and preparation

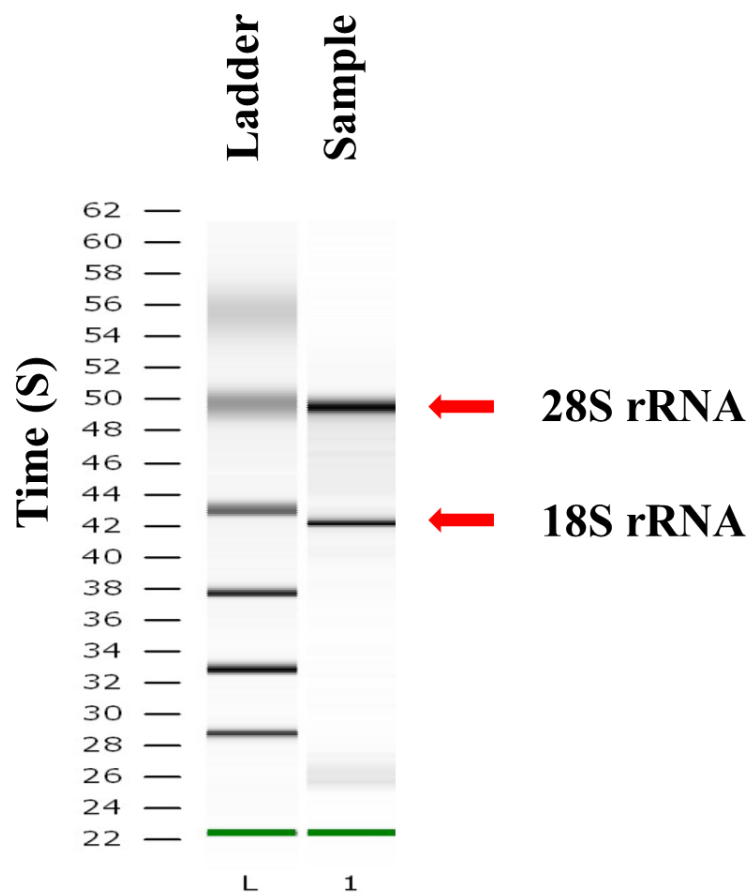
ARPE-19 cell cultures were grown as described in Chapter 4, and after 30 days in serum free medium they were exposed to one of the following interventions; serum free media (control) , 10% human serum (HS) (vol/vol) , 0.2mM PLO and 0.2mM PLO + 10% HS , for 60 days changing media every 2-3 days. Samples were collected from 3 independent experiments (total of n=3

for each condition). Total RNA was extracted and cDNA synthesis was performed as described in **Chapter 2**.

5.3.2. RNA quality assessment prior to microarray analysis

Microarray analysis requires mRNA to be of good quality. The RNA quality was assessed using an Agilent Bioanalyzer **Figure 5.1A, B**. The bioanalyzer software generates a RNA integrity number (RIN). This number ranges from 1-10, with 10 representing the maximum integrity value. Using the highest RIN scores, three samples of each group collected were chosen for microarray analysis. Samples with a RIN score below 7.5 were excluded from the experiment.

A)



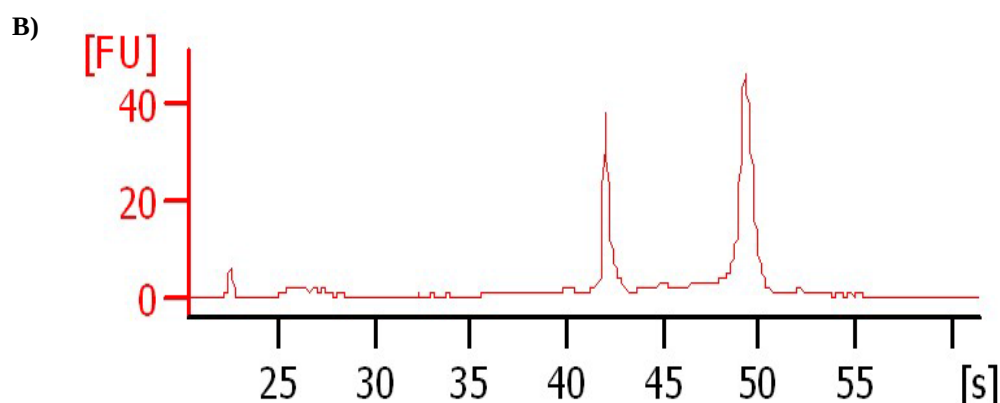


Figure 5. 1 RNA quality assessment. RNA quality assessment using Agilent Pico chip and Bioanalyzer (A) Gel-like image showing ribosomal RNA bands showing, 28S and 18S. (B) Representative electropherogram plotting fluorescence unit (FU) against time (s).

5.3.3. Pre-processing and normalization of microarray data

The RNA labelling and hybridization steps were performed at the Nottingham Arabidopsis Stock Centre (NASC). Each sample was hybridised to an Affymetrix Human Transcriptome Array 2.0 GeneChip® then scanned. This high-resolution array design contains >6.0 million distinct probes covering coding and non-coding transcripts. 70% of the probes on this array cover exons for coding transcripts, and the remaining 30% of probes on the array cover splice variants and non-coding transcripts (285,000 full-length transcripts covered, >245,000 coding transcripts, >40,000 non-coding transcripts, >339,000 probe sets covering exon-exon junctions). CEL raw data files were imported into GeneSpring® GX 13 software. Standard data processing was performed including summarization, log transformation and baseline transformation, which are detailed below.

5.3.3.1. Background correction and Normalization

Background correction was carried out to estimate the background portion of the probe signals and subtract it accordingly to avoid bias estimation. In this work the Robust Multichip Analysis (RMA) algorithm was applied to process the probes signal intensity (Irizarry et al., 2003). The baseline to median was chosen, in which for each probe, the median value of the log summarized signal intensity value from all the samples was calculated and subtracted from each of the samples.

5.3.3.2. Perform Quality Control on Probe sets

Due to the nature of the experiment (chronic cultures of 90 days), variation between replicates is seen due to different media and reagent batches as well as different passage number of ARPE-19 cells. To overcome this issue and to minimize variability raised from biological replication, the coefficient of variation (CV), which is the standard deviation divided by the mean, was calculated between replicas of the same treatment (Tseng et al., 2001, Sterrenburg et al., 2002, Bland and Altman, 1996b, Bland and Altman, 1996a)

5.4. Results

5.4.1 Principal Component Analysis (PCA) and Hierarchical Clustering.

PCA of the samples showed clear separation between PLO treated cultures and other interventions, **Figure 5.2**. The PLO treatment also had the highest variation between replicates, whereas the three samples in the other three interventions (serum free media, 10% human serum and 0.2mM PLO+ 10% human serum) clustered together. PCA analysis of variation between groups (each group represent an independent experiment of all interventions + a control) shows a degree of a higher variation between replicas in some conditions. This variation between replicas in each intervention will affect the general statistical analysis. To reduce this variation, samples were filtered by CV and the PCA was reassessed on the new condition. Filtering by CV revealed a clear separation between interventions **Figure 5.3**.

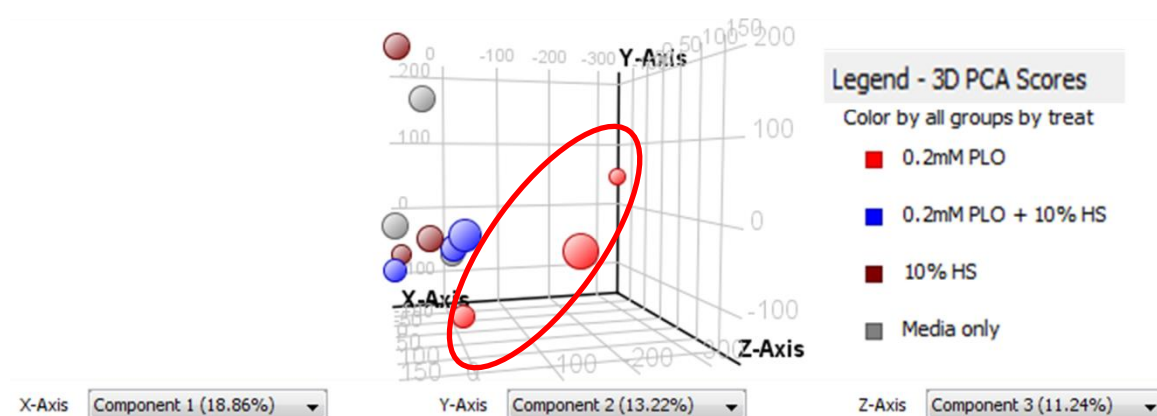


Figure 5. 2 PCA analysis of ARPE-19 culture samples from three biological samples for each intervention. The results are depicted 3-dimensionally with PC1 (18.86%), PC2 (11.24%) and PC3 (13.22%) as the X, Y and Z axes, respectively. Each colour represents each condition.

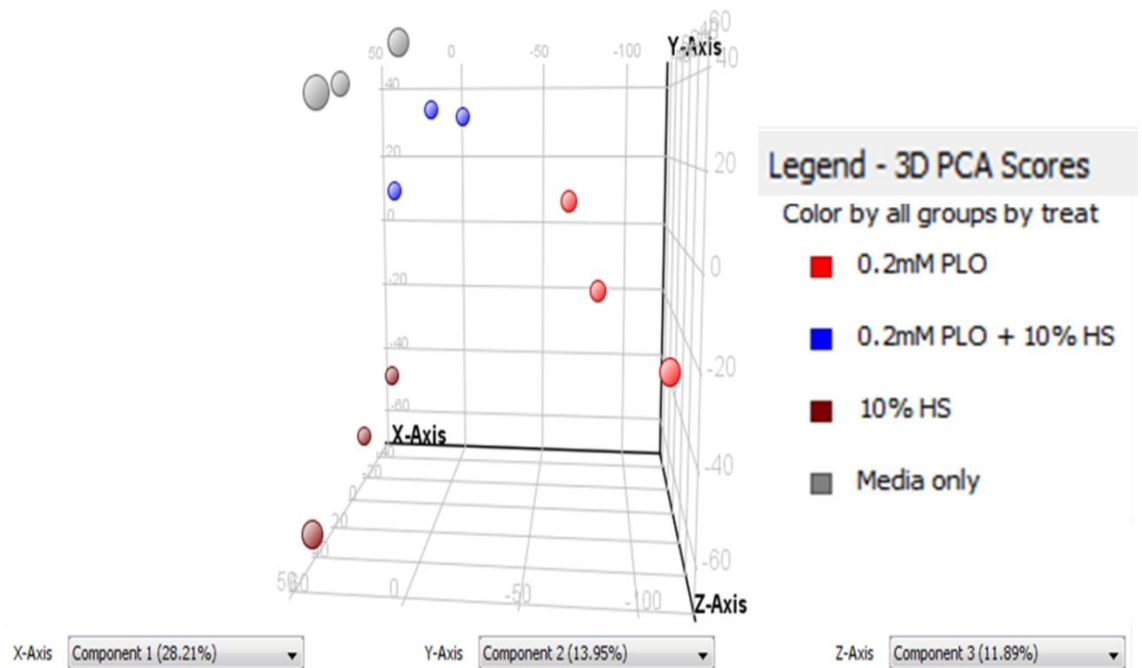


Figure 5. 3 PCA analysis of filtered CV <50%. To reduce the variation between replicates of each condition samples were filtered by their coefficient of variation. The PCA for the samples was then reassessed. The results are depicted 3-dimensionally with PC1 (28.21%), PC2 (13.95%) and PC3 (11.89%) as the X, Y and Z axes, respectively. Each colour represents different treatment.

Two-way hierarchal clustering was performed using Pearson correlation with median centring. Hierarchical clustering indicated that gene expression in the PLO treatment group is clearly separated from the other interventions **Figure 5.4**. The final analysis was performed on the CV filtered list between three biological replicates in each condition.

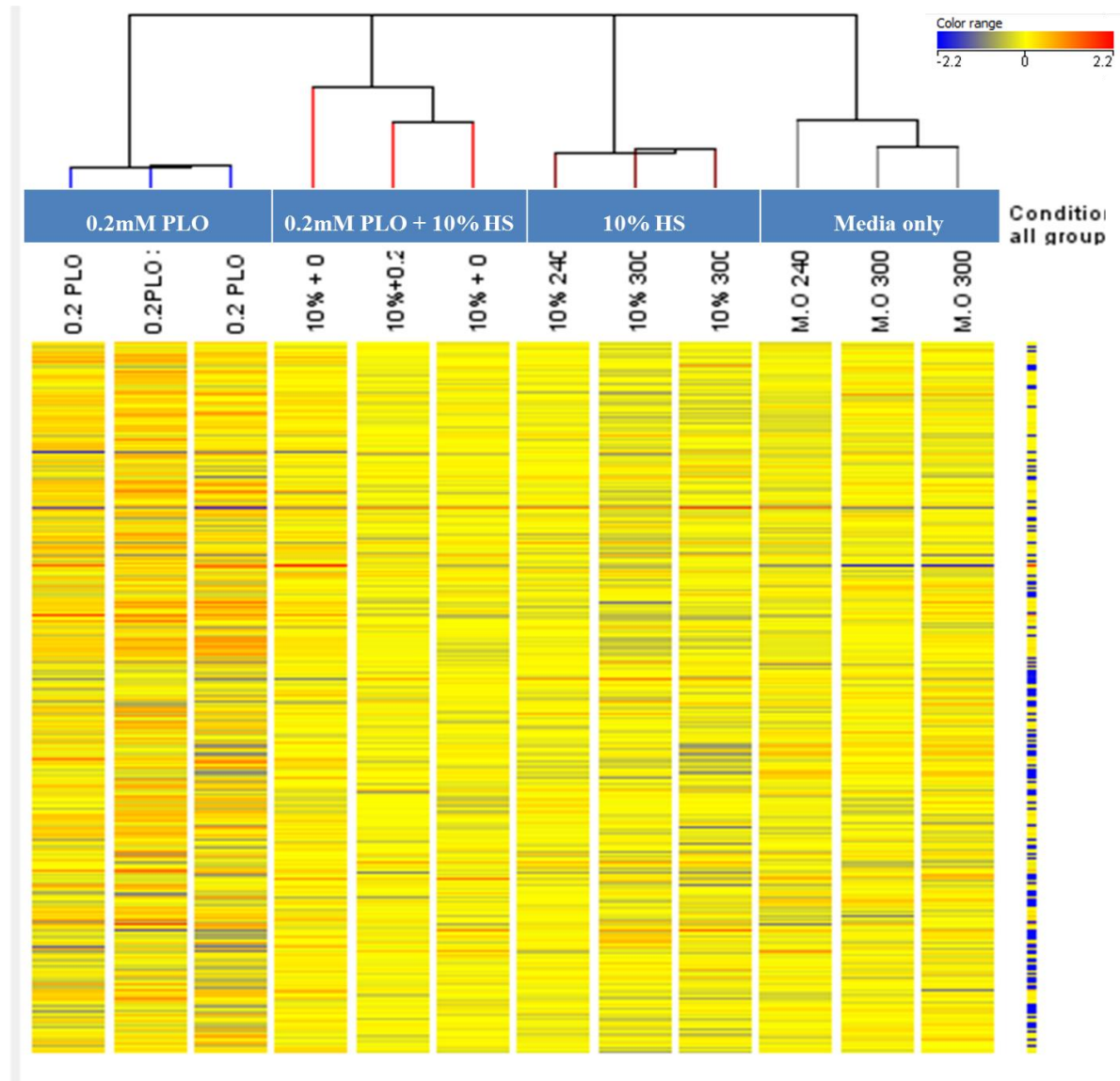


Figure 5.4 Dendrogram of hierarchical clustering from 3 biological samples. Replicates were filtered by >50% CV and a hierarchical dendrogram was drawn using Genespring. The expression patterns for all entities (genes) cross all conditions are shown in colour. The red colour represents high expression and the blue colour low expression, within the -2.2~ 2.2 ranges. A clear separation is seen in PLO treated group based on their gene expression.

5.4.2. The effect of interventions on gene expression

The 10436 entities (genes) out of the initial 67539 remaining following the quality filtering were eligible for statistical analysis. The hybridisation data were analysed by one-way ANOVA, generating a list of 3165 genes with P

<0.05. Significantly altered genes were defined as those with absolute fold change FC > 1.5. The analysis returned a list of 303 differentially expressed genes.

The PLO treated cultures returned the higher list of differently regulated genes (196 entities) compared to control (serum free media), with 102 genes up-regulated (light blue) and 94 genes down regulated (light red) **Table 5.1** summarized the 20 top genes with the highest FC up- and down regulated.

Table 5. 1 Top 20 up and down-regulated genes. Microarray analysis revealed which genes were significantly differentially expressed in PLO treated cultures compared to control (serum free media). Genes listed in fold change (FC) order, light blue signifies genes with up-regulation ≥ 1.5 while light red signifies genes with ≥ 1.5 down-regulation

Top 20 genes up-regulated in PLO treated cultures

Gene symbol	Gene name	Regulation	Fold change
POSTN	Periostin, osteoblast specific factor	up	3.4
PLIN2 LOC100509484	Perilipin 2	up	2.6
SLC7A11	Solute carrier family 7 , member 11	up	2.3
INHBA	Inhibin, beta A	up	2.3
PSAT1	Phosphoserine aminotransferase 1	up	2.2
JAG1	Jagged 1	up	2.2
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	up	2.0
MIR222	MicroRNA 222	up	2.0
RAB27B	RAB27B, member RAS oncogene family	up	2.0
ANGPTL4	Angiopoietin-like 4	up	1.9
SLC39A10	Solute carrier family 39 , member 10	up	1.8
PRSS12	Protease, serine, 12	up	1.8
SGCD	Sarcoglycan, delta	up	1.8
CPT1A	Carnitine palmitoyltransferase 1A	up	1.8
RUNX1-IT1	RUNX1 intronic transcript 1	up	1.7

E2F7	E2F transcription factor 7	up	1.7
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	up	1.7
MIR221	MicroRNA 221	up	1.7
PRUNE2	Prune homolog 2	up	1.7
MIR4737	MicroRNA 4737	up	1.6

Top 20 down regulated genes

CA9	carbonic anhydrase IX	down	-2.9
MT1E LOC100505584	metallothionein 1E	down	-2.8
	submaxillary gland androgen regulated protein		
SMR3B	3B	down	-2.5
MT1F	metallothionein 1F	down	-2.4
MT1X	metallothionein 1X	down	-2.4
PSG1	pregnancy specific beta-1-glycoprotein 1	down	-2.3
MGP	Matrix gla protein	down	-2.3
MT1JP	metallothionein 1J, pseudogene	down	-2.2
GXYLT2	glucoside xylosyltransferase 2	down	-2.0
MT2A	metallothionein 2A	down	-1.9
CP	ceruloplasmin	down	-1.9
MT1L	metallothionein 1L	down	-1.8
MT1CP	metallothionein 1C, pseudogene	down	-1.8
	complement component 1, q subcomponent-like		
C1QL1	1	down	-1.8
PTGIS	prostaglandin I2 synthase	down	-1.8
MATN2 LOC10050655			
8	Matrilin-2	down	-1.7
MT1H	metallothionein 1H	down	-1.7
SLC4A4	solute carrier family 4	down	-1.7
CFH	Complement factor H	down	-1.6
	cytochrome P450, family 24, subfamily A,		
CYP24A1	polypeptide 1	down	-1.6

The Effect of complement activation in the presence of and absence of lipid loading showed fewer changes in gene expression between these interventions and control to those seen in PLO intervention to control. As 39

genes were up-regulated and 35 were down-regulated in the 10% HS treated cultures **Table 5.2**. While cultures that had their complement activated in the presence of lipid loading, showed 24 genes being up-regulated and only 13 genes down-regulated in comparison to control cultures **Table 5.3**.

Table 5. 2 Top 20 up and down-regulated genes. Microarray analysis revealed which genes were significantly differentially expressed in 10% HS treated cultures compared to control (serum free media). Genes listed in fold change (FC) order, light blue signifies genes with up-regulation ≥ 1.5 while light red signifies genes with ≥ 1.5 down-regulation

Top 20 genes up regulated in 10% HS treated cultures

Gene symbol	Gene name	Regulation	Fold change
DHRS3	Dehydrogenase/reductase member 3	up	2.9
CYP24A1	Cytochrome P450, family 24, subfamily A, polypeptide 1	up	2.3
SFRP4	Secreted frizzled-related protein 4	up	2.2
FAT3	FAT atypical cadherin 3	up	2.2
TGM2	Transglutaminase 2	up	2.1
LXN	Latexin	up	2.1
SLC16A12	Solute carrier family 16, member 12	up	2.1
POSTN	Periostin, osteoblast specific factor	up	2.0
MT1X	Metallothionein 1X	up	2.0
AKAP12	A kinase (PRKA) anchor protein 12	up	1.9
SEMA7A	Semaphorin 7A, GPI membrane anchor	up	1.8
ITGB3	Integrin, beta 3	up	1.8
CP	Ceruloplasmin (ferroxidase)	up	1.8
ANGPTL4	Angiopoietin-like 4	up	1.8
PTPRJ LOC100287223	Protein tyrosine phosphatase, receptor type, J	up	1.8
ARHGAP29	Rho GTPase activating protein 29	up	1.8
ARRDC3	arrestin domain containing 3	up	1.7
IL7R	interleukin 7 receptor	up	1.7
LDLR	Low density lipoprotein receptor	up	1.7

Top 20 genes down-regulated in 10% HS treated cultures

TYR	Tyrosinase	down	-3.6
FST	Follistatin	down	-3.5
DSC1	Desmocollin 1	down	-2.9
ABI3BP	ABI family, member 3 binding protein	down	-2.4
TYRP1	Tyrosinase-related protein 1	down	-2.3
MFAP4	Microfibrillar-associated protein 4	down	-2.1
SLC7A11	Solute carrier family 7, member 11	down	-2.0
CTSK	Cathepsin K	down	-2.0
AMTN	Amelotin	down	-1.9
HAPLN3	Hyaluronan and proteoglycan link protein 3	down	-1.9
MFGE8	Milk fat globule-EGF factor 8 protein	down	-1.8
LUZP2	Leucine zipper protein 2	down	-1.8
FAM102A	Family with sequence similarity 102, member A	down	-1.7
GXYLT2	Glucoside xylosyltransferase 2	down	-1.7
PSAT1	Phosphoserine aminotransferase 1	down	-1.7
SYT14	Synaptotagmin XIV	down	-1.6
SERPINF1	Serpin peptidase inhibitor, clade F ,member 1	down	-1.6
ABCA1	ATP-binding cassette, sub-family A , member 1	down	-1.6
SMR3B	Sbmaxillary gland androgen regulated protein 3B	down	-1.6
IFI44L	Interferon-induced protein 44-like	down	-1.6

Table 5. 3 Top 20 up and down-regulated genes. Microarray analysis revealed which genes were significantly differentially expressed in PLO + 10% HS treated cultures compared to control (serum free media). Genes listed in fold change (FC) order, light blue signifies genes with up-regulation ≥ 1.5 while light red signifies genes with ≥ 1.5 down-regulation

Top 20 genes up-regulated in PLO + 10% HS treated cultures

Gene symbol	Gene name	Regulation	Fold change
POSTN	Periostin, osteoblast specific factor	up	2.9
PLIN2 LOC100509484	Perilipin 2	up	2.5
ANGPTL4	Angiopoietin-like 4	up	2.2
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	up	2.0
DHRS3	Dehydrogenase/reductase 3	up	1.9

DDIT4	DNA-damage-inducible transcript 4	up	1.7
ITGB3	Integrin, beta 3	up	1.7
IL7R	Interleukin 7 receptor	up	1.6
C3	Complement component 3	up	1.6
PSAT1	Phosphoserine aminotransferase 1	up	1.6
TGM2	Transglutaminase 2	up	1.6
	Solute carrier family 27 (fatty acid transporter),		
SLC27A2	member 2	up	1.6
AOX1	Aldehyde oxidase 1	up	1.5
TFPI2	Tissue factor pathway inhibitor 2	up	1.5
PTCHD4	Patched domain containing 4	up	1.5
ARRDC3	Arrestin domain containing 3	up	1.5
PLCB4	Phospholipase C, beta 4	up	1.5
LUCAT1	lung cancer associated transcript 1	up	1.5
INHBA	Inhibin, beta A	up	1.5
	ChaC glutathione-specific gamma-		
CHAC1	glutamylcyclotransferase 1	up	1.5
CFH	Complement factor H	up	1.5

Top 20 genes Down-regulated in PLO + 10% HS treated cultures

TYR	Tyrosinase	down	-2.7
	Submaxillary gland androgen regulated protein		
SMR3B	3B	down	-2.5
	Aldehyde dehydrogenase 1 family, member		
ALDH1A3	A3	down	-1.9
DSC1	Desmocollin 1	down	-1.9
FST	Follistatin	down	-1.8
MFAP4	Microfibrillar-associated protein 4	down	-1.7
	Submaxillary gland androgen regulated protein		
SMR3A	3A	down	-1.7
TYRP1	Tyrosinase-related protein 1	down	-1.7
GXYLT2	Glucoside xylosyltransferase 2	down	-1.6
SERPINF1	Serpin peptidase inhibitor, clade F , member 1	down	-1.5
CTSK	Cathepsin K	down	-1.5
CCNA2	Cyclin A2	down	-1.5
SLC30A1	Solute carrier family 30 , member 1	down	-1.5

5.4.3. The effect of long term lipid loading on ARPE-19 cell culture

Analysis of the 0.2mM PLO treated intervention showed higher number of genes exhibiting changes in expression as an outcome of lipid loading, compared to cultures that had 10% human serum intervention (to achieve complement activation), showed a more stable related gene expression in the presence and absence of lipid loading, as fewer changes in gene expression were observed in comparison to control. Thus, lipid toxicity appears to have a greater impact on the transcriptomics of the ARPE-19 cell cultures.

To identify significant biological processes perturbed in response to lipid loading, we used Ingenuity Pathway Analysis® (IPA) software to examine our data in the context of existing knowledge of genome wide interactions. The microarray data set containing the 196 entities probe sets showing greater than 1.5 fold differential expression between genes from lipid loaded cultures and controls culture (serum free media) were uploaded into IPA, in which an interaction networks using the Ingenuity Knowledge Base (IPKB) was built. This identified whether there were any direct or indirect links between genes in each list to a single pathway. It also identified which canonical and cellular functions were most affected in each gene list. The algorithm used to create the networks ensures that as many genes from the dataset are included as is possible. Some genes in each list were classified as ‘unmapped’ and were therefore excluded from the analysis. The IPA analysis was conducted on the 196 differently regulated genes between lipid loaded cultures and controls to identify the most significant pathways, from the IPA library of canonical pathways, using the input data set. The significance of the

association between the data set and the canonical pathway was determined based on two parameters:

1. A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway
2. P value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

Top canonical pathways identified by IPA are summarized in **Table 5.4**, were found to include; p53 Signalling, Super-pathway of Cholesterol Biosynthesis, Aryl Hydrocarbon Receptor Signalling, along with Glycolysis I and Gluconeogenesis I. IPA also identified top tox list **Table 5.5**, tox Lists are gene sets that help you understand mechanistic response of xenobiotic insult. They are based on critical biological processes and key toxicological responses such as adaptive, defensive, or reparative responses to xenobiotic insult and can be used to understand the effect of lipid loading and 10% HS treatments on ARPE-19 cell cultures. The most significantly affected IPA-Tox pathways were also shown to be involved with p53 signalling and Aryl Hydrocarbon Receptor Signalling.

Table 5. 4 Top Canonical pathways identified by IPA. The IPA analysis of the 196 differently regulated genes identified a list of top conical pathways in lipid loaded cultures verses control cultures.

Top canonical pathway	P-value
p53 Signalling	6.88E-07
Super pathway of Cholesterol Biosynthesis	6.86E-06
Aryl Hydrocarbon Receptor Signalling	1.29E-05
Glycolysis I	1.78E-05
Gluconeogenesis I	2.61E-05

Table 5. 5 Top Tox-pathways identified by IPA. The IPA analysis of the 196 differently regulated genes identified a list of IPA-Tox pathways in lipid loaded cultures verses control cultures.

Top Tox list	P-value
p53 Signalling	2.00E-07
Liver Proliferation	7.45E-06
Aryl Hydrocarbon Receptor Signalling	1.71E-05
Cardiac Hypertrophy	8.58E-05
Hepatic Fibrosis	1.19E-04

The IPA analysis also recognized top differently regulated (up and down) between lipid loaded cultures and those cultured in serum free media (control), Table 5.6 summarized this list with light blue signifying up-regulated genes and light red signifying down-regulated ones. Genes associated with lipid concentration and transport such as perilipin 2 (PLIN2), pyruvate dehydrogenase kinase 4 (PDK4) and ATP-binding cassette A1 (ABCA1) were

shown to be as expected due to lipid loading effect, one of the highest up-regulated genes , while several of Metallothionein (MTs) family members were shown to be down-regulated in lipid loaded cultures. Other genes related to the complement cascade such as complement factor H (CFH) and low density lipoprotein receptor (LDLR) were also shown to be down regulated in lipid loaded cultures.

Molecules	Exp. Value	Function
POSTN	3.751	Functions in tissue development and regeneration I.e. wound healing
PLIN2	2.702	Associated with the lipid globule surface membrane material, and maybe involved in development and maintenance of adipose tissue
INHBA	2.236	A FSH secretion inhibitor and regulator
PDK4	2.196	Regulation of glucose metabolism
JAG1	2.192	Play a role in hematopoiesis as well as a mutation of this gene was shown to cause Alagille syndrome
PRSS12	2.058	Studies indicates it might be involved in structural reorganizations associated with learning and memory
ABCA1	1.899	This protein transport various molecules across extra- and intracellular membranes
CPT1A	1.788	Key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane and its deficiency results in a decreased rate of fatty acid beta-oxidation
MT1E	-3.312	This protein functions in primary metal storage, transport and detoxification

MT1F	-3.116	This protein functions in primary metal storage, transport and detoxification
CA9	-2.912	Catalyse the reversible hydration of carbon dioxide that participates in a variety of biological processes.
MT1JP	-2.912	This protein functions in primary metal storage, transport and detoxification
MT1H	-2.234	This protein functions in primary metal storage, transport and detoxification
CFH	-2.204	Regulator of alternative pathway of complement activation
MT1L	-2.194	This protein functions in primary metal storage, transport and detoxification
MT1A	-2.125	This protein functions in primary metal storage, transport and detoxification
LDLR	-1.956	cell surface proteins involved in receptor-mediated endocytosis of specific ligands
MT1G	-1.873	This protein functions in primary metal storage, transport and detoxification

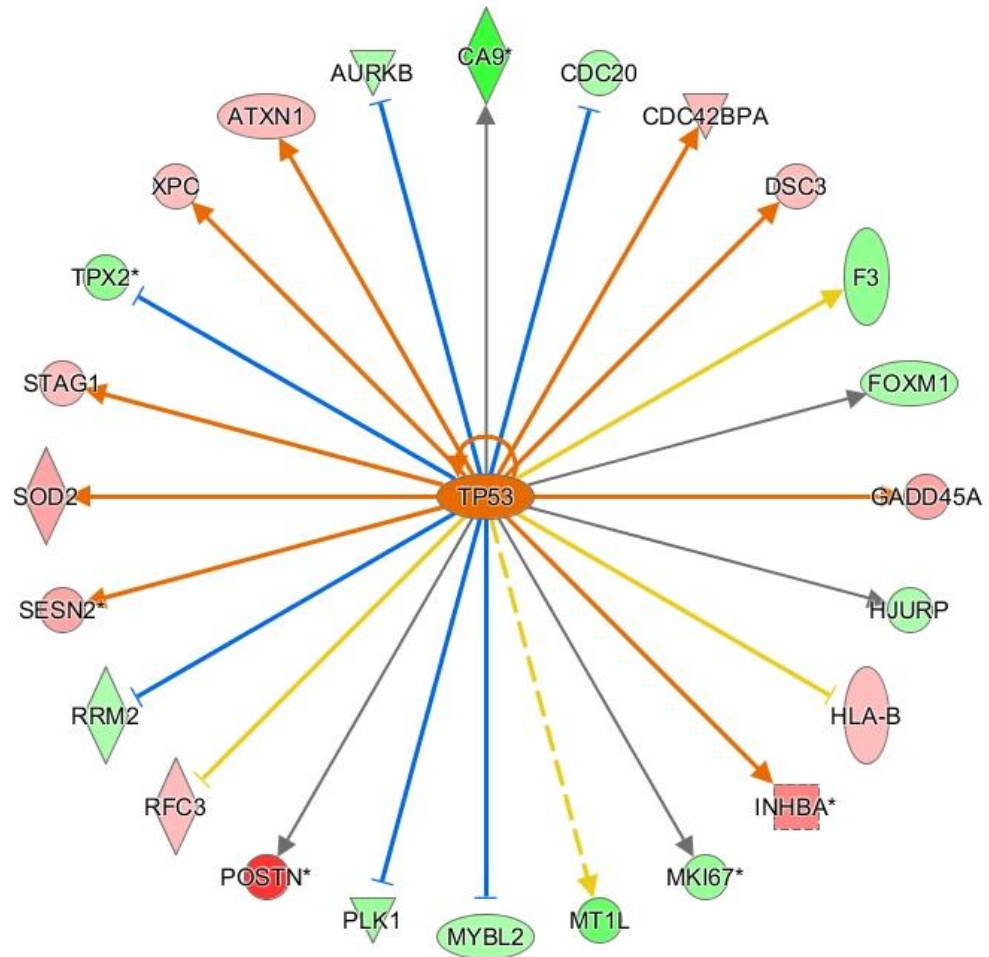
5.4.3.1. Upstream regulators

IPA analysis was used for further analysis of the upstream regulators in lipid loaded cultures. Those upstream regulators included transcription factors that are involved in cell cycle regulations, and induction of apoptosis such as Tumor Protein 53 and 73 (TP53) and (TP 73) **Figure 5.5A** and **B**, while other upstream regulators were found to be associated with ligand-dependent nuclear receptors such as Glucocorticoid receptor (NR3C1) and Peroxisome

Proliferator-Activated Receptor Alpha (PPAR α) both known to modulate several cellular responses such as inflammation, cellular proliferation, glucose and lipid metabolisms **Figure 5.5 C and D**, respectively. Some upstream regulators indicate the presence of inflammatory responses such as tumor necrosis factor (TNF) and interferon gamma (IFNG) and Nuclear Factor Kappa-B complex (NF- κ B complex) while other upstream regulators were found to be associated with other functions such as amyloid beta (A β) precursor protein (APP); this protein is cleaved by secretases to form a number of peptides that include amyloid beta (A β) **Figure 5.5E**, previous work done in **Chapter 3** and **Chapter 4** revealed the presence of positive immune-reactivity for A β 1-42 protein in sub-RPE deposits in lipid loaded cultures and not control.

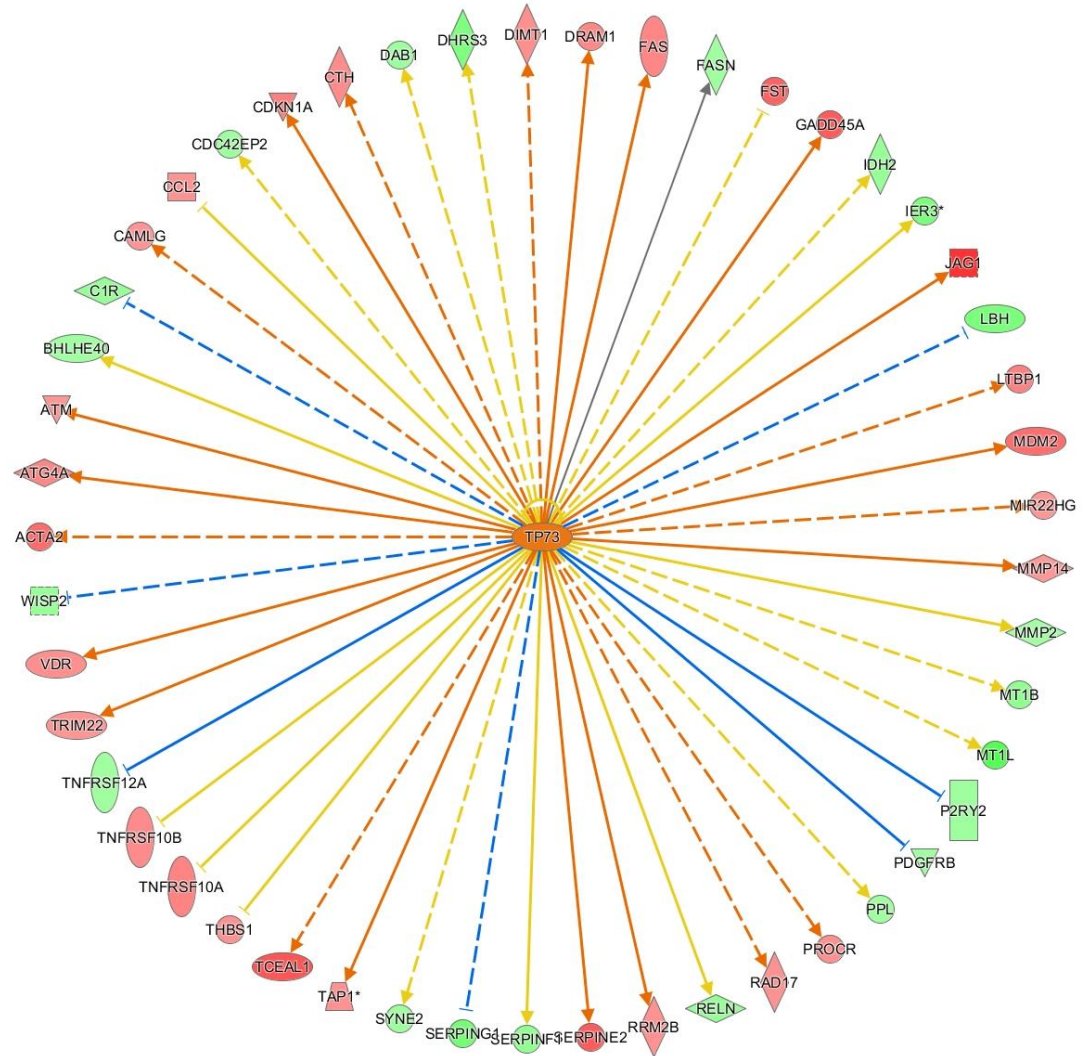
A) T P53

TP53 4

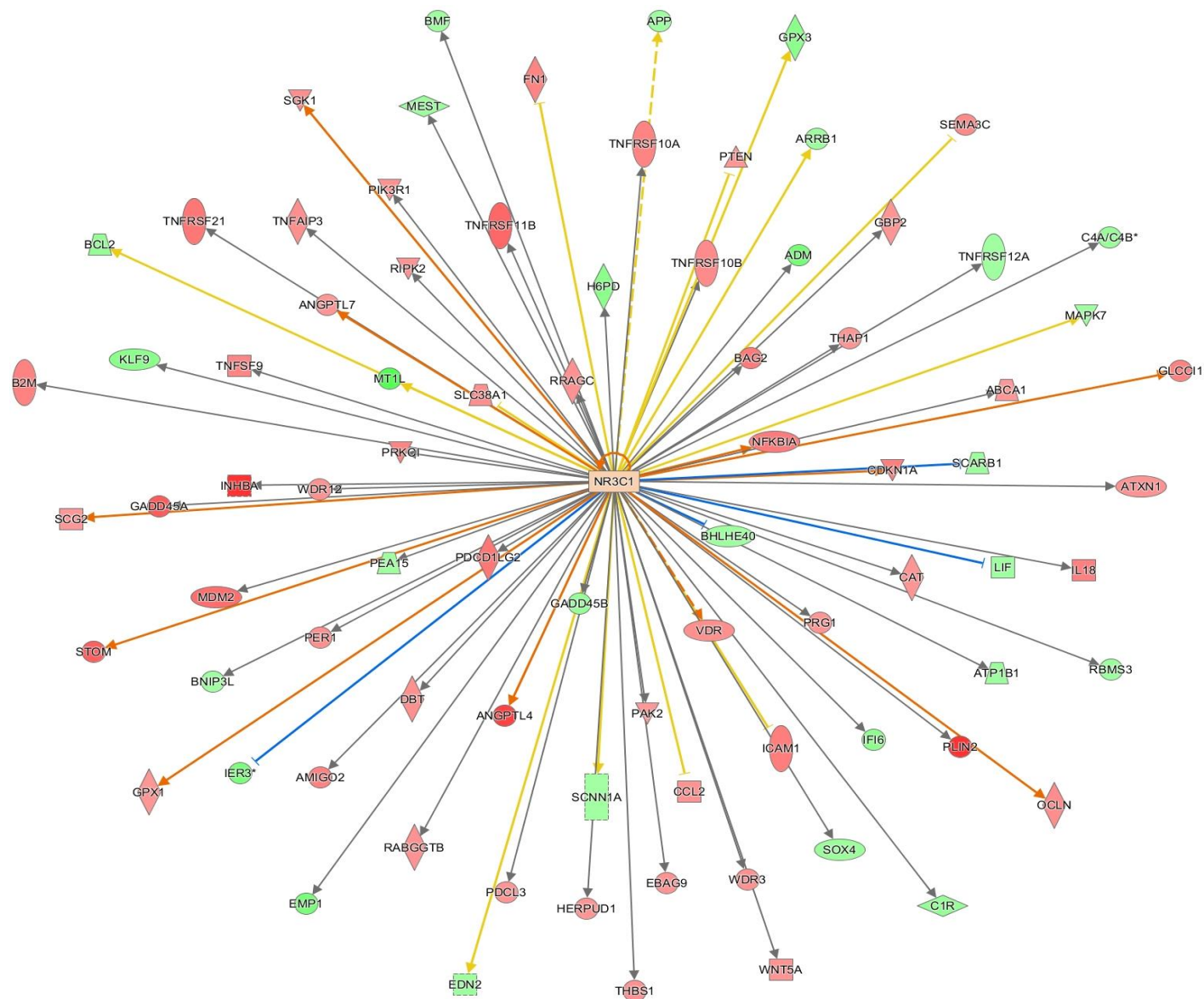


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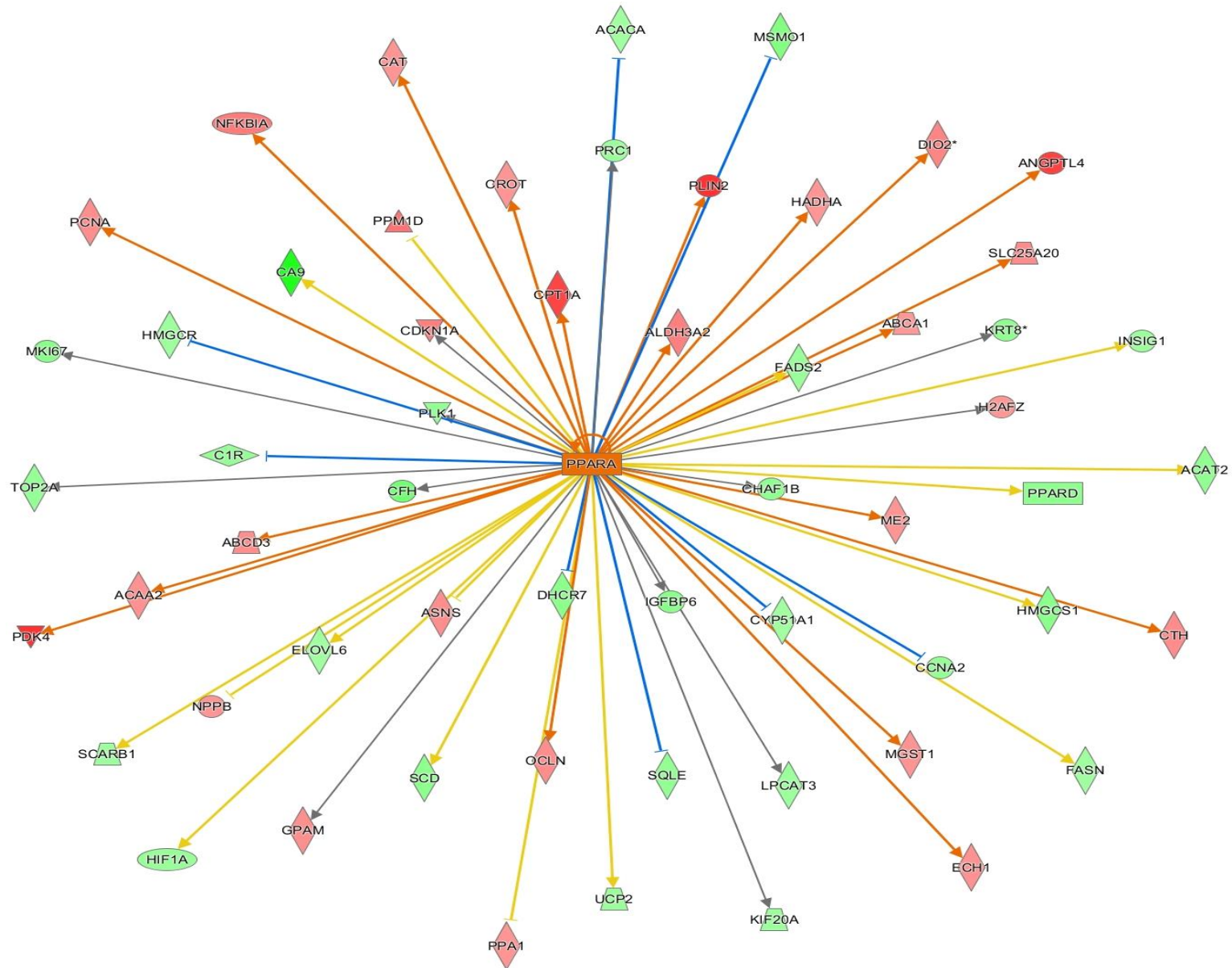
B) TP73



C) NR3C1



D) PPAR α



E) APP

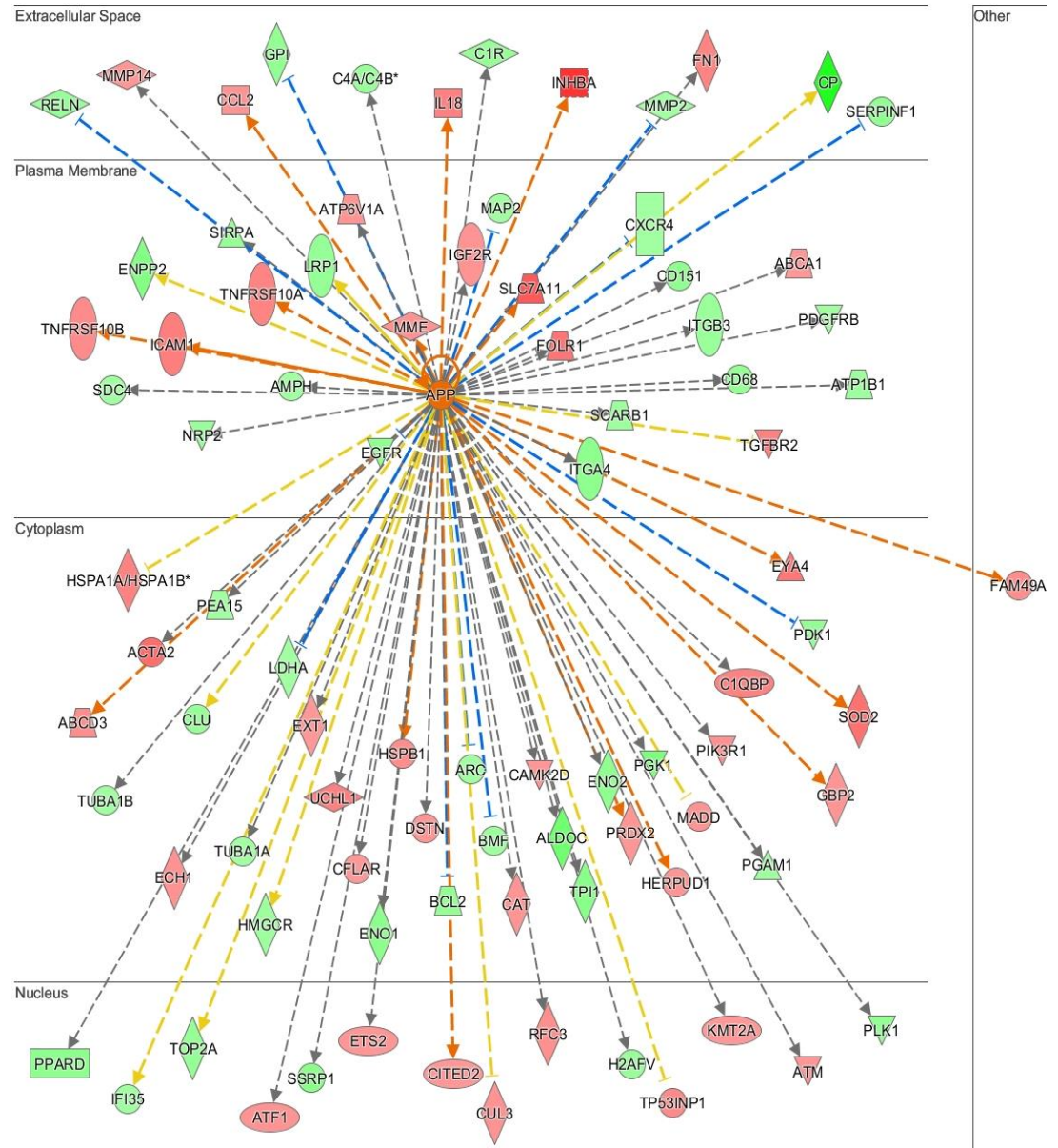
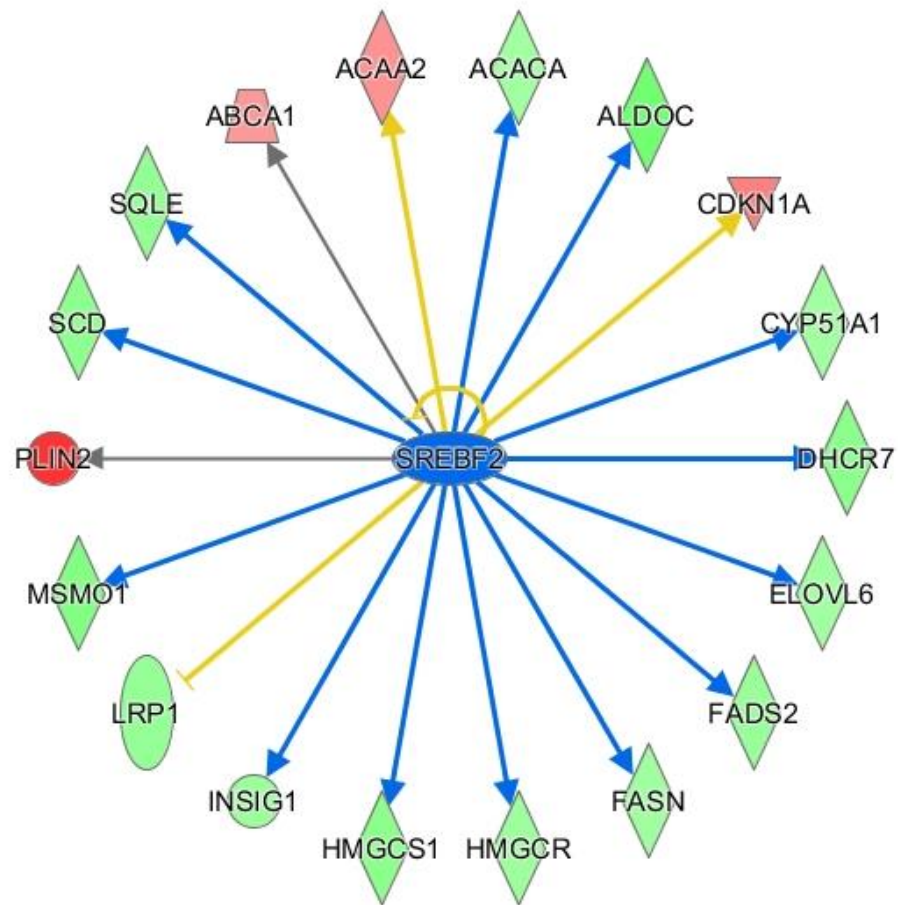


Figure 5. 5 IPA graphical representation of the molecular relationships between differentially expressed proteins in lipid loaded cultures versus control (Serum free media). (A-B) represent upstream regulators of transcription factors, (C-D) represent upstream regulators involved in ligand-dependent nuclear receptors modulation and (E) represent upstream regulators of amyloid beta (A4) precursor protein (APP). The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins

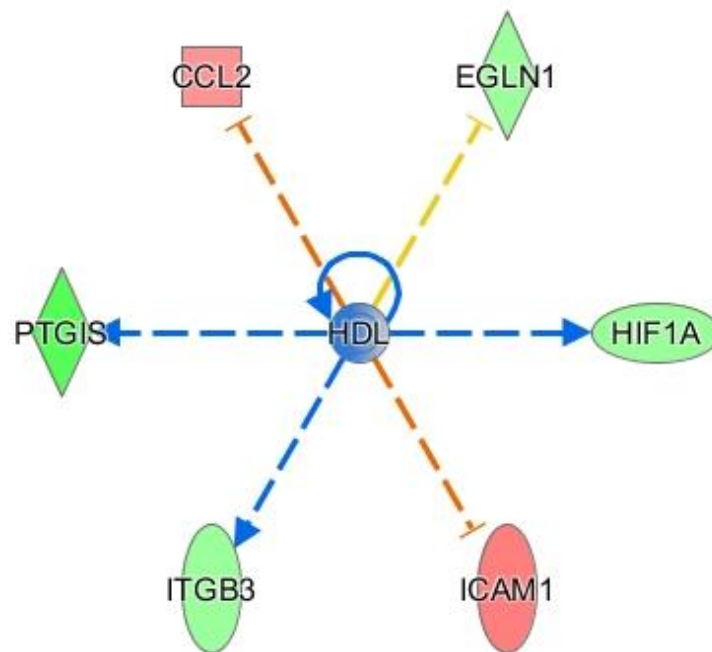
5.4.3.2. Downstream regulators

IPA analysis identified several downstream regulators genes in lipid loaded cultures; those downstream regulators were found to include transcriptional regulators known to be associated in cholesterol homeostasis such Sterol regulatory element-binding protein 2 (SREBF2) **Figure 5.6A** and High Density Lipoprotein (HDL), along with transcriptional regulators such as aryl hydrocarbon receptor nuclear translocator (ARNT) **Figure 5.6B** and the ligand-dependent nuclear receptors PPAR δ all of which are involved in cellular regulation and metabolism. Other downstream regulators were found to be associated with growth factors and cell-cell adhesions such as Angiotensinogen (AGT) **Figure 5.6C**, Transforming Growth Factor, Beta 1 (TGFB1) and hepatocyte growth factor (HGF)

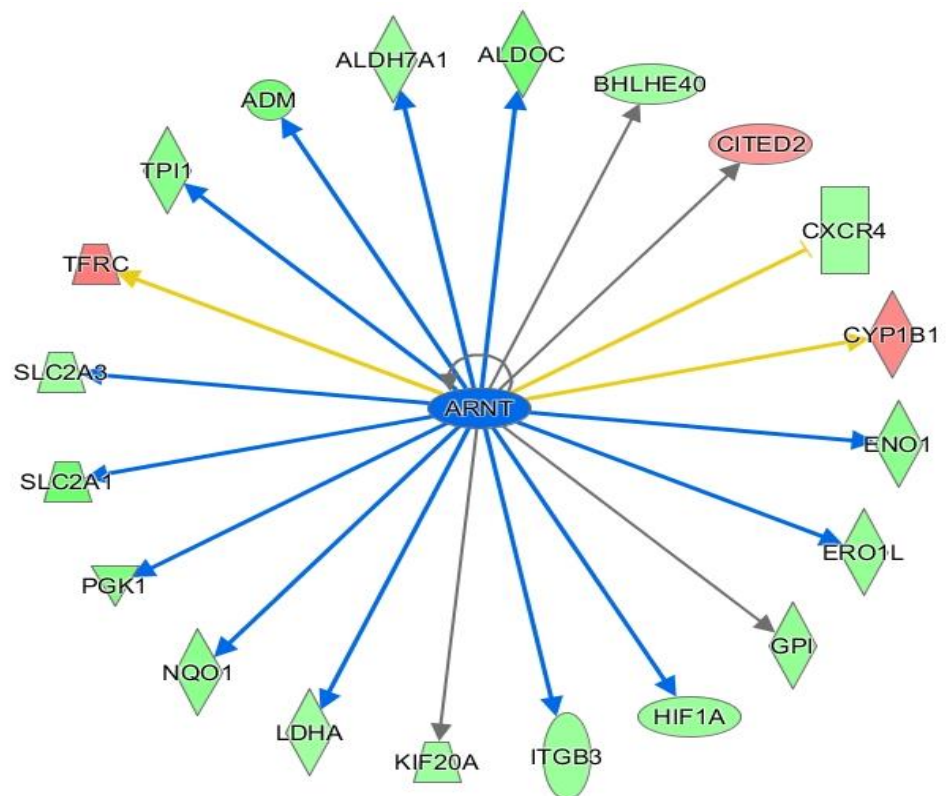
A) SREBF2



B) HDL



C) ARNT



C) AGT

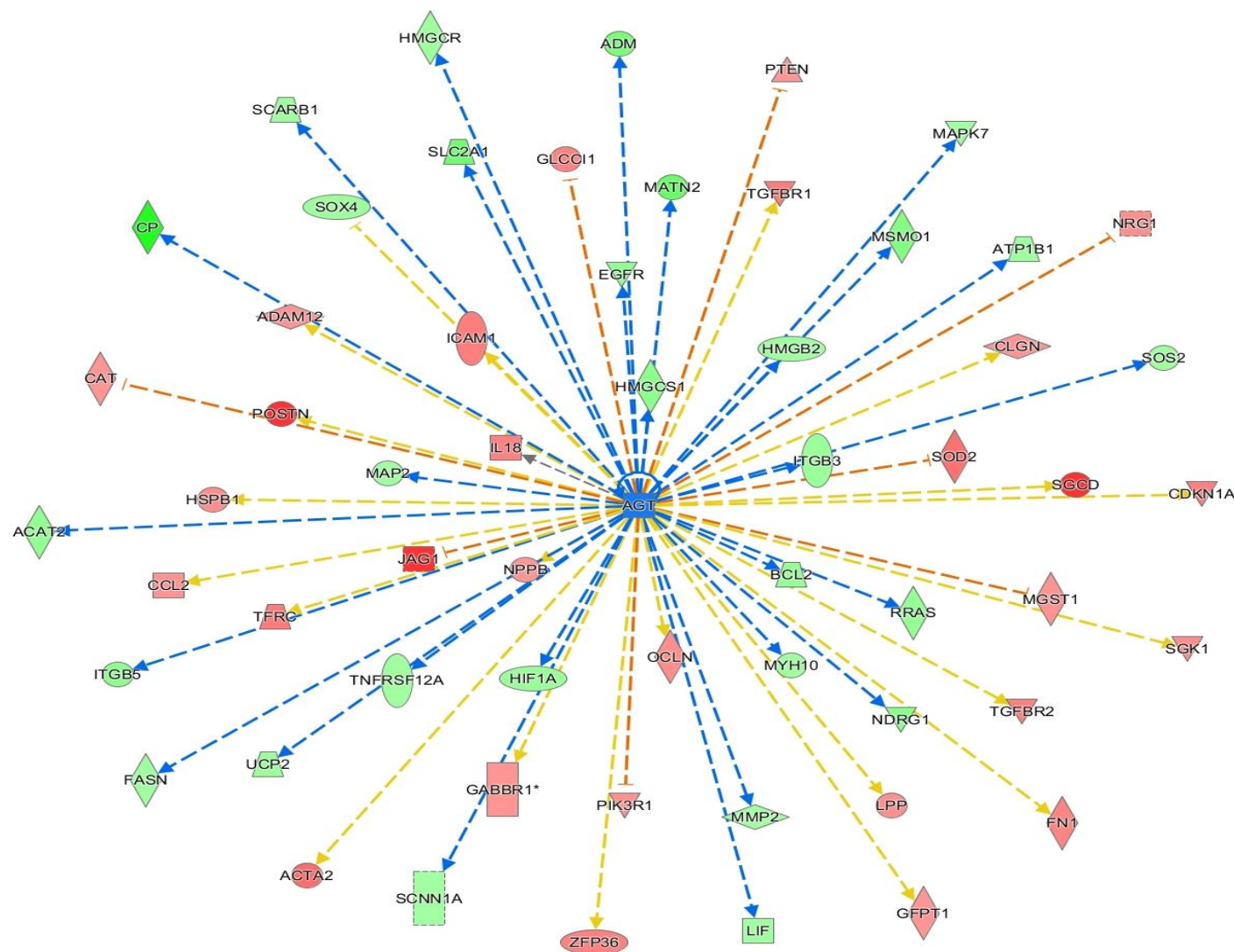


Figure 5. 6 IPA graphical representation of the molecular relationships between differentially expressed proteins in lipid loaded cultures versus control (Serum free media).(A) represent downstream regulators of cholesterol homeostasis, (B) represent regulators involved in ligand-dependent nuclear receptors modulation and (C) represent associated with growth factors and cell-cell adhesions. The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins

5.4.4. The effect of complement activation in the presence of lipid loading

Complement activation in lipid loaded cultures had a modulating effect in lipid accumulation, cultures that were treated with 10%HS showed less intracellular lipid accumulation and intact monolayer integrity. To understand the effect activating the complement cascade in lipid loaded cultures, a further analysis of the data was performed using Gene Spring and Ingenuity Pathway Analysis (IPA) to examine the differences between cultures that had their complement activated during lipid loading and those that did not. A volcano analysis was carried between PLO treated cultures and PLO + 10% HS ones, using a moderated t-test with P-value cut-off 0.05 and fold change cut-off of 1.5 and no correction. The analysis showed 183 entities satisfied the analysis with 91 genes being up-regulated and 92 down-regulated. The obtained data set from GeneSpring GX were then uploaded into IPA for further analysis, this analysis indicated that the majority of the canonical pathways affected are involved in cholesterol biosynthesis, lipid metabolism, Aryl hydrocarbon receptor (AHR) signalling and molecular transport. This analysis also showed that cell-to cell signalling is affected which is relevant to a healthy functioning retina. **Table 5.6** summarizes top Canonical pathways identified by IPA. IPA also identified top tox list **Table 5.7**, that included “cholesterol biosynthesis”, “Aryl Hydrocarbon Receptor Signalling”, “LXR/RXR Activation”, “Hormone Receptor Regulated Cholesterol Metabolism” and “Liver Necrosis/Cell Death”. Together the Tox list and the canonical pathway list indicate the possible protective role the up regulated cholesterol biosynthesis and metabolism.

Table 5. 6 Top Canonical pathways identified by IPA. The IPA analysis of the 183 differently regulated genes identified a list of top conical pathways up regulated in lipid loaded cultures that had their complement activated verses lipid loaded only.

Top canonical pathway	P-value
Superpathway of Cholesterol Biosynthesis	1.51E-10
Cholesterol Biosynthesis I	7.59E-09
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	7.59E-09
Cholesterol Biosynthesis III (via Desmosterol)	7.59E-09
LXR/RXR Activation	8.74 E-05
Aryl Hydrocarbon Receptor Signaling	1.28E-04

Table 5. 7 Top Tox-pathways identified by IPA. The IPA analysis of the 183 differently regulated genes identified a list of top conical pathways up regulated in lipid loaded cultures that had their complement activated verses lipid loaded only.

Top tox list	P-value
Cholesterol Biosynthesis	1.88E-06
Aryl Hydrocarbon Receptor Signalling	2.05E-04
LXR/RXR Activation	6.86E-04
Hormone Receptor Regulated Cholesterol Metabolism	9.22E-04
Liver Necrosis/Cell Death	4.50E-03

A list of top differently regulated (up and down) genes between the two conditions was also generated by IPA Table 5.8. complement activation intervention showed an obvious up-regulation in several members of MTs

family as well as LDLR, which were shown to be down regulated in lipid loaded cultures

Molecules	Exp. Value	Function
MT1X	4.645	This protein functions in primary metal storage, transport and detoxification
MT2A	3.600	This protein functions in primary metal storage, transport and detoxification
MT1JP	3.417	This protein functions in primary metal storage, transport and detoxification
MT1F	3.220	This protein functions in primary metal storage, transport and detoxification
TGM2	3.088	Transglutaminases that catalyse the crosslinking of proteins by epsilon-gamma glutamyl lysine isopeptide bonds
KRT34	2.990	Heterodimerizes with type II keratins to form hair and nails
MT1E	2.876	This protein functions in primary metal storage, transport and detoxification
LDLR	2.456	cell surface proteins involved in receptor-mediated endocytosis of specific ligands
IL7R	2.453	Interleukin receptors involved in inflammation and apoptosis inhibition
MT1A	2.446	This protein functions in primary metal storage, transport and detoxification
DSC1	-5.043	Required for cell adhesion and desmosome formation
TYR	-3.414	Mutations in this gene result in

		oculocutaneous albinism, and nonpathologic polymorphisms result in skin pigmentation variation
ABCA1	-2.899	This protein transport various molecules across extra- and intracellular membranes
HMCN1	-2.552	Involved in the maintenance of extracellular matrix integrity
SERPINF1	-2.521	Inhibits angiogenesis. In addition, this protein is a neurotrophic factor involved in neuronal differentiation in retinoblastoma cells
TYRP1	-2.495	Plays an important role in the melanin biosynthetic pathway
SOD3	-2.248	Antioxidant enzymes
SYT14	-2.243	Mediate membrane trafficking in synaptic transmission
EYA4	-2.224	Transcriptional activator for member of the eyes absent (EYA) family of proteins
STRIP2	-2.057	Plays a role in the regulation of cell morphology and cytoskeletal organization.

5.4.4.1. Upstream regulators

IPA analysis was used for further analysis of the upstream regulators in complement activated cultures. The highest up stream regulators included transcription factors that control cholesterol homeostasis such Sterol regulatory element-binding protein 1 and 2(SREBF1 and SREBF2), along with others that include Peroxisome Proliferator-Activated Receptor Gamma (PPARG) ,and its coactivator 1 beta (PPARGC1B) and low-density lipoprotein (LDL) **Figure 5.7.** Other upstream regulators were found to be associated with growth factors

and cell-cell adhesions such as Angiotensinogen (AGT), Transforming Growth Factor, Beta 1 (TGFB1) and Catenin beta-1 (CTNNB1) **Figure 5.8**

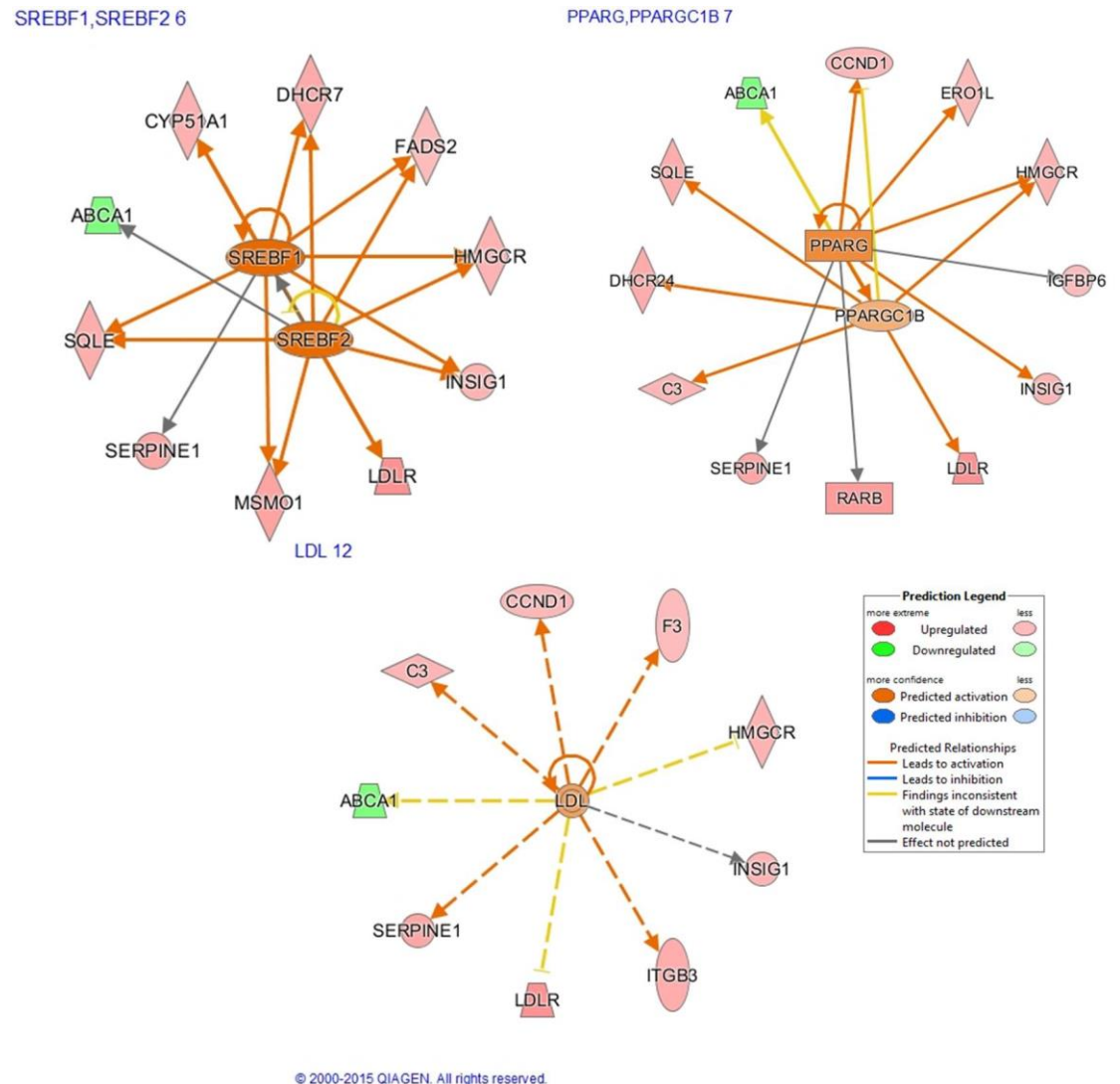


Figure 5. 7 IPA graphical representation of the molecular relationships between differentially expressed proteins in complement activated cultures. The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins.

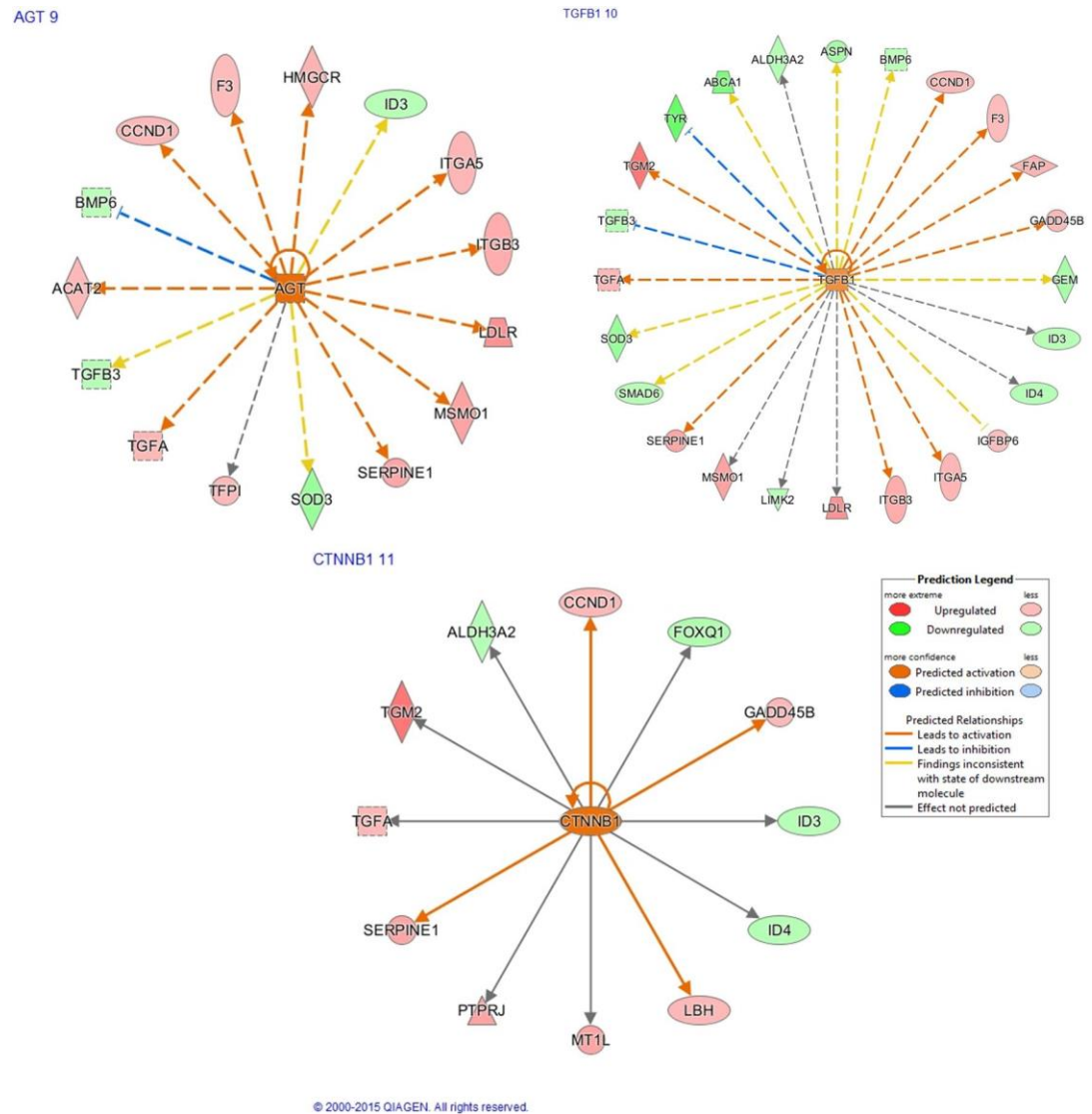


Figure 5. 8 IPA graphical representation of the molecular relationships between differentially expressed proteins in complement activated cultures. The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins. An upstream regulation was seen in function assisted with cellular growth and cell-cell adhesion in complement activated cultures

5.4.4.2. Downstream regulators

Fewer downstream regulators genes were identified by IPA, those downstream regulators were found to be associated with transcriptional regulators such as Peroxisome Proliferator-Activated Receptor alpha (PPARA), Neurogenin 1 (NEUROG1) and brahma-related gene (BRG1 also known as SMARCA4) Figure 5.9. Others downstream regulators were found to be associated other functions such as Phosphatase and tensin homolog (PTEN) and amyloid beta (A4) precursor protein (APP) **Figure 5.10**.

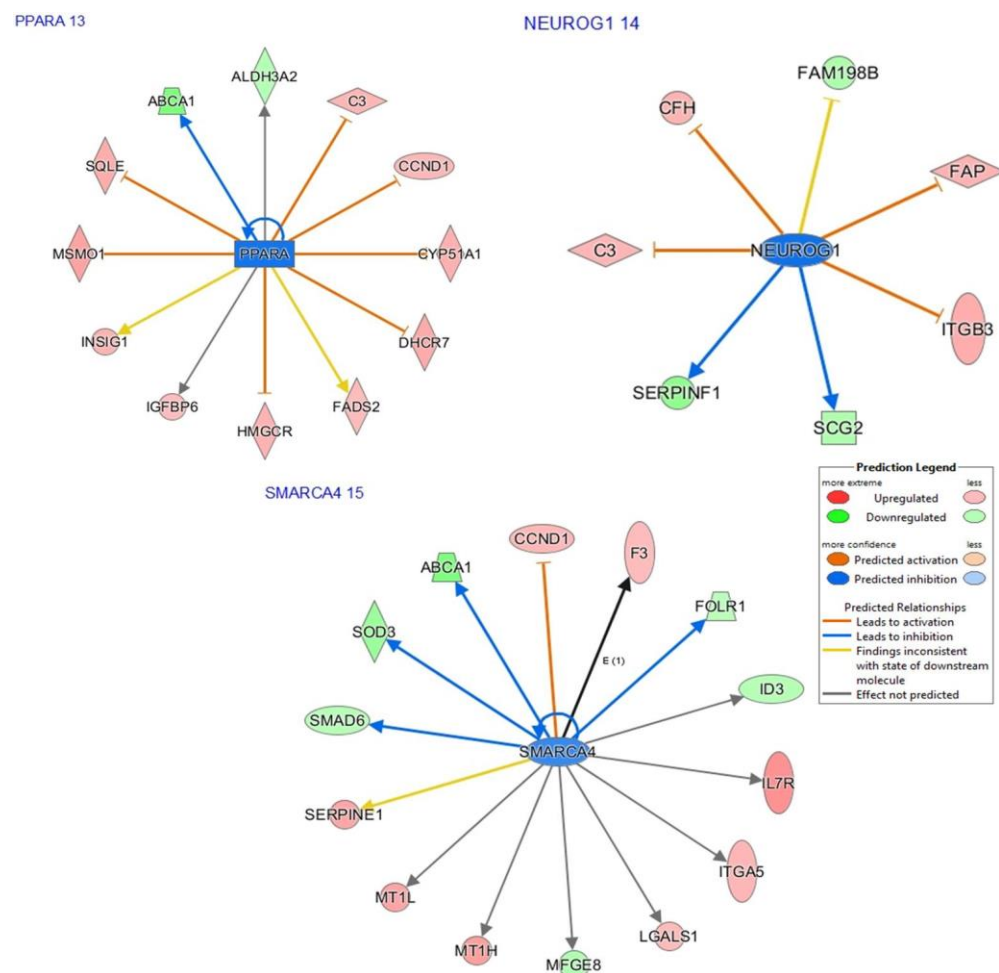


Figure 5. 9 IPA graphical representation of the molecular relationships between differentially expressed proteins in complement activated cultures. The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent

down-regulated proteins. Downstream regulation was seen in function assisted with lipid metabolism and transcriptional regulation.

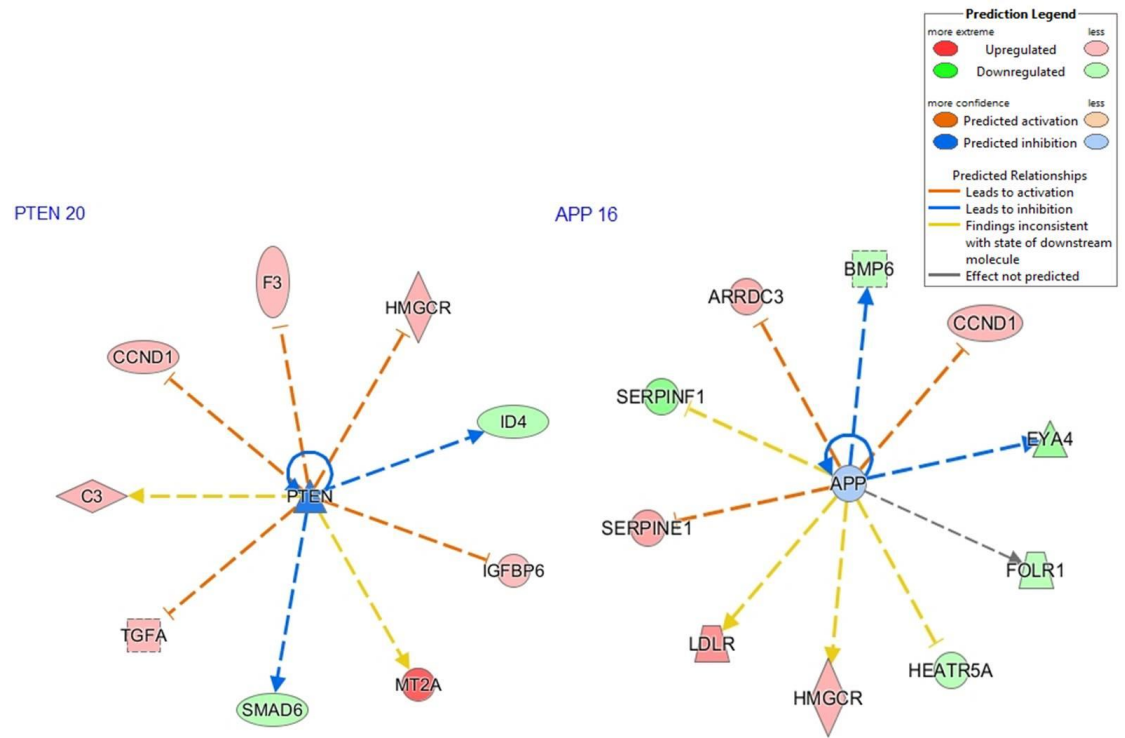


Figure 5. 10 IPA graphical representation of the molecular relationships between differentially expressed proteins in complement activated cultures. The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins. Downstream regulation was seen in function assisted with inhibition of amyloid beta precursor protein

5.4.5. Validation of microarray data using Q-PCR

5.4.5.1. Validation of reference genes

Real time Q-PCR is the “gold standard” technique for validation of microarray data sets. In this study a list of common reference genes were chosen according to Eisenberg & Levanon (2003). Stable expression for the reference genes was seen across interventions **Figure 5.11** with GAPDH having the highest expression.

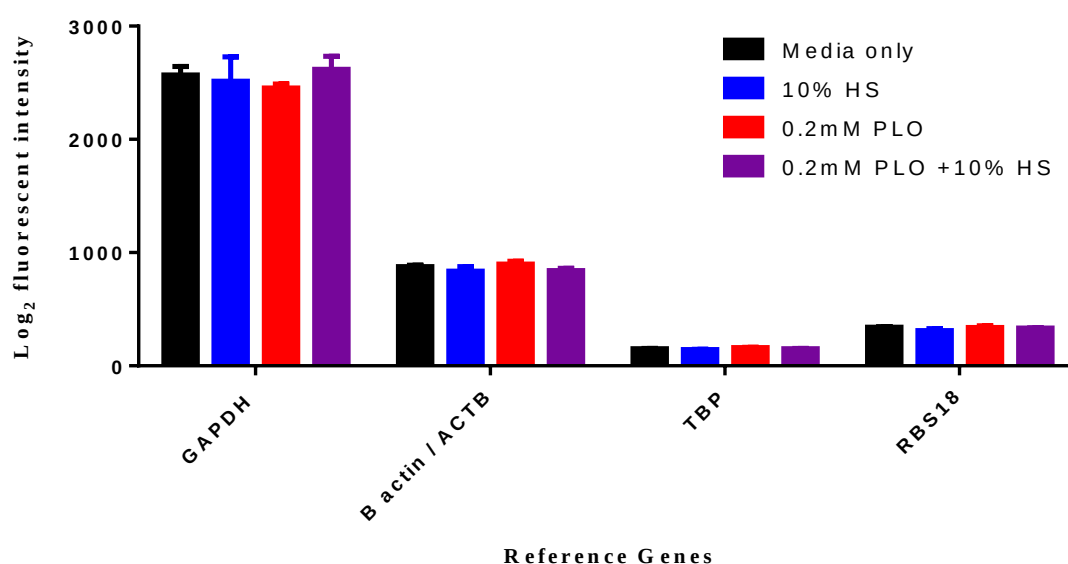


Figure 5. 11 Microarray analysis of reference genes across interventions. Genespring software was used to identify the expression intensity of known reference genes expressed in ARPE-19 cell. Stable expression of reference genes was seen across different interventions.

The expression of reference genes was validated through the use of Taqman qRT-PCR and the cycle threshold (Ct) values were plotted directly. Ct is defined as the number of cycles needed for fluorescence to reach a specific threshold level of detection and is inversely related to the amount of initial RNA template present in the sample (See **Chapter 2, section 2.5**) **Figure 5.12**.

The evaluation of the distribution of CT values obtained from the amplification curves of all samples analysed by qRT-PCR revealed stable range of 24–31 across all candidate reference genes, the overall stability of gene expression was also evaluated providing more validation to the microarray results.

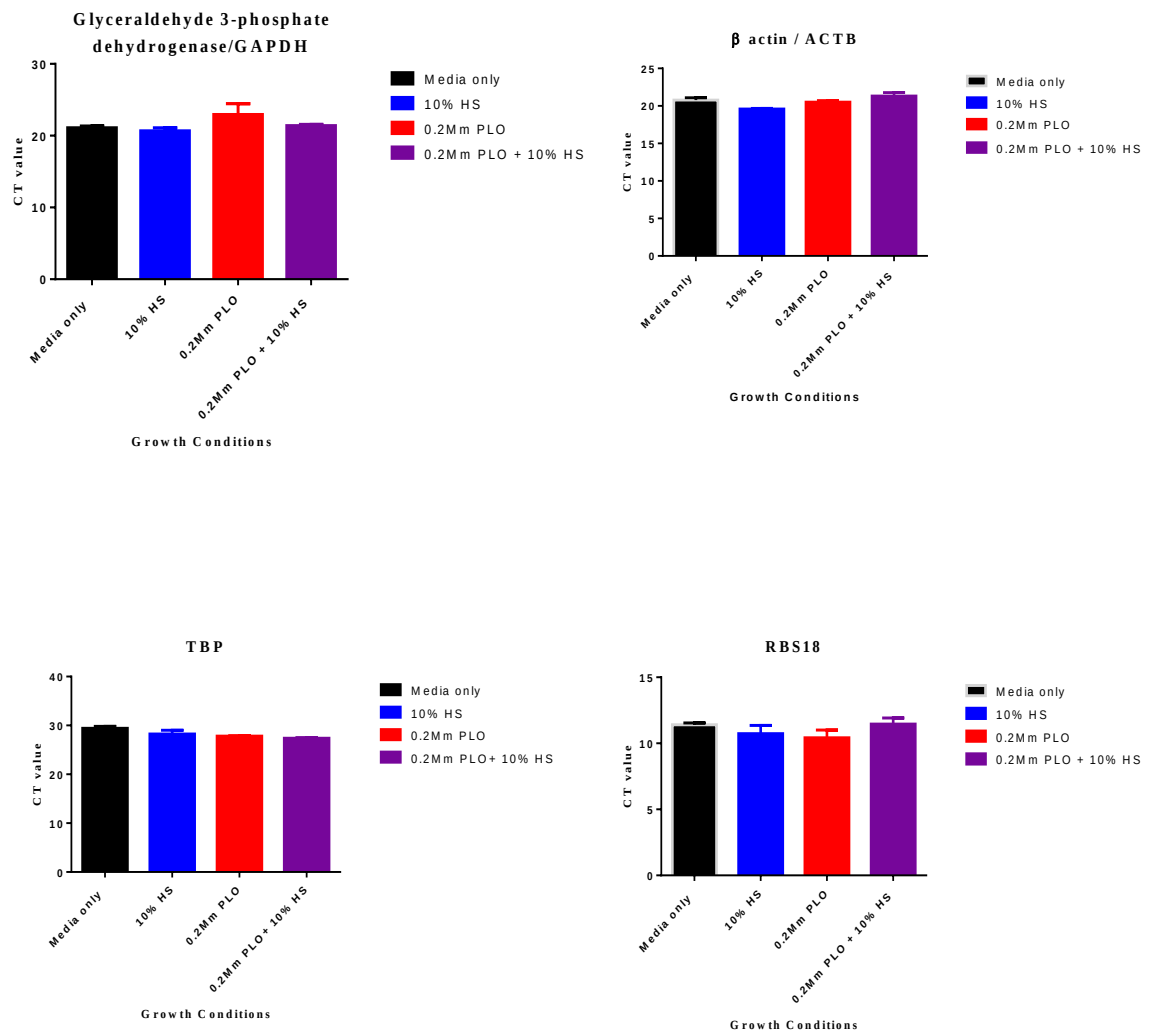


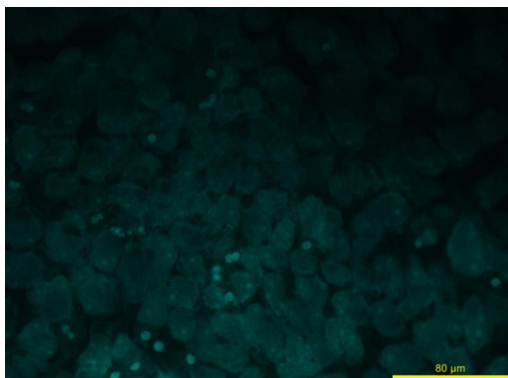
Figure 5. 12 Taqman validation of reference genes. The expression of reference genes was investigated through the use of Taqman. ARPE-19 cell cultures showed stable expression of reference genes across interventions.

5.4.5.2. Cholesterol accumulation analysis

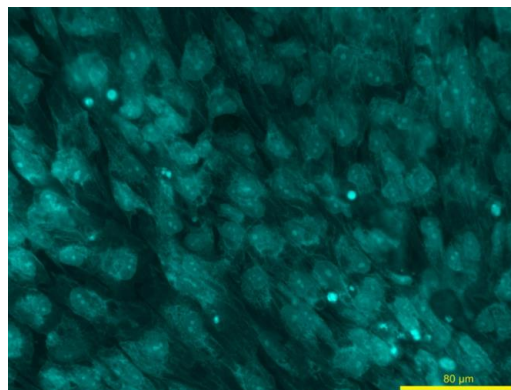
Most of the top canonical Pathways identified by IPA were associated with cholesterol biosynthesis, the effect of complement activation in the presence and absence of lipid loading on cholesterol biosynthesis was investigated by fluorescence analysis. The ability of filipin (excitation 355 nm/ emission 420 nm) to form stable and specific complexes with un-esterified cholesterol (UC) has been utilized to localize the intracellular stores of UC in ARPE-19 cell cultures under different interventions **Figure 5.13A**. Lipid treated cultures showed higher fluorescence of filipin compared to the other interventions. To further validate the results, the total CTCF of UC was calculated using the method used by (Burgess et al., 2010) **Figure 5.13B**. PLO treated cultures showed a significant increase in UC storage. No significant change to the total UC storage was seen in cultures treated with 10% human serum in the presence of PLO treatment.

A)

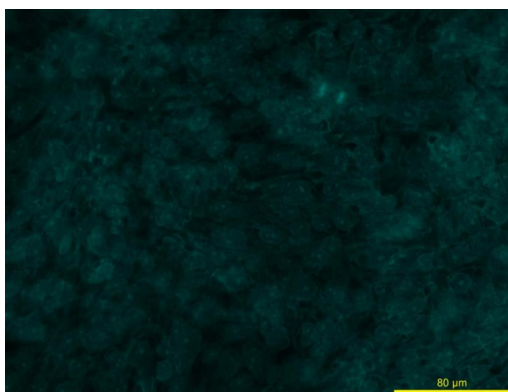
Media only



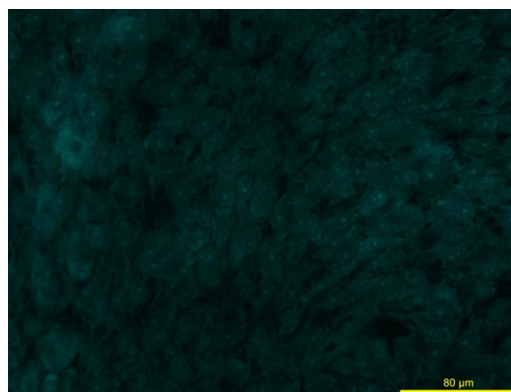
0.2mM PLO



10% HS



0.2mM PLO + 10% HS



B)

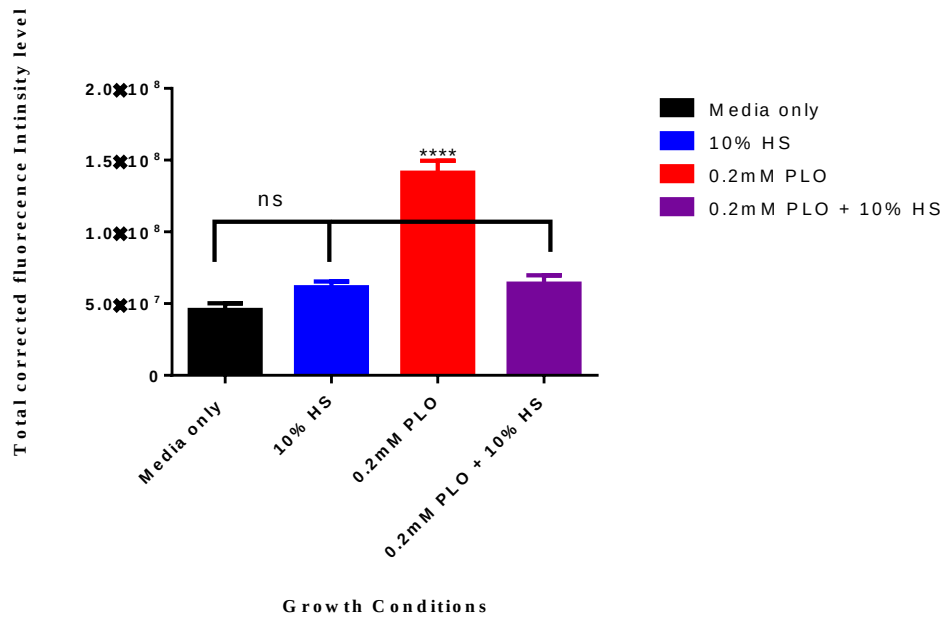


Figure 5. 13 The effect of different interventions on intracellular UC storage. The fluorescent antibiotic filipin stain was used to visualize the intracellular storage of un-esterified cholesterol in ARPE-19 cell cultures that were introduced to different interventions. (A) Fluorescence microscopy images of filipin under different interventions. Cultures that were treated with PLO only showed the highest fluorescence intensity while those that had their complement activated showed no difference to control (serum free cultures). Scale bar = 80µm. (B) Total CTCF was calculated using the ICC images through the use of Image J software (10 pictures per sample). Statistical analysis was done using Oneway ANOVA with Tukey's multiple comparisons test. (ns: no significance, * P < 0.05, ** P<0.01, *** P< 0.001 and **** P < 0.0001).N=3

5.5 Discussion

We have shown that lipid loaded model of ARPE-19 cells captures some aspects of ARMD and that complement activation has a protective role in lipid loaded ARPE-19 cultures. To better understand the response of ARPE-19 cells to lipid loading and how complement activation modulate the cellular response a genome wide microarray analysis was carried to identify differentially expressed genes. Even though microarray data analysis is limited in its reliability, this analysis has identified several interesting genes, PLO treated cultures identified 196 genes to be differentially expressed compared to control, while cultures that had 10% human serum (to achieve complement activation) intervention, showed fewer differentially expressed genes compared to control in the presence and absence of lipid loading, (37 and 74 genes respectively). Thus, activation of complement may be responsible, directly or indirectly, for inhibiting the effects of lipid loading upon gene expression.

Interestingly, the most highly down-regulated genes in PLO treated cultures were associated with genes reported to be down regulated with ageing in humans and were found to be associated with onset of ARMD. Almost all subtypes of isoform 1 of Metallothionein family were down-regulated in PLO treated cultures. Metallothioneins (MTs) are ubiquitous proteins that play a role in zinc and copper homeostasis, to regulate synthesis, assembly, or activity of Zn metalloproteins and to protect against damage caused by reactive oxygen species (ROS), they are also known to provide protection against oxidative stress caused by inflammation (Sato et al., 2000, Blasiak et

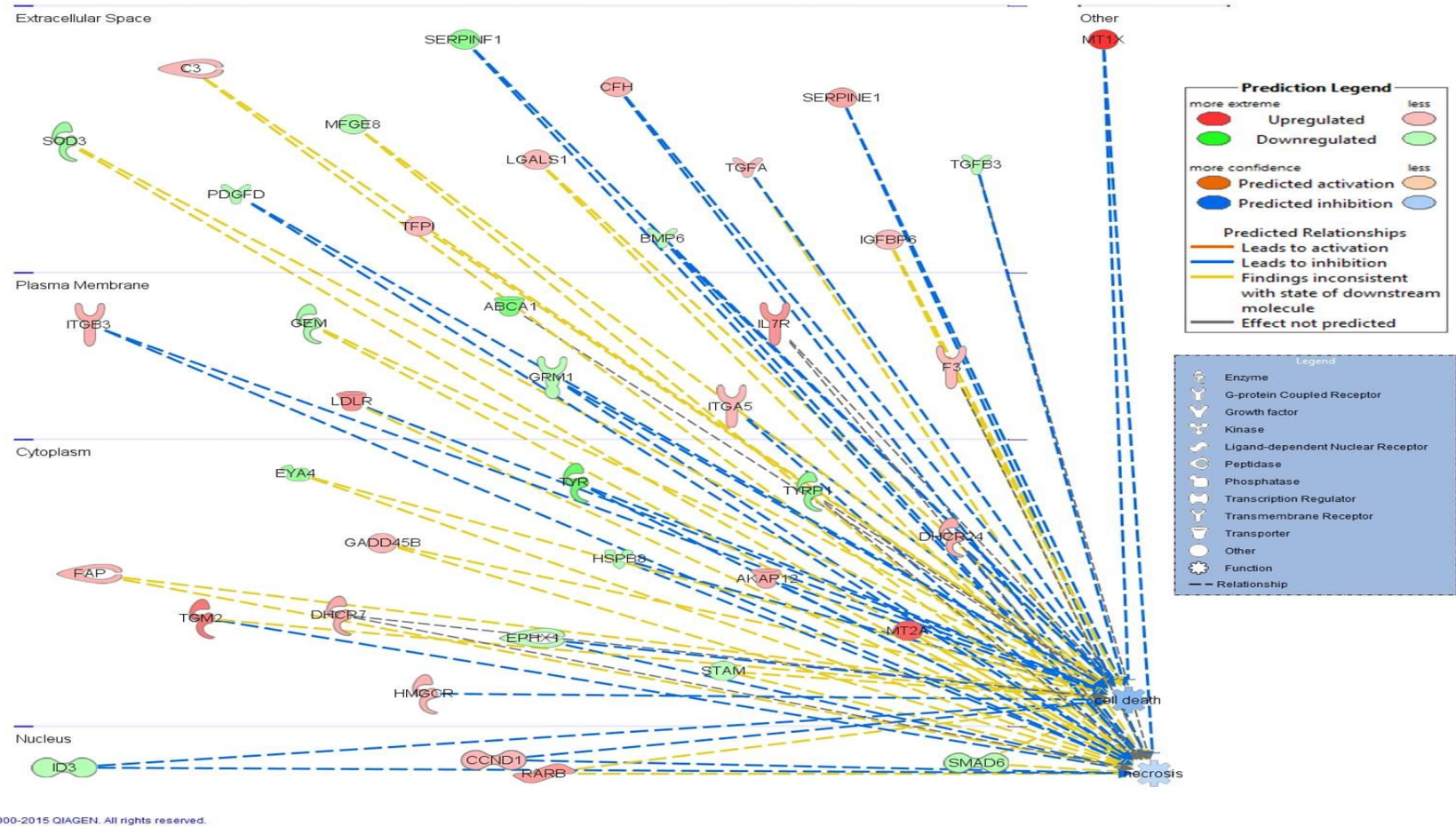
al., 2014, Nicolas et al., 1996a, Tate et al., 1993). Complement factor H (CFH) was also found to be down-regulated after the introduction of lipid loading. The eye is capable of producing its own CFH and in contrast to other organs it may depend on this resource more heavily than the supply from the blood because of the blood-retinal barrier which restricts the movement of proteins from the blood into the retina (Mandal and Ayyagari, 2006). RPE cells are thought to be the main producers of CFH in the eye, as mRNA expression is higher than in any other ocular cell type (Hageman et al., 2005). CHF deficiency is considered a major risk factor for developing ARMD (Clark et al., 2010, Narayanan et al., 2007, Skerka et al., 2007) . The microarray analysis for complement activated cultures identified an up-regulation of CFH gene, C3 gene, which was previously tested in Chapter 4 and noteworthy number of metallothionein family members (MT1X, MT2A, MT1JP, MT1F, MT1E and MT1A).

IPA software identified a shared disease and functional pathway between the up-regulation of C3 protein, CFH and metallothionein family members in the inhibition of cell death and promoting cell survival **Figure 5.14A**, while the down-regulation of C3 protein, CFH and metallothionein family members that was seen in lipid loaded cultures showed an up-regulation of cell death **Figure 5.14B**. This observation along with the work of other research groups highlights the modulatory role of CFH as a potential target in ARMD pathogenicity (Zhang et al., 2015, Nozaki et al., 2006). Together these results are consistent with the observation made in chapter 4, that lipid toxicity

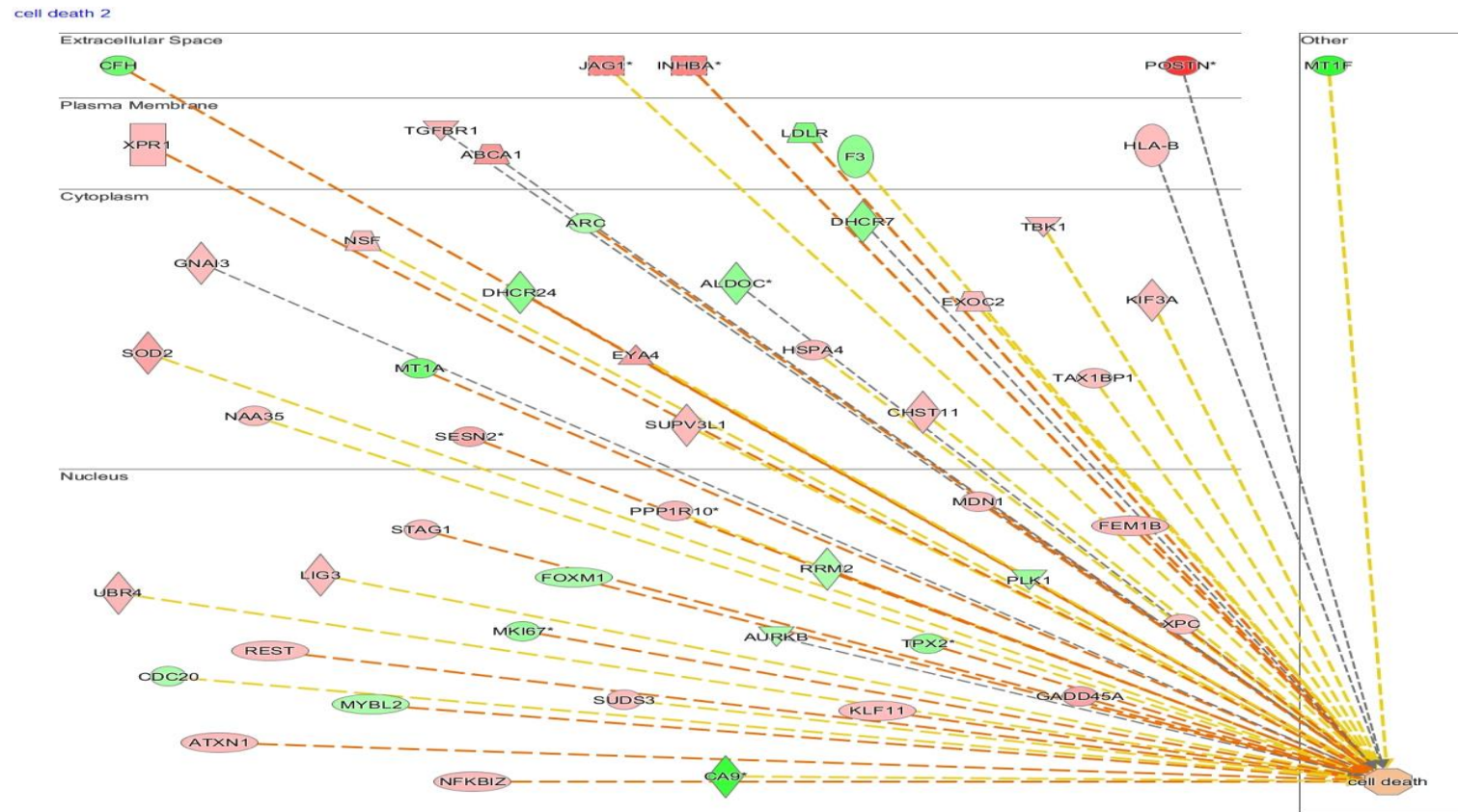
contributes to the changes seen in ARPE-19 cultures that captured aspects of ARMD pathologies and that complement activation modulates the response to lipid loading.



A)



B)



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Figure 5. 14 IPA graphical representation of the molecular relationships between differentially expressed secreted proteins in complement activated cultures. Functional and disease analysis was carried through IPA. (A) 10% HS treatment showed an up-regulation of C3, CFH and metallothionein family members that was found to be associated with predicted inhibition of cell death. (B) 0.2mM PLO treatment showed a down-regulation of C3, CFH and metallothionein family members that was found to be associated with predicted increase of cell death.

To have a better understanding of the effect of lipid loading and complement activation in the presence of lipo-toxicity, IPA analysis was carried between cultures that were treated with PLO only and those that had their complement activated in the presence of PLO treatment. Lipid loaded cultures showed an upstream regulation in several genes involved in cell death and apoptosis as well as genes involved in inflammation, while a downstream regulation was seen in genes responsible for cholesterol biosynthesis and lipid metabolism. In the other hand, cultures that had their complement activated showed an opposite effect in genes involved in cholesterol biosynthesis and lipid metabolism indicated by the upstream regulation seen for SREBF1, SREBF2 and PPAR δ . Cultures that had their complement activated also showed a strong up regulatory expression of MT1 family indicating a protective response to the effect of lipid loading.

Cholesterol biosynthesis and Aryl hydrocarbon receptor (AhR) signalling were among the top canonical pathways identified by IPA for both lipid loaded cultures and those that had their complement activated, lipid loaded cultures showed a downstream activation of AhR. The aryl hydrocarbon receptor (AhR) is a nuclear receptor that regulates a protective response against toxin overload in cells (McIntosh et al., 2010), Upon ligand binding to a specific domain on the AhR, the protein heterodimerizes with a structurally related protein called the AhR nuclear translocator (ARNT). This complex then facilitates expression of a sequence of genes involved in extracellular matrix metabolism, cholesterol biosynthesis, ubiquitination and proteasomal

degradation, and clearance (De Abrew et al., 2010, Hillegass et al., 2006, Sato et al., 2008). A study carried by Hu et al in 2013 found that AhR along with constituents of its canonical signalling pathway are present in human RPE cells and its activity and protein levels in human RPE cells decrease with age. When the group used AhR^{-/-} mice to study the AhR signalling pathway effect, they found that AhR^{-/-} exhibit decreased visual function and develop dry ARMD-like pathology that included disrupted RPE cell tight junctions, accumulation of RPE cell lipofuscin, basal laminar and linear-like deposit material (Hu et al., 2013). This finding was further verified by other groups (Choudhary et al., 2015, Kim et al., 2014) In this study IPA analysis of lipid loaded cultures showed a down regulation ARNT, even though the effect of AhR signalling on the presented cell culture model was not investigated further in this study, these findings emphasize the potential role of AhR as a therapeutic target for ARMD.

5.6 Conclusions

In **chapter 4**, it was proposed that complement activation causes an adaptive response to lipid loading. This chapter investigated the findings in **Chapter 4** through the use of genome wide microarray analysis. Complement activation induced an up-regulation in lipid and cholesterol biosynthesis, which is associated with a decrease in intracellular lipid accumulation and an increase in deposition of extracellular deposits.

6

GENERAL DISCUSSION AND FUTURE ASPECTS

6.1 General Discussion

The complexity of ARMD is reflected by the relative paucity of effective therapies, preventive strategies, and absence of good animal or cell culture models in which can be used to investigate the disease with. Even though current treatments are present for the neovascular form of ARMD (wet ARMD), there is currently still no effective treatment for dry ARMD, which is the more common form of the disease. Animal models, although useful in some respects, fall short of recapitulating all aspects of ARMD; this is in part due to structural differences between animal and primate retinas such as the lack of macula in rodents, as well as the prolonged time needed for the disease to develop and the cost involved in long term of animal care. These highly

specified conditions have limited the use of animal models to those attempting to recapitulate specific genetic disorders associated with dry ARMD (Edwards and Malek, 2007, Marmorstein and Marmorstein, 2007, Zeiss, 2010). Cell culture models are important tools used to study the physiology and pathology of cells and tissues, including RPE cells. One of the advantages of use of cell culture models, is that it allows the creation of a system in which experimental conditions can be controlled and manipulated, with higher reproducibility than those from animal models (Hornof et al., 2005). In the investigation and study of ARMD, the presentation of *in-vivo* phenotype of RPE cells in cultures does not guarantee that it is of physiological relevance to disease (Cristofalo, 1997, Cristofalo, 1976). ARMD is a disease of the old and with age, age related changes transpire. Thus one needs to distinguish between what can be considered normal aging change and what should be considered disease. For example a low level of drusen formation in someone aged 70 years would be considered normal. However, increasing levels of deposits and their size to a point they become associated with regional RPE detachment, will eventually leading to RPE loss and degeneration of overlying photoreceptors. Therefore, the ability to distinguish changes that are normal to the natural aging and those that are pathologically related is of a great importance in understanding ARMD pathology.

A model of aged RPE is presented in this thesis; it showed similarities to the *in vivo* phenotype with the demonstration of continuous monolayer containing ZO-1 and Occludin rich tight junctions. The model captured some

of the features of ARMD including the ability to secrete VEGF in media, as well as the accumulation of intracellular lipids that subsequently contribute to the formation of sub-RPE deposits similar to those found in aged individual. An important observation was made in the process of creating the model, which is only lipid laden cells develop sub-RPE deposits. This observation strongly correlate with reported finding of the correlation between vacuolated and lipid-laden RPE cells (“lipoidal degeneration”) and drusen-like bodies deposits and a macular pigmentary anomaly that are seen in the eye of Rhesus and Cynomolgus macaques (Fine and Kwapien, 1978, El-Mofty et al., 1980, Feeney-Burns et al., 1981, Stafford et al., 1984, Ulshafer et al., 1987, Engel et al., 1988).

We explored the role of lipo-toxicity by exposing ARPE-19 cultures to a mixture of palmitic oleic and linoleic acids (PLO), reflecting the FFA composition in human blood, in order to increase the level of intracellular lipid content of ARPE-19 cell. This chronic lipid loading through the use of PLO treatment (60 days in this study), showed an obvious increase in the level of intracellular lipids, that accompanied morphological changes to the ARPE-19 monolayer as seen in **Figures 3.10 - 3.15**. These changes included the loss of tight junction, increase secretion of VEGF into the culture media and the increased accumulation of sub-RPE deposits that were positive for toxic amyloid beta ($A\beta$) protein. These observed changes reported in Chapter 3 captured some of the important changes seen in ARMD patients, and supports the idea of lipo-toxicity as contributing factor in ARMD pathology (Lozano-

Rechy et al., 1993, Fine and Kwapien, 1978, Stafford et al., 1984) .Even though strong evidence supports the hypothesis of lipo-toxicity, the source of this lipid accumulation remains unknown. Studies that investigated the correlation between plasma lipids and ARMD remain inconsistent (Kozlov et al., 2012, Adams et al., 2011, Reynolds et al., 2013) ; but there is increasing evidence for RPE having an important role in retinal lipid metabolism (Li et al., 2005, Wang et al., 2010b, Rodriguez de Turco et al., 1999, Fliesler et al., 1993, Holz et al., 1994).

The mechanism of drusen formation and accumulation in sub-RPE is still not clearly defined, there have been two major hypotheses suggested for the accumulation of drusen.

- 1) Intracellular lipid accumulation in the RPE cells (lipo-toxicity of the RPE) due to impaired transport and clearance mechanisms (Rudolf et al., 2004, Wang et al., 2010b, Curcio et al., 2001, TU et al., 2010)
- 2) Activation of the complement system, which in turn trigger chronic case of inflammation that damage the RPE cells in the long term (Buschini et al., 2011, Wu et al., 2013, Thurman and Holers, 2006, Johnson et al., 2001)
- 3) We hypothesize a third mechanism, that drusen occur due to increased transport of lipid out of RPE cells- as a response to the cells becoming lipid loaded. This then has drusen formation being associated with ARMD but not causal. Rather the drusen is an adaptive protective response.

To further support the hypothesis presented in this work and to investigate the activation of complement as a pathology risk, the role of long term complement activation was investigated in the presented model. ARPE-19 cultures were introduced to 10% human serum (HS) to activate the complement cascade. The role of the long term complement activation was investigated (60 days) versus the effect of long term lipid accumulation (60 days). Long term treatment with 10% HS showed no damaging effect on the ARPE-19 monolayer, even though an increase in the sub-RPE deposits that were positive for A β were found in complement activated cultures along with significant increase in VEGF secretion, the overall cultures integrity and ARPE-19 viability showed no difference to control unlike the changes seen in lipid loaded cultures. Interestingly, those changes reported in lipid loaded cultures were shown to be reversed when these cultures had their complement activated, indicating a modulatory “protective” effect for the complement activation treatment. As seen in **chapter 4**, lipid loaded cultures that had their complement system activated not only showed preserved tight junctions and stable TEER expression but also showed lower level of intracellular lipid accumulation demonstrating a process of lipid removal and clearance triggered by the complement system activation.

The hypothesis that complement activation has a protective effect was further supported by the observation that despite the increase in APOE positive sub-RPE deposits in complement activated cultures, there was a reduction in the level of A β deposits compared to lipid loaded cultures.

Immunohistochemistry analysis identified a co-localization relation between APOE protein and A β in complement activated cultures only Figure 4.9. This relation is of a particular interest as the affinity of APOE isoforms has been found by others to influence both A β accumulation and ARMD progression (Malek et al., 2005, Calero et al., 2009, Lampropoulou et al., 2012, Verghese et al., 2013, Youmans et al., 2012) . Together these results seen in **Chapter 4** support a hypothesis that considers the complement system as an initial protective mechanism; rather than an instigator of ARMD pathology.

In order to help understand how the complement system influence lipid metabolism, a genome wide microarray analysis was conducted. It was noted that cultures that were exposed to lipo-toxicity showed a down-regulation in genes that are associated with human aging and ARMD; those included substantial number of Metallothioneins (MTs) MT-1 and MT-2 family members which are responsible for the protection against reactive oxygen species (ROS).

Mammalian MTs are comprised of four isoforms designated MT-1 through MT-4. MT-1 and MT-2 (exist in a number of isoforms) are expressed in most tissues including the RPE (Sato et al., 2000, Chen et al., 2004), whereas MT-3 and MT-4 are expressed predominantly in the central nervous system and in keratinizing epithelia, respectively (Hidalgo et al., 2006). All MTs isoforms have been implicated in disparate physiological functions, such as zinc and copper metabolism, protection against reactive oxygen species, or adaptation to stress (Lynes et al., 2014, Ceballos et al., 2003). The level of MTs expression

in the RPE is believed to decrease with aging, leaving the RPE vulnerable for the damage caused by oxidative stress and chronic inflammation (Tate et al., 1993). A work carried by Nicolas et al reported the decreased expression of MTs in the retina of a cynomolgus monkey that showed drusen like deposits, similar to those seen in early onset macular degeneration (Nicolas et al., 1996b). An experiment carried by Sato et al. reported that obese mice that have inactive MTs accumulate fat in their liver when fed high fat diet (Sato et al., 2010). In the other end of the spectrum the activation of MTs family was shown to be protective to the tissue (Vasák, 2005, Chen et al., 2004). Hua et al. reported the increase expression of MTF-1 showed a protective property to the neurodegeneration associated with A β -42 expression in *Drosophila* eye (Hua et al., 2011). These finding along with the microarray data suggest a possible effect caused by the increase of intracellular lipid accumulation on MTs expression, identifying a potential target for ARMD therapy.

Complement factor H (CFH) was also found to be down-regulated after the introduction of lipid loading. CFH deficiency is considered one of strongest genetic risk factor for developing ARMD (Clark et al., 2010, Narayanan et al., 2007, Skerka et al., 2007), CFH is known to be involved in maintaining homeostasis of complement system, when CFH and its cofactor complement factor I (CFI) are bound they degrade C3b to inactive iC3b this action limit the formation of C3bBb also known as “C3 convertase”, thus inhibiting of the alternative pathway of complement (Klein et al., 2005, Luo et al., 2011). CFH also inhibits the deposition and release of C3a and C5a respectively (Nozaki et

al., 2006), both of these complement components are reported to be involved in ARMD and are regularly seen in eye sections of ARMD deceased patients(Heinen et al., 2009). On the other hand, the microarray analysis of lipid loaded cultures that had their complement activated showed an up regulation in CFH that was seen to correlated with the upregulation of MTs and prevent cell death, none the less the interaction between lipid metabolism and CFH is seen in other disorders. Patients with Membranoproliferative glomerulonephritis type II (MPGN II), also termed “dense deposit disease”, showed drusen like accumulation in their eye with dysregulated lipid profile (Mullins et al., 2001). Together these findings suggest the intracellular lipid accumulation in the RPE might influence important gene expression alteration that can lead to the progression of ARMD.

To further elucidate the mechanism leading to the changes seen in lipid loaded cultures the microarray data were further analyses with Ingenuity Pathways Analysis (IPA). Although IPA database is biased toward well-studied genes that are extensively reported in the literature relative to newly discovered genes, it offers a method for rapidly establishing potential associations between genes and functional pathways. IPA was used to analyse the microarray data and to help in identifying effected pathways that might contribute to those changes presented in **Chapter 3**, which are believed to capture some aspect of ARMD. The IPA analysis identify the most significantly affected top canonical (Signalling and Metabolic) and Tox list (cellular response) pathways to be involved with down regulation of cholesterol biosynthesis and removal.

Interestingly, lipid loaded cultures that had their complement system activated showed opposite effect on cholesterol biosynthesis, Genes that are responsible for cholesterol metabolism and removal such as SERBF1, SREBF2, HDL and LDLR were all up regulated in complement activated cultures and not in lipid loaded ones. Immunofluorescence staining of cholesterol also showed that lipid loaded cultures had excess accumulation of cholesterol in cellular membrane, which is can be due to the down regulation of HDL and LDLR, therefore it is suggested that excess accumulation of intracellular lipids cause SREBF to undergoes intramembrane proteolysis that affect both cholesterol and by zinc mediated proteases.

Cholesterol is an essential component of cellular membranes. The liver releases this cholesterol in the form of very-low-density lipoproteins (VLDL) that contain three apolipoproteins (E, C, and B100), which subsequently are transformed to low-density lipoproteins (LDL) with mainly apolipoprotein B100 as their coat protein. Those LDL particles are considered the main source of cholesterol for most cells of the body. However, the NR and the RPE obtains cholesterol via both local biosynthesis as well as uptake of blood-borne cholesterol, as they have been found to contain all the genes necessary for independent cholesterol homeostasis (Fliesler et al., 1993, Fliesler and Bretillon, 2010, Tserentsoodol et al., 2006b, Elner, 2002, Tserentsoodol et al., 2006a).

In the RPE it is suggested that excess cholesterol is stored as esterified cholesterol in lipid droplets within the cell or removed by high-density

lipoproteins (HDL) to be expelled out the blood-retinal barrier to be cleared by the liver (Zheng et al., 2012), this removal process occurs through the low-density lipoprotein (LDL) receptor (LDLR) pathway, and its activity is meticulously governed by sterol regulatory element-binding transcription factors (SREBFs). Sterol regulatory element-binding transcription factors (SREBFs), which are transcription factors that modulate the transcription of genes involved in lipid and cholesterol metabolism (Horton et al., 1998, Pai et al., 1998). The SREBFs consist of three different SREBF isoforms, SREBF-1a, SREBF-1c and SREBF-2. They are produced by two genes, with SREBF-1a and -1c transcribed from a single gene by alternative promoter use and splicing, and SREBF-2 from a separate gene (Hua et al., 1995). In sterol-depleted cells, SREBF forms a complex with SREBF cleavage activating protein (SCAP) to assist in its transportation to the Golgi apparatus where it can be processed by specific proteases that releases the amino-terminal transcription-activation domain of the SREBF. The active form of SREBF can then travel to the nucleus where it binds to the promoters of target genes such as low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase/ HMGCR) where it modulate their activity (Rawson, 2003). Excess accumulation of cholesterol in cellular membrane has a toxic effect (Warner et al., 1995), the age-related accumulation of esterified and unesterified cholesterol in BrM's and choriocapillaris especially under the macula is proposed to lead to the formation of oxidised LDL and oxysterols. This in turn could directly impair

RPE function leading to an inflammatory response triggered by an outcome of para-inflammation.

Impairment of intracellular cholesterol trafficking is also linked to increase the level of beta-amyloid, it was shown by Yamazaki et al to increases amyloidogenic cleavage of amyloid precursor protein (APP) and A β production in animal models of Niemann-Pick type C disease (NPC) (Yamazaki et al., 2001) and in neurons cultures treated with U18666A (3- β -[2 (diethylamino)ethoxy]androst-5-en-17-one), a chemical agent that induces cholesterol sequestration and mimics some aspects of the NPC phenotype (Runz et al., 2002). Recent work carried by Pierrot et al demonstrated that induced increase expression of APP levels in rat cortical neurons decreases cholesterol biosynthesis as well as mRNA levels of several genes involved in cholesterol homeostasis, that include SREBPF1 and SREBPF2, as well as their target genes HMGCR, HMGCS and LDLR, while, acute down regulation of endogenous APP expression in cortical neurons increases SREBPF isoforms, HMGCR and LDLR mRNA levels which reflected increase in cholesterol biosynthesis in general (Pierrot et al., 2013). Furthermore, more evidence supporting that cholesterol has the capacity to modulate A β generation and its clearance were seen in animal model fed rich cholesterol diets as they dramatically exacerbated A β pathology, whereas cholesterol-lowering drugs such as statin decreased the A β burden as well as AD pathology (Höglund et al., 2006). Similar findings were reported in RPE cells, when Wang et al treated RPE cells isolated from senescent (12-month-old)

C57BL/6 mice with cholesterol they reported a significantly increased in A β production while activities of A β -degrading enzyme neprilysin and anti-amyloidogenic α -secretase showed an associated decrease. Moreover, they also reported that senescent C57BL/6 wild-type mice fed with a cholesterol enriched diet developed sub-RPE deposits containing A β (Ratnayaka et al., 2015, Wang et al., 2012a), this effect of rich cholesterol diet was also reported by Dasari *et al* as A β positive deposits accumulation and the presentation of some other aspects of ARMD pathologies were observed in rabbit models fed rich cholesterol diet (Dasari et al., 2011). The alterations in amyloid precursor protein (APP) processing into amyloid-beta (A β) peptides, and deficits in the expression of the innate immune-repressor complement factor H (CFH), not only underlie the amyloidogenic and inflammatory characteristic of Alzheimer's disease but also cause age related degeneration of the retina. Work carried by Alexandrov et al, reported increase retinal abundancy of A β 1-42 peptides along with deficits in complement factor H when they fed murine transgenic models of Alzheimer's disease (Tg-AD) rich cholesterol diet (Alexandrov et al., 2011). Considerable amount of studies further supports the correlation between ARMD pathology and Alzhimers disease, not only due to the similar constitute of drusens and Alzheimer's disease plaques (Anderson et al., 2004, Anderson et al., 2002, Kesler et al., 2011, Johnson et al., 2002) but also due to the impairments in visual function in patients with Alzheimer's disease extending from targeted ganglion cell atrophy and loss in the retina to increased inflammatory neurodegeneration in the primary visual cortex (Cui et al., 2007, Kesler et al., 2011).

In addition to genetic factors, a number of environment factors have been implicated in ARMD and these include smoking (Cong et al., 2008, Woodell and Rohrer, 2014), lack of exercise and obesity (Adams et al., 2014, Chua et al., 2006, Howard et al., 2014) are implicated in increasing the risk of developing ARMD as well as to the risk of its progression to late-stage ARMD. Therefore lifestyle changes, such as weight reduction and increase physical activity may be beneficial. Even though high dietary intake of cholesterol (Cougnaud-Grégoire et al., 2014, Dasari et al., 2011) and saturated fat has been regarded as ARMD risk factors for a long time, there has been no consistency in the reports on plasma lipids in ARMD pathology (Klein et al., 2003, Howard et al., 2014). None the less regardless to source of cholesterol in the retina, these reported findings of the correlation between fat in-take, and obesity (reflecting long term exposure) and ARMD, along with the observations presented in this work, implicate interplay between A β , inflammatory responses and cholesterol regulation in ARMD pathology **Figure 6.1**. Further understanding this interaction between cholesterol overload (lipotoxicity) and transcription factors controlling different lipid pathways may offer a better understanding of early disease mechanisms in dry form of ARMD.

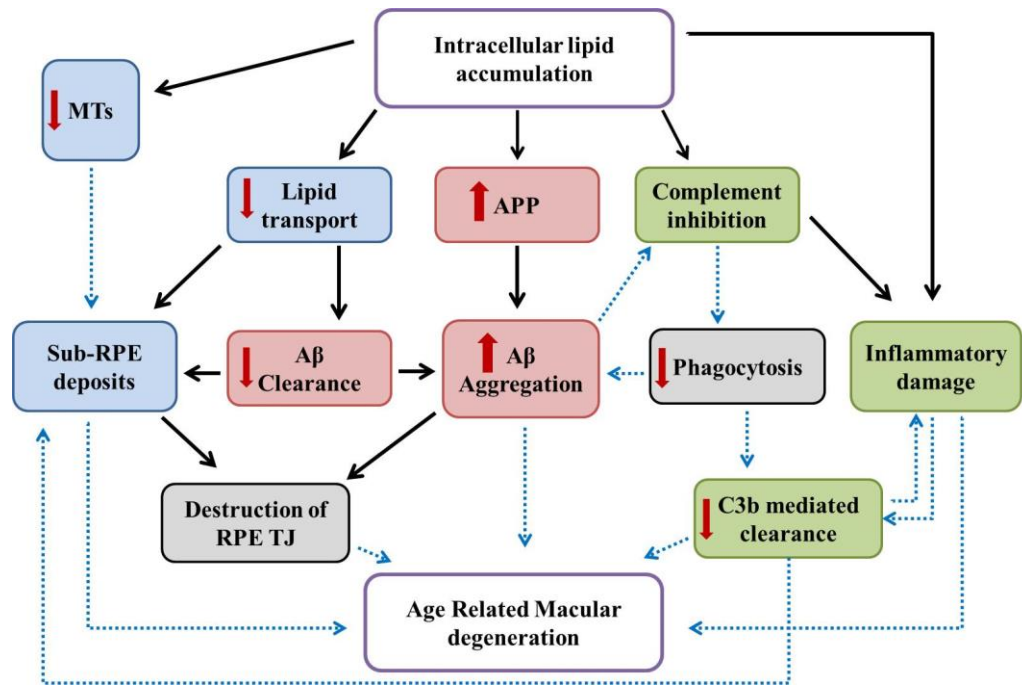


Figure 6. 1 An overview of how intracellular lipid accumulation is implicated in ARMD. These findings are based on the current experimental or observational evidence (solid black arrows), with hypotheses shown by (blue arrows). Several pathophysiological pathways thought to contribute to disease such as Aβ aggregates (light red), chronic inflammation (green), sub-RPE deposits (blue) and others (grey). Abbreviations: MTs (Metallothioneins), APP (Amyloid Precursor Protein), Aβ (Amyloid Beta), RPE (Retinal Pigment Epithelium) and TJ (Tight Junctions).

6.2 Limitation

This work has a number of limitations. The use of whole human sera in systemic complement activation is generally used in cell culture studies (O'Shaughnessy et al., 2012, Johnson et al., 2011, Lee et al., 2014) . As it was shown in Chapter 4 the use of 10% human serum did increase the expression of C3 complement component at gene level while serum that was in-activated by heating didn't. However, this doesn't represent the complete complement cascade and the complement membrane attack complex C5b-9, in particular was not investigated. Immunoreactivity analysis was excluded due to the fact that a whole serum is used to activate the complement. This method increases the possibility of complement deposition (not true activation) therefore reflecting incorrect positive results. There is a fine line between activating the complement for the purpose of homeostasis (anti-inflammatory action) and triggering damaging inflammation (pro-inflammatory action). The microarray data did indicate that inflammatory pathways were being activated in PLO treated cultures.

Another limitation of the microarray study is the long culture protocol that was required. Samples were collected from three different experiments that conducted in the period of two years, the difference in ARPE-19 passage number and the changes in reigned and media consignments might all contribute to the variation that was seen between replicates. The produced ARPE-19 cell cultures in this study may serve as a partial model for RPE. In the retina, the RPE support the photoreceptors of neuroretina. Limiting the

culture to just RPE cells limits what can be achieved. For example, another toxic deposit, A2E, occurs in ARMD which is formed as a by-product from the visual cycle but as our model excludes photoreceptors, this cannot be modelled by our system.

6.3 Future Work

It was shown how Sub-RPE deposits formed under both interventions (PLO and 10% HS). Future work should include investigation of how such deposits could be cleared. This model could be improved by co-culture techniques and particularly including the endothelium which is likely to be involved in the clearance pathway. Hamilton et al introduced a model of tri-layer model to capture RPE, Bruch's membrane and choroid complex (Hamilton et al., 2007). Complement activation was showed to express protective properties to lipid loaded ARPE19 cells in culture. The activation of the complement system can occur via three pathways; classic, alternative and the manan binding pathway. The alternative pathway is believed to be the main associated pathway in ARMD as CFH is the most implicated complement component reported so far. In a study carried by Johnson et al. the used of a serum depleted of C1q showed a reduction in deposits positive for C5b-9 while depleting the serum of CFB "complement component required for alternative pathway activation" had no effect. The expression of C1q was found to be down-regulated in lipid loaded cultures and up-regulated in the presence of active complement system (results are not shown) indicating a possibility of mixed activation of different complement pathways. The implication of the complement system in ARMD is far from simple, to better define the specific role of the activation of each pathway of the complement cascade in this lipo-toxic model of ARMD might shade a new light on the effect of each pathway on the pathologies reported by the model.

One of the main observations seen in this work is the role of lipid metabolism and transport in the formation of sub-RPE deposits. Peroxisome proliferator-activated receptors (PPARs) are members of the steroid/thyroid nuclear receptor superfamily of ligand-activated transcription factors. They can be activated either exogenously by fatty acid derivatives or endogenously by prostaglandin derivatives, these ligands form a complex with the PPARs and translocate to the nucleus where they form heterodimers with retinoid-X receptors (RXR) that binds to PPAR Response Elements (PREs) located on the target gene. Once bound, transcription is enabled (Berger and Moller, 2002, Kota et al., 2005). In the human eye all subtypes of PPAR (α , δ and γ) are expressed in the RPE cells and the neuronal retina (Herzlich et al., 2008, Bishop-Bailey, 2011). PPARs are believed to play an important role in lipid metabolism, immune regulation, oxidative stress, vascular endothelial growth factor (VEGF) regulation and docosahexaenoic acid (DHA) turnover, all of which are known to be implicated in the pathophysiology of ARMD (Malek et al., 2010, Ciudin et al., 2013, Herzlich et al., 2009). The role played by PPARs is of particular importance, investigating their expression in the presented model of ARPE-19 cultures might show a possibility of modulation in intracellular lipid accumulation and clearance of sub-RPE deposits, a further investigation of the effect could have a potential for therapeutic target. The use of agonist for PPAR δ was shown previously to influence lipid metabolism and reverse cholesterol transport (Kang et al., 2007, Oliver et al., 2001). To

investigate the effect of PPAR and their agonist in the lipo-toxic model of ARMD may serve as a potential investigatory tool.

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