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Genetic and Environmental Risk factors in

Age-related Macular Degeneration

in vitro, in vivo and population studies

By

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M.B., B.Ch., BaO, Dipl Stat., MRCSI Ophth.

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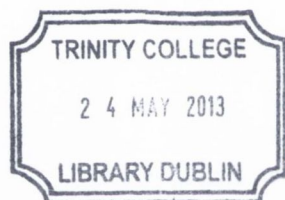
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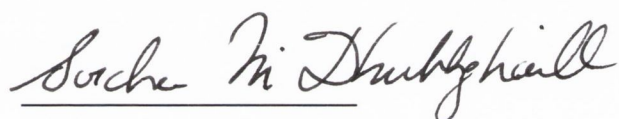
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Supervised by Professor Lorraine Cassidy, Mr. Mark Cahill, and Dr. Matthew Campbell



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Sorcha Ni Dhubhghaill

October 2011

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"Age doesn't matter, unless you're a cheese"

Billie Burke

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Additional supportive material

Appendix 1: SOD1-/- pathology report

Appendix 2: Consent form for AMD study

Appendix 3: Patient questionnaire

Abbreviations

ACE	Angiotensin Converting Enzyme
AGEP	Advanced Glycation End Product
AOPP	Advanced Oxidation Protein Product
ALEP	Advanced Lipid End Product
AMD	Age-Related Macular Degeneration
APOE	Apolipoprotein ε
AREDS	Age-Related Eye Disease Study
ARM	Age-Related Maculopathy
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblastic Growth Factor
BCA	Bichinichoninic Assay
BlamD	Basal Laminar Deposits
BlinD	Basal Linear Deposits
BMI	Body Mass Index
BrdU	Bromodeoxyuridine
CC	Choriocapillaris
CEP	Carboxyethylpyrrole Protein
CFB	Complement Factor B
CFH	Complement Factor H
CNV	Choroidal Neovascularisation
CRALBP	Cellular Retinaldehyde Binding Protein
CRP	C-reactive Protein
CSE	Cigarette Smoke Extract

DAPI	4'-6-Diamidino-2-phenylindole
DHA	Docosahexanoate
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
EDCC	Eye Disease Case Control Study
ELISA	Enzyme-linked Immunosorbant Assay
EPA	Eicosapentaenoic Acid
ERG	Electroretinogram
EUREYE	European Eye Study
FAZ	Foveal Avascular Zone
FFA	Fluorescein Fundus Angiography
GA	Geographic Atrophy
GSH	Glutathione
GSSH	Glutathione Disulphide
HMW	High Molecular Weight
HRP	Horseradish Peroxidase
ICG	Indocyanine Green
IntARM	International Classification and Grading System for Age-Related Maculopathy and Age-Related Macular Degeneration
IVT	Intravitreal Injection
L-ORD	Late-onset Retinal Degeneration
LALES	Los Angeles Latino Eye Study
LAST	Laboratory Animals in Science and Teaching
LOD	Logarithm of Odds
LPS	Lipopolysaccharide
LMW	Low Molecular Weight
MAC	Membrane Attack Complex
MCP1	Monocyte Chemoattractant Protein 1
MESA	Multi-ethnic study of atherosclerosis
MGNII	Membranoproliferative Glomerulonephritis Type II

MPSMacular Photocoagulation Study

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nm	Nanometres
NHANES	National Health and Nutrition Examination Survey
NSAIDs	Non-Steroidal Anti Inflammatory Drugs
oBRB	Outer Blood Retinal Barrier
OCT	Optical Coherence Tomography
OR	Odds Ratio
PAH	Polycyclic Aromatic Hydrocarbons
PAMDI	Prevalence of Age-Related Macular Degeneration in Italy
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PCV	Polypoidal Choroidal Vasculopathy
PEDF	Pigment Epithelium Derived Factor
PFA	Paraformaldehyde
PLEKHA1	Pleckstrin homology domain-containing family A, member 1
PUFA	Polyunsaturated Fatty Acids
RAP	Retinal Angiomatous Proliferation
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
RPED	Retinal Pigment Epithelial Detachment
RR	Relative Risk
RVEEH	Royal Victoria Eye and Ear Hospital
SDS	Sodium Dodecyl Sulphate
SFD	Sorsby Fundus Dystrophy

SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
STGD	Stargardt Macular Dystrophy
TIMP-3	Tissue Matrix Metalloproteinase 3
TMB	Tetra-methylbenzidine
VEGF	Vascular Endothelial Growth Factor
WARMGS	Wisconsin Age-Related Maculopathy Grading System

Chapter 1: Introduction

1.1. General ocular anatomy and development

The eye is a highly developed organ optimized for the transmission of visual signals via the optic nerve to the visual cortex. It is composed of three layers: the sclera, the choroid and the retina. These layers surround the central cavity which is filled with by the vitreous humour.¹ A portion of the globe is transparent and forms the cornea, which allows light to enter the eye (Fig. 1.8.1). Light is refracted at the air-cornea junction as well as at the crystalline lens to focus it to a point on the retina. The photoreceptors in the neural retina convert light photons into an electrical potential that is conveyed through the optic nerve to the brain.

The eye begins in the fifth week of neonatal life. The primitive optic vesicle develops from the neuroectoderm and differentiates into a double-layered optic cup. This cup forms the neural retina, the retinal pigment epithelium and the fibres of the optic nerve.¹ An invagination of the overlying surface ectoderm and mesoderm forms the lens, vitreous, ciliary body and cornea. The retina further develops into an outer neuroblastic zone and an inner neuroblastic zone. Cells of the outer layer give rise to the rod and cone retinal photoreceptors, bipolar cells and horizontal cells. The inner layer forms the amacrine cells and the ganglion cells. The layers of the retina are, from the choroidal layer to the vitreous surface (outer to inner retina), as follows: The retinal pigment epithelium (RPE), the outer segments of the photoreceptors (rods and cones), the external limiting membrane, the outer nuclear layer - the nuclei of the photoreceptors, the outer plexiform layer, the inner nuclear layer, the inner plexiform layer, the ganglion cell layer, the nerve fibre layer - axons of the ganglion cells and finally the internal limiting membrane (Fig.1.8.2). The blood supply to the outer third of the retina is derived from the choroidal circulation, whereas the central retinal artery supplies the inner two thirds of the retina. The central retinal artery supplies 5% of the oxygen requirements to the retina. The choroid supplies the remaining 95%, which is indicative of the high metabolic demands of the photoreceptor layer and retinal pigment epithelium (RPE).²

The RPE is a tight layer of hexagonal epithelial cells located between the photoreceptors and Bruch's membrane. There are approximately 4 to 6 million RPE cells in each eye. RPE cells are post-mitotic and therefore lack the ability to proliferate under normal conditions. Each RPE cell develops tight intercellular complexes with its neighboring cell. These points of contact are enforced by the expression of junctional proteins (e.g. occludin). The formation of a barrier system restricts the free flow of molecules from the choriocapillaris (CC) to the neural retina. This restriction in flow forms the outer blood-retinal barrier (oBRB). The basal surface of the RPE is in contact with the photoreceptor layer and provides a number of supports to visual function including the absorption of light, maintenance of the subretinal space and phagocytosis of the rod and cone outer segments.³ Over the course of 70 years a single RPE cell will phagocytose an estimated 3 billion rod outer segments.⁴ The process of outer segment phagocytosis permits the recycling of retinal and polyunsaturated fatty acids back into the visual cycle. Each RPE cell supports the nutritional requirements of approximately 45 photoreceptors and RPE death leads to the death of the overlying dependent photoreceptors.⁵

The Bruch's membrane is a five-layered structure located between the RPE and the choriocapillaris (CC). The membrane is acellular, composed of the basement membrane of the RPE, two layers of collagenous matrix sandwiching a central elastin layer, and the basement membrane of the choriocapillaris. The membrane is approximately 2µm thick. It is thinnest centrally in the area of the fovea and macula and becomes thicker towards the peripheral retina. The choroid is a highly vascular tissue and is located beneath Bruch's membrane. The endothelial cells of the choriocapillaris are highly fenestrated and permit the free flow of molecules from the blood to the Bruch's membrane. The fenestrations of the vessels are preferentially orientated towards the membrane and RPE suggesting a paracrine functional relationship between RPE and choroid.⁶

1.2. Macular anatomy

The macula lutea, or yellow spot, is an area visible on the retina, located approximately 3mm lateral to the optic nerve¹ and can be found between the arterial arcades (Fig 1.8.3). This region has evolved as the point of highest visual resolution in the retina and is only found in species of the 'Order primates', which includes Homo sapiens. The average human adult macula is 5mm in diameter. The retina dips into a small depression approximately 1.5mm in diameter called the fovea at the centre of the macula.

The fovea is the central focus point for the refractive apparatus of the eye and is the point of greatest visual acuity. The depression visible at the fovea is due to the displacement of the ganglion cell layer peripherally providing the direct access of light to the photoreceptor layer in this area. Within the fovea there is a further central pit of 0.35mm in diameter known as the foveola.¹

The blood supply to the macula is derived from the choroid and the retinal vasculature. The central fovea is devoid of overlying blood vessels and is known as the foveal avascular zone (FAZ). This region is dependent on the underlying choroid for metabolic support. The photoreceptors in the macula are predominantly cones and are found at the highest density in the fovea (147,000/mm²). The fovea contains cones exclusively, the majority of which are red (long wavelength) and green (medium wavelength) cones. The yellow color of the macula is due to an increased concentration of pigments derived from the carotenoid family such as xanthophyll, zeaxanthin and lutein.⁷ These pigments are concentrated in the Henle fibre layer that forms the macular portion of the outer plexiform layer. With a peak absorbance of 460nm this pigment concentration is thought to act as a filter which serves to restrict high-energy blue light damage to the macula.⁸ The concentration of the pigment in the macula provides an antioxidant effect as well as contributing to the hypofluorescence observed in fluorescein angiography.⁹

The macula differs from the peripheral retina both anatomically and physiologically. The specific features of the macula that set it apart from the peripheral retina may have an aetiological connection to the development of macula specific diseases. Structurally, the neural retina in the region of the macula becomes thinner at the umbo to permit the passage of light. The peripheral photoreceptors are predominantly rods whereas the macula contains high concentrations of cones peaking at the fovea. The blood supply in the foveal avascular zone is derived from the choroidal circulation with a reduced contribution from the retinal vasculature. The Bruch's membrane region of the macula is 55nm thick, approximately one third of the thickness of the membrane in the periphery, and the porosity of the membrane increases from 0% in the periphery to 35% in the foveal area.¹⁰

Physiologically, the macula is the central focal point of the visual axis and is thus exposed to the highest concentration of light energy.¹¹ This subjects the macula to the highest amounts of photochemical and oxidative stress. The accumulation of macular pigment may provide additional protection against the

effect of this additional oxidative stress. The porous and thinned structure of the Bruch's membrane in this area is consistent with the high metabolic demands of this tissue. These adaptations result in a significant reduction in resistance to diffusion from the choriocapillaris. The resulting diffusion gradient allows rapid delivery of nutrients and removal of waste products from the RPE, provided the oBRB remains intact. It allows for the unimpaired passage of fluid through to the neural retina in the absence of the oBRB. Accumulation of waste products within the Bruch's membrane and impairment of the diffusion gradient has been implicated in retinal disease.

1.3. Age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive, degenerative eye condition affecting the macula,¹² and is the leading cause of visual impairment in people over 65 in the entire Western world.¹³ AMD can affect the choriocapillaris, Bruch's membrane, RPE and the neurosensory retina but damage is almost exclusively confined to the central macular region of the retina.¹⁰ The disease was first described by Hutchinson and Tay in 1875¹⁴ and Needleship in 1884 as central choroidal atrophy but it was Otto Haab who coined the term Senile Macular Degeneration in 1885.¹⁵ At that time pathological findings were attributed to: "sclerosis and obliteration of the choriocapillaris in the macular area". The treatment options available for macular degeneration were so limited in fact that the advice given by the prominent ophthalmologist Sir Steward Duke Elder in the last century was that: "Such people can only be helped in so far as every effort should be made to assist them to accommodate their interests with philosophy and resignation to their limited visual efficiency".¹⁶ Until recently, AMD had been described as a "spectator's sport". Nevertheless, the past two decades have yielded newer treatment modalities and the current prognosis for these patients is more optimistic.

Though the macula comprises only 4% of the retinal surface it conveys the vision of highest resolution and thus, the loss of central visual clarity impacts heavily upon activities of daily living e.g. reading, recognizing faces etc.¹⁰ There is a wide spectrum of visual disability observed in AMD. In early or mild cases there may be no accompanying visual impairment. If the disease progresses central vision may become profoundly affected. Patients may be reassured though, that even in advanced stages, AMD rarely affects peripheral vision and the patient is not likely to be rendered completely blind.

1.4. Clinical classifications of AMD

There is a wide range of phenotypic appearances observed in AMD that has resulted in some difficulties in developing classification systems. Clinically, age-related macular changes may be described as atrophic or neovascular (Table 1.8.1). These classifications have also been described as either: "dry" (atrophic) or "wet" (neovascular).¹⁷ Both disease categories contain a wide spectrum of disease manifestations.

1.4.1. Atrophic AMD

The atrophic presentation is the most common form of AMD and affects approximately 85% of persons with AMD.¹⁸ Early signs of age-related changes in the presence of minor visual impairment have been described as age-related maculopathy (ARM). As clinical findings increase and visual impairment increases, these patients transition to the later stages of AMD. The hallmarks of atrophic disease are drusen and RPE abnormalities. These are typically the earliest findings in patients with AMD (Fig 1.8.4B).

Drusen are identified as pale yellow extracellular deposits located beneath the RPE and are described as hard or soft based on the morphological appearances. Over time, drusen may become calcified and appear glistening. These collections typically cluster at the posterior pole and may coalesce to form basal laminar deposits (BlamD) or basal linear deposits (BlinD)¹⁹. Based on a histological examination of 378 eyes, Sarks et al described a clinical spectrum of appearances spanning the normal degenerative changes of the RPE to the late changes of AMD (Fig1.8.5).²⁰ This classification describes not only the clinical characteristics, but also exemplifies the temporal evolution of drusen. Drusen display autofluorescent features on fluorescein angiography and this property of drusen is due to the lipofuscin component and it has been suggested that autofluorescent imaging may be a means of monitoring disease progression in dry AMD.²¹ Small, hard drusen (<63µm) are features of normal ageing and are not a significant risk factor for the advancement of AMD.¹⁰ Elderly patients with small drusen and no clinical symptoms do not require regular clinical assessment. In the absence of other pathological changes drusen alone are rarely associated with severe visual impairment. They may however be associated with subtle defects in macular function such as contrast sensitivity and central

visual field sensitivity.²² The size, number and degree of confluence of drusen must be considered as larger drusen is a significant clinical risk factor for progression of AMD.²³

Both hard and soft drusen may lose their clear boundaries, as the disease progresses. Drusen can become more confluent and may form retinal pigment epithelial detachments (RPED) over time and clinically it may be difficult to differentiate large drusen from small RPEDs. Similar to drusen, RPEDs typically appear as hyperfluorescent regions of rapid filling on angiography. Drusen are seen in a number of other ocular conditions including membranoproliferative glomerulonephritis type II (MGNII), Malattia Leventinese, Doyne's honeycomb dystrophy and Sorsby's fundus dystrophy.

In addition to drusen and RPEDs, RPE abnormalities occur in AMD that may appear as either hyper (or hypo) pigmentation at the macula. In advanced cases, the RPE at the macula may atrophy in a well-delineated pattern with a scalloped edge. This form of degeneration is known as geographic atrophy (GA) and involves widespread atrophy of the RPE (Fig.1.8.4B). In the absence of the RPE, the overlying photoreceptors die which results in exposure of choroidal vessels. Geographic atrophy often develops in the parafoveal regions and may spare the fovea until late progression.¹⁰ When the fovea is involved however this is the most visually devastating form of atrophic AMD occurs and accounts for approximately 10% of blindness associated with AMD.¹⁸ The factors that determine the progression of ARM to either geographic atrophy or neovascular AMD are not yet known and recent data suggests that the loss of photoreceptors in geographic atrophy may in fact precede the RPE changes.²⁴

1.4.2. Neovascular AMD

In the process of neovascular AMD, new blood vessels grow from the choriocapillaris and breach the Bruch's membrane. The infiltrating vessels may penetrate deeper through the RPE and enter the neural retina. The pathological growth is known as choroidal neovascularisation (CNV), as they arise from the choriocapillaris vasculature.^{12, 25} CNV typically occurs against a background of atrophic AMD but in many cases it may be the first clinical manifestation. The neovascular vessels may have a grey appearance with a boggy subretinal tissue that typically leaks on fluorescein angiography.¹⁰ Although it only accounts for approximately 10% of AMD cases, neovascular AMD may lead to a rapid

deterioration in vision and accounts for 75% of cases of significant visual impairment associated with AMD.²⁶

CNV consists of fragile ectopic membranes that may proceed to hemorrhage, subretinal fluid accumulation, retinal pigment epithelial detachment, retinal atrophy and disciform scarring which results in rapid visual loss (Fig 1.8.4C).²⁶ Clinically CNV may be described on the basis of fluorescein appearance as follows:

- Classic CNV
- Occult CNV
- Fibrovascular CNV

Classic CNV has a clearly defined “lacy” pattern in the early phase followed by leakage into the subretinal space. Occult CNV appears as an ill-defined hyperfluorescence in the early phase and lacks the definition of classic CNV. The majority of CNV lesions (>80%) are of the occult class.¹⁰ Fibrovascular PED displays a mixed appearance of both CNV and PED. These distinctions are important in some of the older forms of treatment (photodynamic therapy) but are less so in the more common biological treatment strategies in use today. In the absence of treatment, neovascular membranes progress with over 65% of eyes suffering severe visual loss (a drop in 6 or more lines of acuity) within 5 years.²⁷ Retinal angiomatous proliferation (RAP) is a subcategory of neovascular AMD. The proliferating vessels extend to form retinochoroidal anastomosis in these patients. They may be recognized by an increased number of intraretinal haemorrhages, telangiectasia and microaneurysms.¹⁰

CNV is a response to tissue injury and is similar in nature to the granular tissue of wound healing.¹⁵ In general, the wound healing response is characterized by; clot formation, deposition of fibrin matrix, infiltration of inflammatory cells, angiogenesis, extracellular matrix destruction and re-formation, scarring and re-epithelialization.²⁸ Fibrin is deposited from the choriocapillaris leading to degradation and remodeling of the Bruch’s membrane in the case of CNV. Vascular endothelial cells and proliferating fibroblasts originating in the choroidal vessels migrate through Bruch’s membrane and grow beneath the retinal pigment epithelium and retina. The tendency for CNV to penetrate Bruch’s

membrane in the region of the macula rather than in the peripheral retina may be related to the thinned structure and higher porosity of the membrane in this area.

The natural history of untreated CNV is one of progressive growth and leakage.²⁰ After a few months the lesion enters a cicatricial stage that results in a disciform scar. These scars form dense central scotomas that cause significant central visual impairment, though peripheral vision is typically spared. It must be noted that both GA and CNV may be present simultaneously. Approximately 2-4% of patients with bilateral GA may develop CNV over the course of two years²⁹ and any underlying GA may be obscured in the presence of CNV. Therefore it is likely that the two conditions coexist in more instances than has been recognized.

1.4.3. Standardised systems of AMD classification

The application of a standard classification system allows for direct comparison and is conducive to meta-analyses of multiple epidemiological studies. The two most widely used systems are the Wisconsin Age-Related Maculopathy Grading System (WARMGS),³⁰ and the International Classification and Grading System for Age-related Maculopathy and Age-related Macular degeneration (IntARM).³¹

WARMGS³⁰

Wisconsin Age-Related Maculopathy Grading System was described by Klein et al in 1991 and is based on 30° stereoscopic photographic pairs of fundus images.³⁰ A grid pattern is superimposed over the centre of the image (Fig 1.8.6). The central ring corresponds to a circle of 500µm radius in an average eye. The middle and outer rings correspond to 1500µm and 3000µm respectively. The template divides the macular area into nine subfields. A series of circular templates are also provided to estimate the size of drusen and pigmentary changes (Fig1.8.6). The grid patterns contain templates for 63µm, 125µm and 250µm (C₁, C₂ and C₃ respectively). The WARMGS describes drusen on the basis of maximum size, predominant type, area and degree of confluence (Table 1.8.2).

WARMGS provides grading systems for retinal pigment epithelial degeneration based on the area of the subfield affected in addition to drusen classification. The grading of the late stage AMD is less detailed than that of drusen as the requirements of the WARMGS do not include the use of fluorescein

angiography. Sensory retinal detachment and RPE detachment are placed in the same grade. Subretinal and sub-RPE haemorrhages are similarly lacking in strict definitions and are graded on the basis of 4 categorisations: absent, present, questionable or cannot grade. Geographic atrophy (GA) is defined as absent, questionable, less than 50% of subfield or greater than 50% of subfield.

Int(ARM)

This alternative classification system was devised by the International ARM Epidemiological study group in 1995.³¹ Stereoscopic photographic transparencies are initially graded with the same central subfield grid used by the WARMGS, and the grading is performed in a similar manner, by dividing the retina into three concentric circles. The templates provided for photographic grading (C₀-C₄) encircle areas of 63µm, 125µm, 175µm, 250µm and 500µm. The circles are used to measure drusen size and the templates may also assist in sizing areas of RPE pigmentation abnormalities and regions of geographic atrophy. This classification system is effectively an extension of the WARMGS and provides greater clarification of pigmentary changes, geographic atrophy and neovascular changes. AMD is defined as late ARM that includes either neovascular or geographic atrophic disease manifestations (Table 1.8.3).

1.5. Epidemiology of AMD

The prevalence and incidence of AMD are both increasing with the ageing population, and are resulting in an increase in debilitation as well as social burden. Over two thousand people are registered as blind or partially sighted and receive assistance from the National Council for the Blind in Ireland.³² These patients benefit from the provision of visual aids and other support services, however the estimated number of those eligible for registration is approximately 7,000. The prevalence of the disease has increased in Ireland by 113% between the years 1996 and 2003 and now accounts for 25% of all blindness registrations where 44% of all new registrations are as a result of AMD.³³ In the United Kingdom, there are an estimated 214,000 patients entitled to blind or partial sight registration due to AMD and this number is expected to rise.³⁴ Similarly, there are over 1.75 million people affected by AMD in the United States and this is expected to increase to 3 million by 2020 and the number of people affected by AMD globally is approximately 50 million.³⁵

The prevalence of AMD has been widely studied among the Caucasian population. Three large population-based epidemiological studies established the prevalence of AMD over three continents by working collaboratively. The Beaver Dam study³⁶, the Blue Mountains Eye study³⁷ and the Rotterdam study³⁸ all collectively utilized a common grading system (WARMGS) which facilitated the comparative analysis of a total of 14,752 participants.³⁹ The results showed that AMD is rarely seen before the age of 55 and becomes very common after the age of 75. The prevalence of AMD at each age range was derived from these three studies and has been summarized (Table 1.8.4). The findings from eight large studies on the prevalence of AMD in the Caucasian population are summarized (Table 1.8.5). The prevalence of AMD in the Caucasian population is 1.51-5.4%. These studies were selected, as they are the largest studies performed to date that employ either the WARMGS or the IntARM grading criteria. It illustrates the variability in the prevalence of AMD, even within the Caucasian subgroup. The rates of progression to CNV and GA also differ between reports. The Reykjavik Eye Study and the Baltimore Eye Study reported that GA was more prevalent than CNV in Icelandic and American Caucasian populations. Conversely it was found that CNV was far more prevalent than GA in the Beaver Dam, Blue Mountains, Rotterdam, the European Eye (EUREYE), Melbourne VIS and the PAMDI studies. The Salisbury eye study reported a similar rate for both GA and CNV. The prevalence and the incidence of AMD in the southern Irish population have not yet been established.

It has been noted that while large-scale studies of AMD in Caucasian populations are plentiful, the prevalence of AMD in the Black population has not been as widely assessed. Only four studies are available for inclusion in this summary (Table 1.8.5). The Barbados Eye Study was conducted prior to the adoption of the WARMGS and the IntARM grading systems, and it is therefore not directly comparable to the other reported studies.⁴⁰ This study is included in this summary, however, as the largest reported study on the prevalence of AMD in a 100% Black population sample group. The Salisbury Eye Evaluation,⁴¹ Baltimore Eye Study⁴² and the Multi-ethnic study of atherosclerosis (MESA) assessed the Black population as a smaller subgroup of a sample population. The prevalence of AMD was found to be lower among the Black population than in the Caucasian population, in all studies, at 0.21-2.4%. GA and CNV appear far less commonly among the black population. The AREDS trial found the odds ratio of developing AMD in the Caucasian population was 3.93 when compared with non-Caucasians. This was calculated from a study population of 4,519 participants, of which only 201 were

non Caucasian.²³ It has been proposed that the reduced risk of developing AMD is due to a protective effect conferred by denser retinal melanin pigmentation.⁴³ Melanin may act as a free radical scavenger, which in turn reduces the oxidative stress burden on the RPE.

The Los Angeles Latino eye study (LALES)⁴⁴ is the largest study undertaken to date on the prevalence of AMD in the Hispanic population. This study showed that both GA and CNV occur with similar frequency in Hispanics and significantly less frequently than among the white population (Table 1.8.5). The MESA study reported a higher prevalence of all forms of AMD (4.2%) but a similar rate of late AMD (0.2%) to that of the LALES study. This may be accounted for by the categorization of early ARM changes into the AMD category of the MESA study.

The prevalence of AMD has been widely studied in the Asian populations, when contrasted to the studies undertaken on the black and Hispanic populations (Table 1.8.5). The prevalence of AMD is 0.3-6.7% with the highest prevalence detected in the Andhra Pradesh eye study⁴⁵ and the India Eye Study.⁴⁶ AMD is more prevalent in Asian men than Asian women.⁴⁷⁻⁴⁹ The Singapore Eye Study,⁴⁹ Handan Eye Study⁵⁰, Beijing Eye Study,⁵¹ Funagata Eye Study⁵² and the Hisayama Eye Study⁴⁸ reported a similarly low prevalence rates of 0.3-0.87% AMD in Singapore, China and Japan. The Funagata, Handan, Beijing and MESA studies on the prevalence of AMD in the Asian population are less informative on the progression to late stage AMD as they reported all forms of late AMD in a single statistic rather than detailing specific rates of GA and CNV. The available data indicates that CNV (0.46-0.67%) is more common than GA (0.2-0.31%) in the Asian population (Table 1.8.5). Polypoidal choroidal vasculopathy (PCV), commonly categorized as exudative AMD, is also more common in Asians, which potentially skews the observed prevalence of late AMD.⁵³ CNV is more prevalent than GA in the Asian population. This may be due to either the higher prevalence of PCV or a potential genetic resistance to the development of GA.

There was a significant gender bias noted in the prevalence of AMD in the Asian population, which has not been detected in other ethnic subgroups. In the Handan eye study, the prevalence of CNV was higher in Asian men than Caucasian men but lower in Asian women than Caucasian women. This may be due to the higher ratio of male to female smokers in Asia. In the Handan province of China the

prevalence of cigarette smoking among men is 58.7% whereas the prevalence among women is as low as 0.3%.⁵⁰

AMD appears to be most prevalent among the Caucasian population and among the Asian male population. The significant differences observed in the prevalence of AMD between different ethnic groups may be due to a number of factors: smoking, alcohol-intake, background histories of cardiovascular disease and a multitude of other factors all serve to confound results, in turn impairing direct comparison of prevalence rates. PCV is more common in the Asian and Black populations and also presents a significant confounding factor.^{53, 54} The classification of PCV in terms of AMD is currently debated. The two arguments may be stated as follows: AMD may be either a different phenotypic appearance of AMD or an entirely different pathological entity. A clearer classification system to account for the influence of PCV will assist in illustrating the true prevalence of AMD. It may also reflect variability in the underlying genetic risk factors that cause a person to be predisposed to AMD.

Of the AMD classification systems that have been described, the WARMGS and the IntARM are the most frequently used. Similarities between the two systems permit pooling of epidemiological data across the largest studies. The routine use of grading systems in clinical practice is not currently recommended. The disadvantages associated with these grading systems are chiefly in the time required to perform them and the limited applications of grading. Complete grading of retinal images requires overlaying the grid template and measuring the retinal features of interest to the study. Secondly, clinical applications to the grading system are limited. Progression of ARM may be slowed by antioxidant vitamin preparations in 20-25%.⁵⁵ Neovascular AMD is treated primarily with intravitreal anti-VEGF antibodies. There are no accepted dosing regimens based on the grade of neovascular AMD nor has the grade of neovascular AMD been successfully correlated to prognosis therefore grading does not currently play a role in risk stratification. In the future, it should become possible to plan dosing regimens and predict long-term outcome based on the grade of lesion at the time of presentation when long-term follow up data for the use of anti-VEGF treatments becomes available. Routine grading of AMD images would then be beneficial.

1.6. Aetiology of AMD

The exact aetiology of AMD is not yet known. Evidence has been gathered from epidemiological data, histological assessments and animal models to account for the presentation of the disease. Based on current knowledge a number of hypotheses have been proposed but no single theory accounts for all of the risk factors and clinical findings in AMD. The disease process is likely to be due to a number of collaborating factors that culminate in a wide array of phenotypic presentations, and so the true aetiology of AMD may include features from all of the proposed theories.

Aetiological theories in AMD may be divided into:

- The oxidative stress hypothesis
- The vascular hypothesis
- The inflammatory hypothesis

1.6.1. *The oxidative stress hypothesis of AMD*

Oxidative stress is defined as oxidizing chemical damage resulting from an imbalance between reactive oxygen intermediates (ROI) coupled with a reduced capacity to detoxify these molecules. ROIs are highly reactive oxygen-based molecules that have unpaired electrons.⁵⁶ These molecules include the superoxide anion ($O_2^{\bullet-}$), singlet oxygen (1O_2), hydroxyl free radical ($OH^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$) and lipid peroxyl radical and hydrogen peroxide (H_2O_2).⁵⁷ Under normal physiological conditions, aerobic tissues are subjected to constant oxidative stress from the mitochondria. Oxidative stress is countered by antioxidant mechanisms (Fig.1.8.8). In the absence of this protection, however, ROIs readily interact with amino acids, carbohydrates, lipids and nucleotides (Fig.1.8.9).⁵⁷

Generation of ROIs in the retina

The retina has the highest tissue oxygen consumption in the body and is therefore particularly susceptible to oxidative stress.⁵⁸ Reactive oxygen species in the retina are generated through a number of chemical processes, which include oxidative phosphorylation, photochemical reactions and direct generation of oxidants. All aerobic cells are subjected to a basal level of oxidative stress from the mitochondria. During oxidative phosphorylation, free electrons are shuttled through the mitochondrial membranes to generate energy in the form of adenosine triphosphate (ATP). Approximately 5% of the

electrons generated leak into the cytoplasm, subjecting the surrounding cell organelles and membranes to oxidative stress. Secondly, the photochemical reactions of the retina generate ROS within the photoreceptor cell layer.⁵⁷ Finally the accumulation of chromophores (photosensitizers) e.g. rhodopsin, lipofuscin and melanin in the RPE can also directly generate reactive oxygen species.

Generation of antioxidants

Antioxidant protective mechanisms may be divided into enzymatic and free radical scavengers. Superoxide dismutase (SOD), catalase, glutathione peroxidase, and heme peroxidase are present in all cells and catalyse the conversion of ROS into oxygen, water and glutathione disulphide (GSSG) (Fig.1.8.8). Free radical scavengers are small molecules that interact with ROIs to form less harmful complexes. Vitamin C (ascorbate) and vitamin E (α -tocopherol) are antioxidants that provide secondary protection against oxidative stress. Vitamin C is the most potent soluble antioxidant derived from dietary sources⁵⁷ and *in vitro* studies suggest that vitamin E in particular plays a role in preventing the oxidation of polyunsaturated fatty acids (PUFA) in the photoreceptor outer segments.⁵⁹ Carotenoids also possess free radical scavenger potential and are present in high concentrations at the macula, contributing to localized antioxidant protection.⁶⁰ Reduced glutathione (GSH) is another potent free radical scavenger that is produced endogenously by the NADPH-glutathione reductase system. GSH is found in plasma as well as intracellularly and reacts with ROIs to generate GSSG, which in turn may be recycled back to GSH (Fig. 1.8.8).

Damage from ROIs in the retina

ROS may react with proteins, carbohydrates, lipids and nucleic acids (Fig.1.8.9). The oxidation of protein forms advanced oxidation protein products (AOPP) which contain cross linked disulfide bonds and predisposes the protein to fragmentation, misfolding and aggregation.⁶¹ Carbohydrates interact with ROIs to form advanced glycation end products (AGEs). The unsaturated hydrogen bonds within polyunsaturated fatty acids (PUFA) are highly susceptible to oxidative stress. These interactions form lipid peroxides and lipid peroxy radicals. PUFA are present in high concentration in the retina, particularly in the membranes of the photoreceptor's outer segments. The outer segments are

metabolized and accumulate in the RPE layer. Both nuclear and mitochondrial DNA are susceptible to oxidative insult. Bases may become modified, DNA strands broken or cross-linked. ROS have therefore been linked to mutations, activation of proto-oncogenes and alterations in tumor suppressor genes.⁶² Oxidative stress may therefore affect all components in the retinal photoreceptors and RPE. It culminates in the deposition of drusen, the promotion of RPE atrophy and the development of CNV. Oxidative stress has also been found to cause deposition of extracellular matrix along the Bruch's membrane.⁶³ In an animal model of the oxidative stress mechanism, carboxyethylpyrrole (CEP), a product oxidized docosahexanoate (DHA), was sufficient to cause retinal lesions similar to drusen and GA.⁶⁴ CEP is found in higher abundance in tissues from AMD eyes than in controls, and is localized to the photoreceptor rod outer segments. Serum samples taken from patients with AMD showed 2.3-fold concentrations of anti-CEP antibodies supporting a role for CEP in the pathogenesis of AMD.⁶⁵

Clinical evidence to support the oxidative stress theory of AMD

Increased levels of oxidative stress markers have been observed in both the serum^{61, 66} and retina^{67, 68} of patients with AMD. Patients with AMD display increased serum levels of oxidized LDL when compared with controls.⁶⁹ Protective antioxidant mechanisms in retina decrease with the onset of both age and AMD.⁷⁰ Catalase and superoxide dismutase activity are significantly lower in the serum of patients with AMD⁶¹ and retinal concentrations of protective pigments^{9, 60} and antioxidants are similarly reduced.⁷¹ In contrast to this, Frank et al have observed an increase in the activity of antioxidant enzymes (haeme oxygenase 1 and 2) in RPE of eyes with CNV.⁷² This increase may be due to larger pathological stress exerted by the newly developing vessels.

Increased dietary intake of antioxidants vitamins C and E appeared to be protective against AMD in both the NHANES⁷³ and Beaver Dam studies. Assessments of serum concentrations of these vitamins have been inconsistent and have not supported the dietary observations as to their efficacy. To overcome daily fluctuations in antioxidant levels the Eye Disease Case Control Study (EDCC) trial constructed an index to assess a more global antioxidant capacity of serum samples.⁷¹ The index, which included vitamins C and E, carotenoids and selenium was inversely related to the risk of developing AMD.⁷¹ Based on these epidemiological observations the Age-Related Eye Disease Study (AREDS) was

constructed to assess the effects of antioxidants and zinc on the development of AMD (Table 1.8.6).⁵⁵ Over the course of the study, 4,757 participants between the ages 55-80 were recruited. The treatment group received a preparation that included antioxidants, copper and zinc. (Table 1.6.1) The benefits conferred by this preparation were found to be modest. There was no role for antioxidants in the prophylaxis of AMD and there were no benefits to be found in mild AMD.⁵⁵ However the antioxidant plus zinc preparation was found to be of benefit in patients with intermediate bilateral AMD or unilateral advanced AMD. It also reduced this risk of progression from moderate dry AMD to wet AMD by 27% over 10 years when compared with a placebo. The follow- on study, AREDS 2, includes macular pigments lutein and zeaxanthin as well as omega 3 long chain polyunsaturated fatty acids, docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA). The results of AREDS 2 are expected to be available in 2013.

The oxidative stress model was the leading hypothesis for the aetiology of AMD prior to the development of the immunological hypothesis. In this model the primary site of insult is the photoreceptor and RPE complex. Clinically, the modest clinical effects of antioxidants and the reduced levels of circulating support the hypothesis and macular antioxidants associated with AMD.

1.6.2. The vascular hypothesis of AMD

The vascular hypothesis or haemodynamic model of AMD, originally proposed by Friedman, is based on the observations that AMD shares a number of common risk factors with atherosclerosis.⁷⁴ The model proposes that an increased deposition of lipid at the level of the sclera is the primary insult in AMD (Table 1.8.6). Lipid infiltration progressively hardens the sclera, in a manner similar to atherosclerosis, and leads to an increase in choroidal vessel resistance. The subsequent impaired choroidal perfusion leads to an increase in capillary hydrostatic pressure and a reduction in the clearing of metabolic products at the level of the RPE. The accumulation of these retinal waste products may lead to the thickening of Bruch's membrane, drusen formation and RPE atrophy. Therefore in the vascular model of AMD, RPE dysfunction is thought to be a secondary effect of the degeneration rather than a primary event.

Evidence in support of the vascular hypothesis of AMD

There are significant similarities in both the epidemiology and the risk factors associated with AMD and atherosclerosis. Age, hypertension, obesity and cigarette smoking have all been associated with both AMD (Chapter 1.7) and atherosclerosis.⁷⁵ The use of antihypertensive medications has been associated with a reduced risk of developing AMD, linking the two pathological processes.⁷⁶ In addition, the vascular model is the only hypothesis that can account for the association of hyperopia with AMD.⁷⁷ The smaller hyperopic eye may be more susceptible to increased vascular resistance and impaired choroidal flow.⁷⁸

There are, however, a number of weaknesses to the model. It is not currently possible to directly measure choriocapillary pressure, and assessments of scleral rigidity are not sufficiently robust for clinical application.⁷⁸ Pathologically, the lipid deposition at the level of Bruch's membrane does not appear to be derived from the systemic circulation. This is found to be in contrast with atherosclerosis. Analysis of the cholesterol content of basal laminar deposits and drusen indicates that these lesions are deposited by the dysfunctional RPE.⁷⁹ This is supported by the lack of protective effect of statins in the treatment of AMD.⁸⁰

In conclusion, despite the number of epidemiological associations between AMD and atherosclerosis, the vascular hypothesis has least clinical data and histological evidence in support of it. It is possible that there is a significant vascular component to the development of AMD, but it is unlikely to be the sole mechanism through which the disease develops.

1.6.3. The inflammatory hypothesis of AMD

The inflammatory hypothesis of AMD expands on the oxidative stress model by suggesting that AMD is caused by inappropriate inflammation in susceptible individuals (Fig.1.8.11).⁸¹ The initial inflammatory trigger may be one of a number of events that includes, but is not limited to, oxidative stress. There is mounting evidence from genetic, histological and molecular studies to indicate that AMD is mediated through a dysregulation of inflammation. Hageman et al proposed the inflammatory model of AMD in 2001.⁸² In this model drusen are thought to represent cellular debris from

degenerating RPE cells trapped within the layers of the RPE and Bruch's membrane. RPE dysfunction precedes the accumulation of debris subretinally. The cause of the initial dysfunction may be oxidative stress, chronic chemical stress of other aetiology or a sub-clinical infection not yet isolated.

Drusen as markers of inflammation

Drusen contain remnants of cell organelles, melanin and lipofuscin which are most likely derived from RPE debris,⁸³ but they appear to be more complex than simple foci of extracellular material extruded from the ageing RPE. The RPE components within drusen may be due to blebbing of the cell membrane in response to cellular injury and chronic inflammation. Dendritic cell processes have been identified in many drusen central cores.⁸² Therefore it has been proposed that drusen may represent by-products of an underlying chronic inflammatory process occurring between the RPE and Bruch's membrane.⁸² The inflammatory proteins that have been isolated to drusen are listed (table 1.8.7). These indicate the active role of the inflammatory pathway in the development of AMD. Based on the composition of drusen both the innate and adaptive arms of the immune system are involved in AMD (Fig. 1.8.11). The prevalence of complement cascade associated proteins, in particular, within drusen is significant.

Inflammatory cell mediators and AMD

Leukocytic activation is instrumental in the development of CNV and inflammation. Inflammatory mediators also govern the progression from atrophic to neovascular AMD. Depletion of macrophages in a laser-induced animal model of CNV inhibits the immune response and the subsequent neovascularisation.^{84,85} Genetic knockout mice deficient in key inflammatory proteins CD18 and ICAM-1 also show a decrease in CNV volume.⁸⁶ It is possible that macrophages promote angiogenesis through cytokine-induced upregulation of VEGF in RPE cells.^{87,88} Monocyte chemoattractant protein 1 (MCP1) is expressed by RPE cells in AMD that may attract macrophage infiltration. Both I-CAM1⁸⁹ and MCP-1⁸⁷ have been detected in CNV co-localized with leucocytes. The influence of these inflammatory mediators has been reported in CNV only and any contribution to atrophic AMD has not yet been described.

Autoimmunity and autoantibodies

Serum markers of inflammation and antibodies have been inconsistently linked with AMD (Table 1.8.8). Anti-astrocyte⁹⁰ and anti-retinal⁹¹ auto-antibodies have been detected at an increased level in the serum of patients with AMD but have been detected in normal controls. It is also unclear whether these are a cause or an effect of retinal inflammation.⁹¹ The antibody associations are neither specific nor sensitive for the condition. As such, there is no role for routine serum assessment in AMD.

Complement pathway activation and AMD

The complement system is a cascade of over 30 proteins that interact in response to pathogen invasion or host cell injury.⁹² It involves the generation of enzymes, many of which are serine proteases that result in immune system activation, promotion of phagocytosis and cell membrane attack.⁹³ There are three established methods of complement pathway activation; i) the classical pathway, ii) the alternative pathway and iii) the lectin mediated pathway (Fig: 1.8.12). Drusen contain deposits of complement proteins. Immunostaining of retinal sections from patients with AMD for complement factors C5, C5a, C5b-9 have consistently shown to be associated within the deposits.^{83, 94-97} Complement factors C3, C3c, iC3b have been reported but with less consistent intensities.^{94, 95, 98} Complement regulatory factors have also been shown to be present in drusen. Vitronectin^{94, 95, 97, 99-101} and clusterin (Apolipoprotein J)^{95, 97, 101} are complement factor modulators and are found in large quantities within drusen. Factor B has also been localized to drusen.¹⁰² C-reactive protein (CRP), an acute phase reactant of the immune system with the potential to activate the classical complement pathway has also been detected in drusen.⁹⁴

Anaphylotoxins C3a and C5a

Enzymatic cleavage of factors C3 and C5 produces both large molecules and smaller molecules. The larger molecules C3b and C5b, respectively, bind to surface membranes of pathogens and host cells. This binding directs the action of the complement system to membrane surfaces and limits the spread of the cascade. The smaller products C3a and C5a freely diffuse and initiate general inflammatory cell responses. C5a in particular acts as a potent chemokine to promote the migration of phagocytic cells to the site of injury and may be responsible for macrophage and the dendritic cell infiltration of Bruch's

membrane in AMD. To support this, C3a and C5a have been identified in drusen deposits¹⁰³ and have the potential to increase the expression of VEGF by inflammatory cells.

The membrane attack complex (MAC)

Complement factor 5b binds to factors C6, C7 and C8 to form the C5b678 complex. This complex catalyses the polymerization of complement factor 9 to form transmembrane pores. These pores insert themselves into the target membranes and allow free passage of water. Cellular homeostasis is impaired, which results in complement-mediated cell lysis. Components of the MAC have been recorded in drusen and BLD and the suppression of the membrane attack complex reduces choroidal neovascularisation *in vivo* suggesting a role for the MAC in the pathogenesis of both dry and wet forms of AMD.^{104, 105}

Inhibition of Complement

The complement system, and the alternative pathway in particular, requires tight regulation to prevent inappropriate immune system activation and collateral damage to viable host cells. A number of inhibitors exist in soluble and membrane bound forms to suppress particular complement proteins. Circulating inhibitors include: i) Complement factor H (CFH), ii) Factor H-like 1 (FHL-1), iii) Complement factor I, iv) C4 binding protein, v) C1 inhibitor (C1INH), vi) Vitronectin, vii) Clusterin. Complement factor H CFH regulates complement activation by binding to C3b and competing with factor B to displace any attached Bb. It also acts as a cofactor to Factor I to inactivate C3b. It is the most abundant inhibitor in plasma and has been shown to co-localise with C3b in drusen. CFH also directly binds lipid oxidative stress products.¹⁰⁶ The role of factor H in the pathogenesis of AMD is strongly supported by genetic evidence (Chapter 2.3).^{103, 107-110}. Congenital deficiency in CFH results in type 2 membranoproliferative glomerulonephritis (MPGNII).¹¹¹

In addition to significant renal sequelae, patients with this disease demonstrate retinal accretions that highly resemble drusen, both clinically and biochemically.¹¹² Human retinal pigment epithelial cells have been shown to express complement factor H constitutively, perhaps as a means of self-preservation from complement-mediated attack. Serum levels CFH are elevated in AMD patients.¹¹³

This may be response to an increase in complement activity. Patients expressing genetic defects in factor H may not suppress complement activation as effectively leading to the increased risk. CFH knockout mice (*cfh*^{-/-}) develop changes in the retina and display increased complement activation.¹¹⁴ Since the discovery of the initial mutation in complement factor H, four additional mutations associated with complement system factors have been linked to macular degeneration.^{102, 115-117} Complement factor proteins affected by the AMD associated SNPs are illustrated in (Fig.1.8.13).

The inflammatory cascade must be initiated and there are a number of potential priming events for the inflammatory response in AMD. Oxidative stress at the level of the RPE has been shown to activate the complement pathway *in vivo* and produce a phenotype similar to atrophic AMD in mice.⁶⁴ Oxidised lipid products have been show to activate the serum complement proteins¹¹⁸ and oxidized by-products of photoreceptor cells reduce the expression of the protective factor H in retinal pigment epithelial cells.^{119,120} This implies that the inflammatory hypothesis is an extension of the oxidative stress hypothesis. Smoking is the most significant lifestyle risk factor associated with AMD¹²¹ and can directly activate C3 in plasma.¹²² Activated C3 also displays impaired binding to factor H in the presence of cigarette smoke.¹²³ In addition, smoke contains a high concentration of free radicals and oxidants and it is likely that it contributes to oxidative stress in the retina.¹²⁴ Low grade infections may also act as a trigger for the development of AMD. Prior infection with *Helicobacter Pylori*, Cytomegalovirus and *Chlamydia Pneumoniae* have been documented as more frequent in patients with AMD.¹²⁵ It is therefore possible that AMD represents a common biological response to a wide variety of pathological insults.

Anti-inflammatories and AMD

It is evident that inflammation plays a significant role in the pathology of AMD. As such, a number of therapies targeting inflammation have been investigated. Classical anti-inflammatory agents, immunosuppressive agents and biological agents have all been explored in AMD.¹²⁶ To date there have been no associations between either non-steroidal anti-inflammatory drugs (NSAIDs) or oral corticosteroid treatment and the prevention of AMD in humans.¹²⁷ In fact, aspirin has been associated with an increased risk of developing AMD. The cause for this is currently unknown.⁸²

Steroids exert anti-inflammatory effects through inhibiting the transcription of inflammatory cytokines, inhibit the expression of I-CAM, reducing permeability and expression of bFGF. The role of oral corticosteroids in the suppression of CNV was described Dees et al in cases of posterior uveitis.¹²⁸ Fourteen patients (17 eyes) with CNV in endogenous posterior uveitis were treated with a single dose of oral corticosteroid followed by low-dose regimens of oral corticosteroids and/or cyclosporine. At a mean follow up time of 15 months, 9 of 17 eyes had improved and 6 remained stable indicating a role for oral immunosuppression in the treatment of active CNVs.¹²⁸ In cases of AMD, intravitreal corticosteroids have been explored more thoroughly though systemic dexamethosone and intravitreal triamcinolone have suppressed the volume of laser induced CNV in rats.^{129, 130} A regimen of intravitreal dexamethosone combined with intravitreal anti-VEGF and PDT has been described as “triple therapy”. The relatively short action of dexamethosone may confer a reduced risk of intraocular pressure spikes. Several reports indicate that triple therapy can stabilize CNV and reduce the number of treatments required in the long term though a large scale RCT has not yet been performed.^{131, 132} It was noted, however, that patients treated previously with anti-VEGF agents did not respond as well to the triple therapy as treatment naïve patients.

Triamcinolone has approximately one fifth of the potency of dexamethosone but has a considerably longer duration of action.¹³³ There is conflicting evidence on the role of intravitreal triamcinolone in CNV treatment with Gilles et al reporting no improvement¹³⁴ and Jonas et al reporting improvements of over 2 or more Snellen lines in 37.4%.¹³⁵ The improvements appeared transient, with a return to pre-treatment vision or worse after one year.¹³⁶ Steroid treatments have since been surpassed,

however, by superior anti-VEGF antibody medications for the treatment of neovascular AMD.¹³⁷ Since 2007, there have been no further reports of large studies on the use of intravitreal triamcinolone alone in the management of CNV however it still plays a role in triple therapy combined with anti-VEGF and PDT treatment modalities. Implantable intravitreal steroid devices that allow a sustained release (fluocinolone acetonide) had progressed to Phase 2 clinical trials but have not yet progressed to phase 3.¹³⁸

Immunosuppressive agents in the treatment of AMD have also been explored. Methotrexate is an inhibitor of dihydrofolate reductase. It is commonly used as a corticosteroid-sparing agent in inflammatory conditions. A case report and a small pilot study of 7 patients have described the potential for intravitreal methotrexate in cases of CNV refractory to anti-VEGF treatments.^{139, 140} This treatment did not show any toxic effects on the retina however the numbers were not sufficiently large enough to show a beneficial effect. Systemic administration of methotrexate has not been evaluated in the treatment of AMD. Rapamycin (Sirolimus) is an immunosuppressive agent that inhibits mammalian target of rapamycin (mTOR) that mediates cell survival and biological agent growth, proliferation and hyperpermeability.¹³³ A phase I clinical trial of systemic immunosuppression of 3 AMD patients with rapamycin supplemented with intravitreal anti-VEGF has been reported.¹⁴¹ Clinical trials investigating the benefit of rapamycin in geographic and neovascular AMD have been registered but not yet reported.¹³³

Treatment with systemic administration of biological agents has also shown promise in treating neovascular AMD. Markomichelakis et al reported membrane regression in three patients with CNV undergoing treatment with regular infusions of infliximab to treat coexisting rheumatoid arthritis.¹⁴² A case series of three patients who were refractive to anti-VEGF treatments were treated with two monthly intravitreal infliximab injections.¹⁴³ All three patients showed improvement during treatment but two regressed after treatment cessation. Giganti et al reported four patients treated with intravitreal infliximab that displayed worsening vision in all measured parameters.¹⁴⁴ The reporters concluded that intravitreal infliximab administration was not effective and may be retinotoxic.

Daclizumab is an IgG monoclonal antibody that binds to the α -chain of the IL-2 receptor. Suppression results in decreased activation and proliferation of T cells. Nussenblatt et al reported four patients treated with daclizumab to supplement standard anti-VEGF treatments however the numbers were insufficient to detect a benefit.¹⁴⁵

In conclusion, the inflammatory hypothesis of AMD has the strongest basis, encompassing the oxidative stress data, drusen biochemistry and genetic evidence. Inflammatory modulators and suppressors are currently the most widely studied area in the treatment of AMD.

1.7. Risk factors associated with AMD

Age-related macular degeneration is a typical example of a disease with a multifactorial aetiology. In multifactorial genetic diseases, inherited risk or protective factors influence disease development by raising or lowering the threshold of developing AMD (Fig. 1.8.14). A genetic predisposition to the disease, in concert with the aging process and destructive environmental risk factors lowers the threshold to the greatest effect and these patients are likely to present earlier. People with a protective genotype and protective environment will have a threshold so high that they may never manifest any pathology. Genetic factors, non-modifiable environmental and modifiable environmental risk factors therefore influence the development of AMD. The risk factors that contribute to the likelihood of developing AMD may be divided into genetic, demographic and environmental (Table 1.8.7).

1.7.1. Genetic Risk Factors

The genetic risk factors associated with the development of AMD may be divided into risk conferring genes and protective genes, and will be discussed in the review of the literature on genetics in AMD (Section 2.2).

1.7.2. Demographic Risk Factors

Age

Age is the most significant factor in the development of AMD.¹⁶ Every study on the epidemiology of AMD has correlated the prevalence AMD with age¹⁴⁶ however, the development of AMD must be regarded as a distinct pathological process rather than a process of normal ageing. A number of changes within the retinal structure occur that are associated with normal ageing.⁴ Ageing changes do not inevitably lead to AMD but in combination with additional AMD risk factors they may facilitate the development of the disease (Table 1.8.8). Over time macular pigment density in the neural retina¹⁴⁷ and melanosome density in the RPE¹⁴⁸ both decrease. These molecules provide antioxidant protection to the highly metabolic retinal tissue. The reduction of this protection may exacerbate the effects of oxidative stress.⁹ The ability of the RPE cells to phagocytose rod outer segments is impaired with age.¹⁴⁹ This leads to the accumulation of the end products of metabolism in photoreceptors and RPE cells. Lipofuscin is a non-degradable substance that accumulates in lysosomes, and has been observed in ageing tissues including the RPE.¹⁵⁰

In the retina, lipofuscin accumulates as a derivative of the visual cycle metabolic pathway. At ten years of age 1% of RPE cells display evidence of lipofuscin accumulation. This increases to over 19% at 90 years of age.¹⁵¹ Lipofuscin, originally thought to be inert, reacts to light and oxygen to produce reactive oxygen intermediates that exert an additional oxidative stress on the macula.¹⁵² Finally, a number of changes occur in the retinal-choriocapillaris complex over time. Ageing RPE cells become pleomorphic and deposit extracellular material in the Bruch's membrane and the sub-retinal space. The activity of matrix metalloproteinases (MMP) decreases with age leading to thickening of the Bruch's membrane.¹⁵² It may increase by up to 135% over 100 years.¹⁵³ With age the choriocapillaris decreases in both thickness and lumen diameter. By the tenth decade it may have only retained less than half of its original thickness (43%).¹⁵³ The thickened Bruch's membrane and the reduced ocular blood flow may impair the diffusion of oxygen and nutrients from the choriocapillaris and the removal of the waste products of the RPE. There is considerable debate as to when the transition point between normal age-related changes of the retina become eligible to be considered as a pathological entity.¹⁵⁴ It is possible that the combination of reduced antioxidant protective mechanisms, increased oxidative

stress and compromised blood flow and diffusion between the choriocapillaris and RPE may all combine to form a predisposition to AMD with age.

Race

There is a higher risk of developing AMD among the Caucasian population compared to the Black or Hispanic populations (See chapter 1.4). The prevalence of AMD in the Asian population is higher in men but lower overall when compared to their Caucasian counterparts. Increased melanin pigmentation has been associated with reduced accumulation of lipofuscin *in vitro*¹⁵⁵ and pigmented animals, *in vivo*, are less prone to the development of CNV.¹⁵⁶

Sex

The pooled findings of three large Caucasian patient cohorts found that there was no significant gender predisposition in the Caucasian population.³⁹ In the Asian population, there is a significant difference, with men being more likely to develop AMD than women in China.⁵⁰ This may be due to the increased smoking rates in Asian men as discussed previously. In the Western population there is a very slight increased prevalence in women over 75; however this may be confounded by the longer life span and greater uptake of medical services by the female group.¹⁵⁷

Hypertension

AMD has been associated with both systemic hypertension and treatment with antihypertensive medications.²³ In the ten-year follow up data of the Beaver Dam eye study, patients with uncontrolled hypertension were over three times as likely to develop neovascular AMD (3.29) than normotensive participants. Similar results were found among the Latino population.¹⁵⁸ Participants with controlled hypertension reduced this to a relative risk of 2.29 (CI 1.12-4.69).⁷⁶ The AREDS trial showed a more modest relative risk for the development of exudative AMD of 1.5 in patient with controlled hypertension.²³ In the presence of established unilateral CNV, the Macular Photocoagulation Study found systemic hypertension to be a significant factor in the progression to CNV in the fellow eye.¹⁵⁹

Atherosclerosis

The association between clinical cardiovascular disease and AMD is not consistent. The Beaver Dam trial did not find an association between macular degeneration and myocardial infarction or stroke.

Three cohort studies and six case control studies have not found any consistent connection with cerebrovascular accident.¹⁴⁶ In the Rotterdam eye study, participants underwent carotid ultrasound assessment to determine whether there was a predisposition to atherosclerosis in patients with AMD.³⁸ Participants with carotid plaques were five times more likely to have an advanced form of AMD. These observations support the vascular hypothesis of AMD which compares the pathological changes of the Bruch's membrane in AMD with the vascular changes noted in atherosclerosis.⁷⁸ Although it is consistently associated with cardiovascular disease, diabetes has not been associated with AMD in the Beaver dam, Blue Mountains, Rotterdam or the Framingham Eye studies.

1.7.3. Clinical Risk Factors

Soft Drusen and RPE changes

The presence of moderate to large-sized drusen was identified by the AREDS trial as a significant risk factor for the development of both forms of late AMD, GA and CNV.²³ Small well-circumscribed drusen are typical features of the ageing retina and are not risk factors for AMD. The AREDS study described a progression rate of early AMD (few isolated hard drusen) to advanced AMD of 1.3% over 5 years. This increased, however, to 18% in the presence of multiple intermediate or large drusen. The Macular Photocoagulation Study (MPS) established that the presence of five or more drusen confers a relative risk of 2.1.^{159, 160} Areas of focal hyperpigmentation are associated with a relative risk of 2.0.^{159, 160}

Hyperopia

In the Rotterdam study of 497 affected patients, it was found that the risk of developing AMD is increased by 5% ⁷⁷ for each increase of +1 dioptre of hypermetropia but these findings were not borne out in either the Beaver dam or Blue mountains study.

CNV in the fellow eye

The presence of unilateral CNV is a highly significant risk factor for developing CNV in the second eye. It confers an annual incidence of 5-14% of developing CNV with a peak incidence of 4 years after the initial onset.¹⁶¹ and the progression to legal blindness within 5 years is 12%.

Raised White Cell Count, fibrinogen and CRP

The Beaver Dam study found an association between elevated white cell count at baseline assessment and an increased 10-year incidence of AMD.⁷⁶ Increased serum fibrinogen has been associated with

late AMD (OR 1.45).¹⁴⁶ C-reactive protein is also a non-specific marker of inflammation. A recent meta-analysis demonstrated that elevated levels of CRP (3>mg/L) were associated with an odds ratio of 1.69 in AMD.¹⁶² These serum findings are non-specific however consistent with a state of low-grade inflammation and the inflammatory hypothesis of AMD (Chapter 1.6.3).

Previous Cataract Surgery

History of previous cataract surgery is also a significant risk factor for the development of neovascular AMD with an estimated relative risk of 3.05 (CI 2.05-4.55).¹⁴⁶ It was also associated with a threefold increased risk of GA.¹⁶³ It is possible that the removal of cataract and replacement with an intraocular lens permits greater penetration of high-energy light wavelengths to the retina. This may result in a greater oxidative stress to an aged retina with reduced antioxidant capacity that results in the promotion of the development of AMD.

1.7.4. Environmental Risk Factors

Cigarette Smoke Exposure

Cigarette smoking is a risk factor for a number of ophthalmic conditions, and has been consistently shown to be the leading environmental risk factor in the development of AMD.⁵⁸⁻⁶⁰ Active smokers are at an increased risk of developing AMD of two to four-fold. The pathophysiology of cigarette smoke in AMD will be reviewed (Chapter 2.4).

Blue/UV light damage

The visible light spectrum extends from approximately 400nm to 770nm. Light of a wavelength less than 40nm is in the high-energy ultraviolet (UV) spectrum whereas wavelengths greater than 750nm are in the infrared range. Most of the UV light is absorbed by the cornea and lens. Some radiation does, however, penetrate through to the retina. Based on the oxidative stress hypothesis of AMD, it is possible that prolonged exposure to high energy light leads to the development of AMD.¹⁶⁴ When images are refracted by the eye, they fall on to the fovea, and this area is subject to the greatest amount of irradiance. An increased risk of developing AMD has been observed in aphakic eyes compared with phakic eyes.¹⁶⁴ This may be attributed to the absorption of high-energy blue wavelength light by the natural lens. There is a small correlation between long term UV light exposure and risk of AMD.¹⁶⁵

Accurate assessments of the contribution of light damage to AMD are hampered by estimates of lifetime exposure to light. It is not possible to objectively measure the amount of light exposure accumulated by an individual over the course of a lifetime.

Diet, fat consumption.

The contribution of dietary fat to the development of AMD was assessed both by the Nurses' Health Study and the Health Professional Follow-Up study. Total fat intake is associated with AMD, with the highest quintile of fat consumption associated with a relative risk of 1.54.¹⁶⁶ High amounts of alcohol intake have been associated with early AMD, though not late AMD.¹⁶⁷ Cho et al have also demonstrated that intake of 4 or more servings of fish a week was associated with a 35% lower risk of developing AMD when compared with an intake of 3 servings or fewer per month.¹⁶⁶ Patients with AMD may benefit from anti-oxidant vitamin supplementation and dietary fish oils. These claims are currently under assessment in the AREDS2 trial.

Body mass index

Body mass index (BMI) assessment has correlated a higher BMI with an increased risk of developing both atrophic and neovascular AMD.^{23, 168} In a prospective study in a Caucasian population, obesity (BMI<30) conferred an odds ratio of developing neovascular AMD of 1.28.¹⁴⁶ It was also noted that patients in the Indian population with extremely low BMIs were at an increased risk of atrophic AMD changes.¹⁶⁸

Serum cholesterol and use of statins

There has been no significant association between elevated serum levels of total serum cholesterol, high-density lipoprotein or low-density lipoprotein cholesterol.¹⁴⁶ HMG Co Reductase medications (statins) exhibit cholesterol lowering, anti-inflammatory and anti-oxidant effects. The use of statin medication has suppressed CNV development in a rat model.¹⁶⁹ There is no association, however, with use and a reduction in the progression of AMD.¹⁷⁰ There have been two randomized control trials on the effects of statins in preventing the progression of age-related macular degeneration. Neither trial provides evidence to support the use of statins though the final reports will not be available until 2012.¹⁷¹

Figures and tables

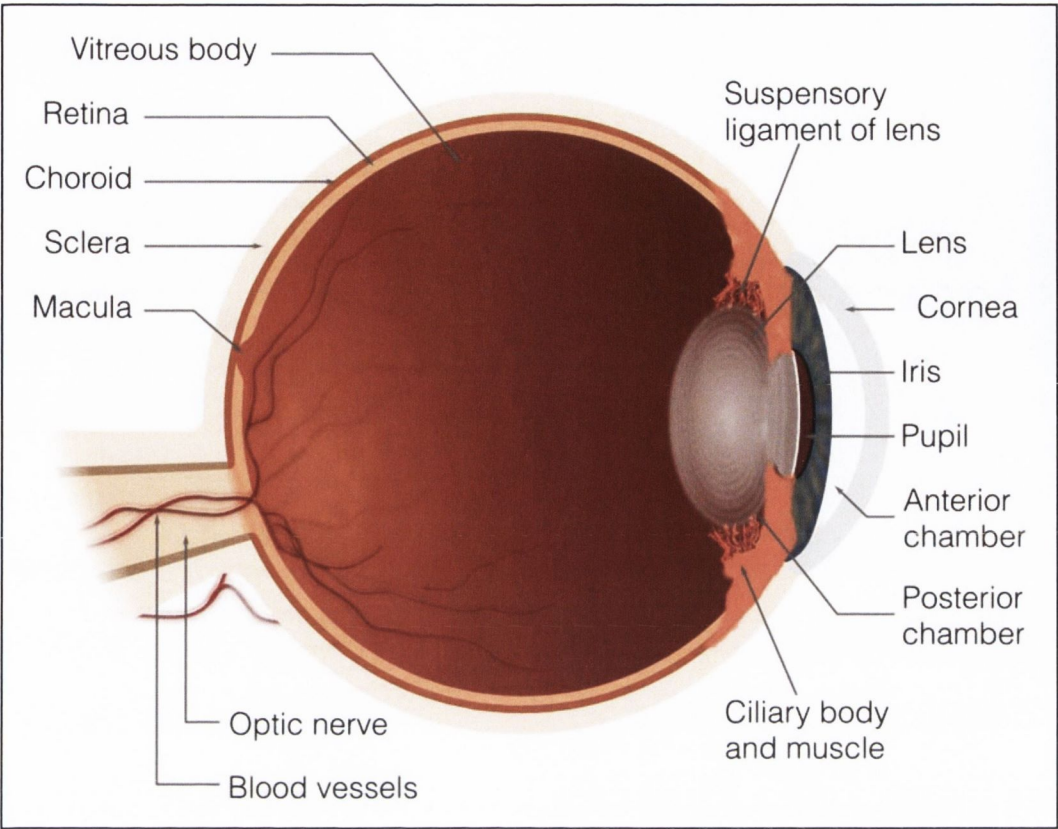


Figure 0.1: General anatomy of the eye

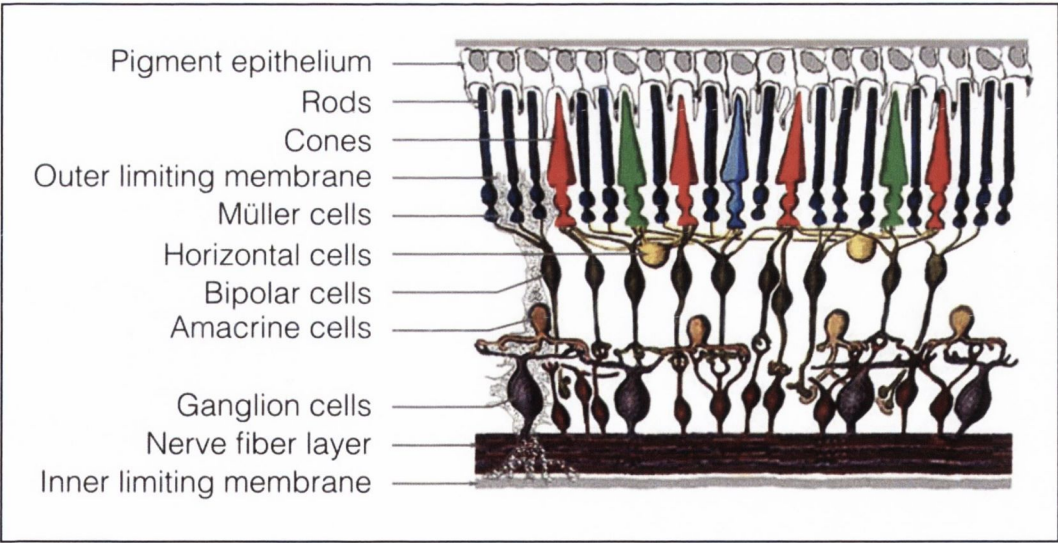


Figure 0.2: Layers of the retina (www.theness.com/neurologicablog)¹⁷²

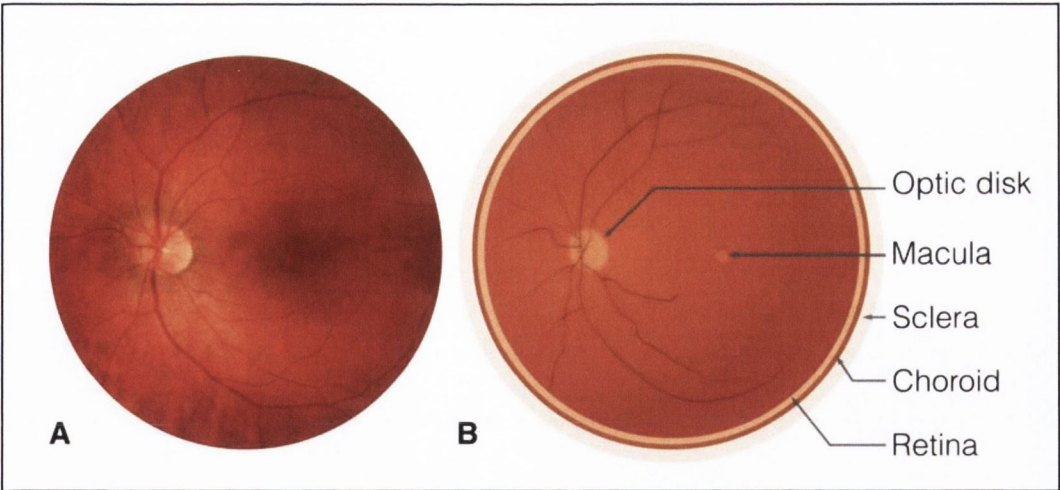


Figure 0.3: A. Normal retina (researcher's own). B. Schematic representation

Classifications of AMD		
	Atrophic (Dry)	Neovascular (Wet)
% of AMD	85-90% of all AMD	10-15% of all AMD
% of blindness associated with AMD	10% of all visual acuity <6/60 associated with AMD. Predominantly due to Geographic Atrophy.	90% of all visual acuity <6/60 associated with AMD.
Mild	Age-related maculopathy (ARM) Early AMD	-
Intermediate	Intermediate AMD	-
Advanced	Geographic Atrophy	Choroidal Neovascularisation

Table 0.1: 1.8.1: Classifications of AMD

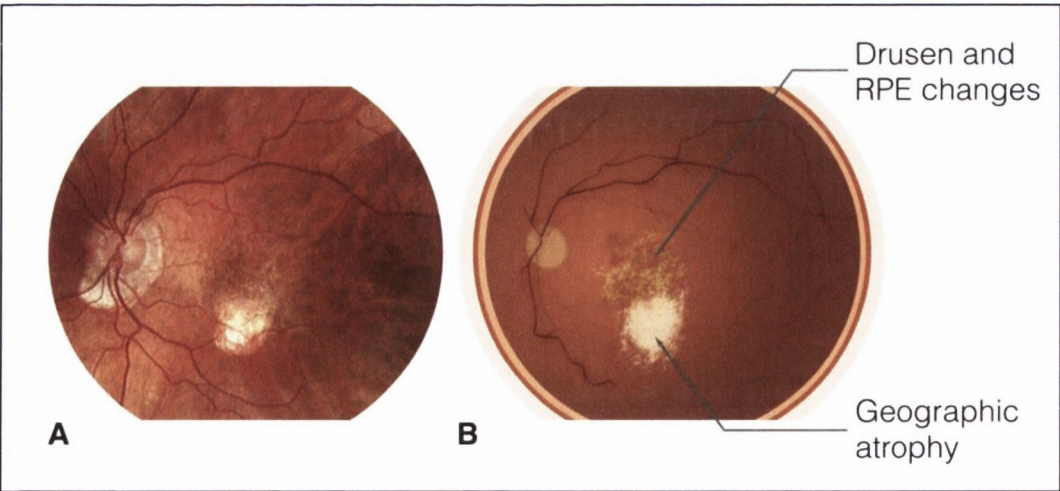


Figure 0.4: A. Atrophic AMD. B. Schematic representation

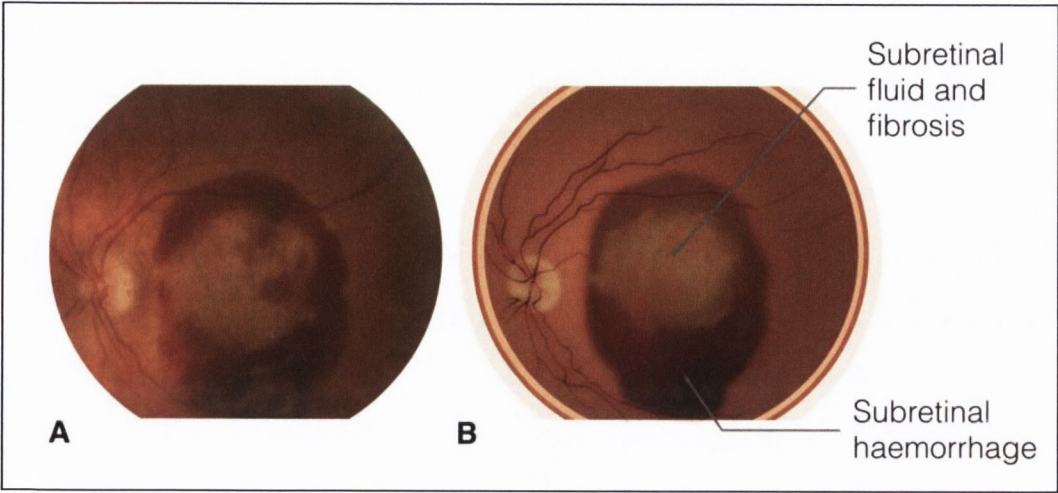


Figure 0.5: A. Neovascular AMD. B. Schematic representation

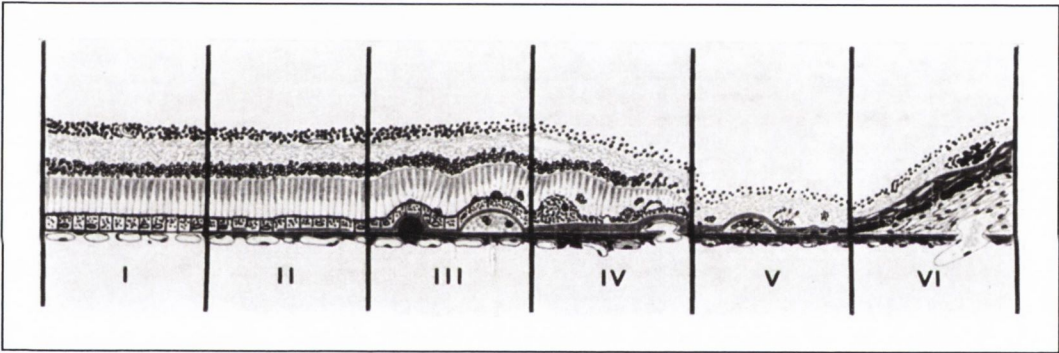


Figure 0.6: Histological grading of RPE changes Sarks et al²⁰

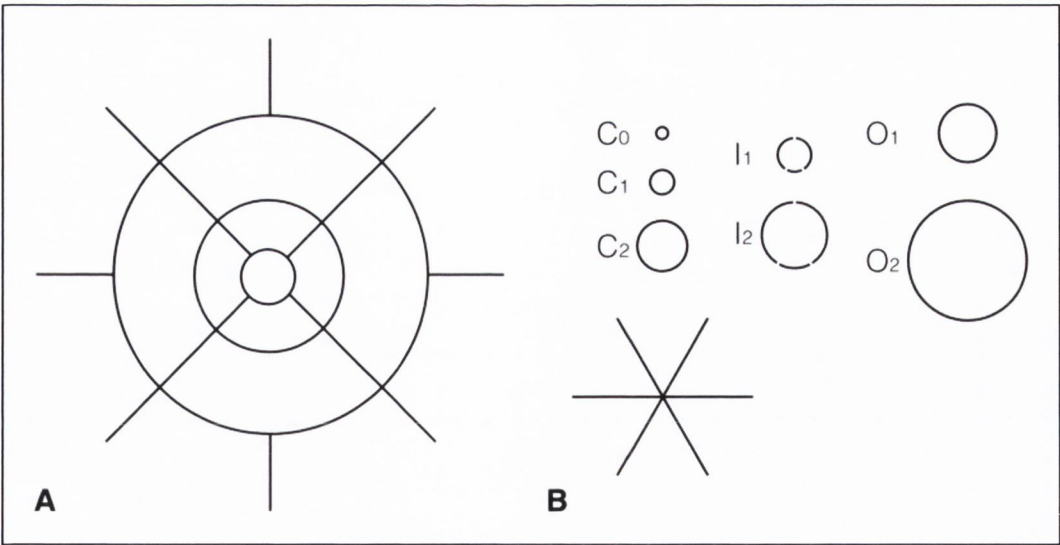


Figure 0.7: A WARMGS Subfield definition grid B: Drusen grid³⁰

Wisconsin Age-Related Maculopathy Drusen Grading Definitions

Criterion	Grade	Definition
Size	0	No Drusen
	1	Drusen Indistinct
	2	Drusen < 63µm
	3	Drusen 63-125µm
	4	Drusen 125-250µm
	5	Drusen >250µm
	6	Reticular Drusen
	7	Cannot grade- obscuring lesion
Type	8	Cannot grade- photographic quality
	0	Hard - indistinct
	1	Hard - distinct
	2	Soft - distinct
	3	Soft - indistinct
	4	Reticular
	5	Faded
	6	Cannot grade- obscuring lesion
	7	Cannot grade- photographic quality

Wisconsin Age-Related Maculopathy Drusen Grading Definitions		
Criterion	Grade	Definition
Area Covered	0	Area covered by drusen < 63µm
	1	Area covered by drusen <125µm
	2	Area covered by drusen <1.6% of subfield
	3	Area covered by drusen <3.1% of subfield
	4	Area covered by drusen <6.3% of subfield
	5	Area covered by drusen <12.5% of subfield
	6	Area covered by drusen <25% of subfield
	7	Area covered by drusen <50% of subfield
	8	Area covered by drusen >50% of subfield
Drusen Confluence		None
		Questionable
		< 10%
		10-24%
		25-49%
		50% or more

Table 0.2: WARMGS Drusen classification (Klein et al³⁰)

International Classification and Grading for Age-related Maculopathy and Age-related Macular Degeneration

Drusen		Pigmentary Changes		Geographic Atrophy		Neovascular AMD	
Stage	Morphology	Stage	Hyperpigmentation	Stage	Presence	Stage	Presence
0	Absent	0	Absent	0	Absent	0	Absent
1	Questionable	1	Questionable	1	Questionable	1	Questionable
2	Hard drusen <125µm	2	Present <63µm	2	Present >125µm	2	Present
3	Intermediate soft 63-125µm	3	Present >63µm	7	Cannot grade obscuring lesions	7	Cannot grade obscuring lesions
4	Large soft distinct >125µm	7	Cannot grade obscuring lesions	8	Cannot grade photo quality	8	Cannot grade photo quality
5	Large soft indistinct >125µm	8	Cannot grade photo quality				
7	Cannot grade obscuring lesions				Location		Typifying features
8	Cannot grade photo quality		Hypopigmentation	1	Outside outer circle	1	Hard exudates
		0	Absent	2	In outer subfield	2	Serous neuroretinal detachment
	Number	1	Questionable	3	In middle subfield	3	Serous RPE detachment
0	Absent	2	Present <63µm	4	In central subfield	4	Haemorrhagic RPE detachment
1	Questionable	3	Present >63µm	7	Cannot grade obscuring lesions	5	Retinal Haemorrhage

International Classification and Grading for Age-related Maculopathy and Age-related Macular Degeneration

Drusen		Pigmentary Changes		Geographic Atrophy		Neovascular AMD	
2	1 to 9	7	Cannot grade obscuring lesions	8	Cannot grade photo quality	6	Scar/glial/fibrosis tissue
3	10 to 19	8	Cannot grade photo quality			7	Cannot grade obscuring lesions
4	>19				Area Covered	8	Cannot grade photo quality
7	Cannot grade obscuring lesions		Location of pigmentary changes	1	175-250 μ m		
8	Cannot grade photo quality	1	Outside outer circle	2	250-500 μ m		Location
		2	In outer subfield	3	500-1000 μ m	1	Outside outer circle
	Size	3	In middle subfield	4	1000-3000 μ m	2	In outer subfield
1	<63 μ m	4	In central subfield	5	3000-6000 μ m	3	In middle subfield
2	63-124 μ m	7	Cannot grade obscuring lesions	6	>6000 μ m	4	In central subfield
3	125-175 μ m	8	Cannot grade photo quality	7	Cannot grade obscuring lesions	7	Cannot grade obscuring lesions
4	175-250 μ m			8	Cannot grade photo quality	8	Cannot grade photo quality
5	>250 μ m						
7	Cannot grade obscuring lesions						Area Covered
8	Cannot grade photo quality					1	175-250 μ m
						2	250-500 μ m
	Location					3	500-1000 μ m
1	Outside outer circle					4	1000-3000 μ m

International Classification and Grading for Age-related Maculopathy and Age-related Macular Degeneration							
Drusen		Pigmentary Changes		Geographic Atrophy		Neovascular AMD	
2	In outer subfield					5	3000-6000µm
3	In middle subfield					6	>6000µm
4	In central subfield					7	Cannot grade obscuring lesions
7	Cannot grade obscuring lesions					8	Cannot grade photo quality
8	Cannot grade photo quality						
	Area						
1	<10%						
2	<25%						
3	<50%						
4	>50%						
7	Cannot grade obscuring lesions						
8	Cannot grade photo quality						

Table 0.3: Summarised International ARM/AMD grading system³¹

Prevalence of AMD by age	
55-64 years	0.21%
65-74 years	0.85%
75-84 years	4.59%
> 85 years	13.05%

Table 0.4: Prevalence of AMD by age³⁹

Summary of prevalence of AMD based on large population studies							
Study	Country	Number of participants	Total prevalence of AMD (%)	Prevalence of GA (%)	Prevalence of CNV (%)	Grading classification	Year of publication
Caucasian population studies							
Beaver Dam Eye Study ³⁶	United States	4756	1.51	0.44	0.88	WARMGS	1992
Blue Mountains Eye study ³⁷	Australia	3585	1.81	0.66	1.2	WARMGS	1995
Rotterdam Eye Study ³⁸	Netherlands	6411	1.62	0.69	0.95	WARMGS	1995
Reykjavik Eye Study ¹⁷³	Iceland	1021	3.5	3.2	0.7	WARMGS	2003
EUREYE Study ¹⁷⁴	European	4753	3.32	1.2	2.29	IntARM	2006
Baltimore Eye Study ⁴²	United States	2518	3.47	2.51	1.82	IntARM	2003
Salisbury Eye Evaluation ⁴¹	United States	1854	1.2	1.8	1.7	Modified WARMGS	2008
PADMI EyeStudy ¹⁷⁵	Italy	845	4.1	1.61	2.1	IntARM	2011
MESA Study ¹⁷⁶	United States	1125	5.4		0.8	WARMGS	2006
Black population studies							
Baltimore Eye Study ⁴²	United States	1843	0.21	0.1	0.11	IntARM	2003
Barbados Eye Study ⁴⁰	West Indies	3444	0.59	0.12	0.48	Specific to the study	1995
Salisbury Eye Evaluation ⁴¹	United States	666	0.5	0.3	1.1	Modified WARMGS	2008

Summary of prevalence of AMD based on large population studies							
Study	Country	Number of participants	Total prevalence of AMD (%)	Prevalence of GA (%)	Prevalence of CNV (%)	Grading classification	Year of publication
MESA Study ¹⁷⁶	United States	725	2.4		0.2	WARMGS	2006
Hispanic population studies							
Los Angeles Latino Eye Study ⁴⁴	United States	6357	0.21	0.1	0.11	WARMGS	2004
MESA Study ¹⁷⁶	United States	615	4.2		0.2	WARMGS	2006
Asian population studies							
Singapore Malay Eye study ⁴⁹	Singapore	3265	0.70	0.31	0.46	WARMGS	2008
Hisayama Eye Study ⁴⁸	Japan	1486	0.87	0.20	0.67	WARMGS	2001
Funagata Eye Study ⁵²	Japan	1758	0.5	-	-	WARMGS	2008
Andhra Pradesh Eye Disease Study ⁴⁵	India	3723	1.8	-	0.11	IntARM	2005
Beijing Eye Study ⁵¹	China	4376	0.3	-	-	IntARM	2006
Handan Eye Study ⁵⁰	China	6581	0.4	-	0.4	WARMGS	2011
MESA Study ¹⁷⁶	United States	347	4.6		1.4	WARMGS	2006
The India Eye study ⁴⁶	India	585	6.7		1.2	WARMGS	2010

Table 0.5: Summary of prevalence of AMD based on large population studies

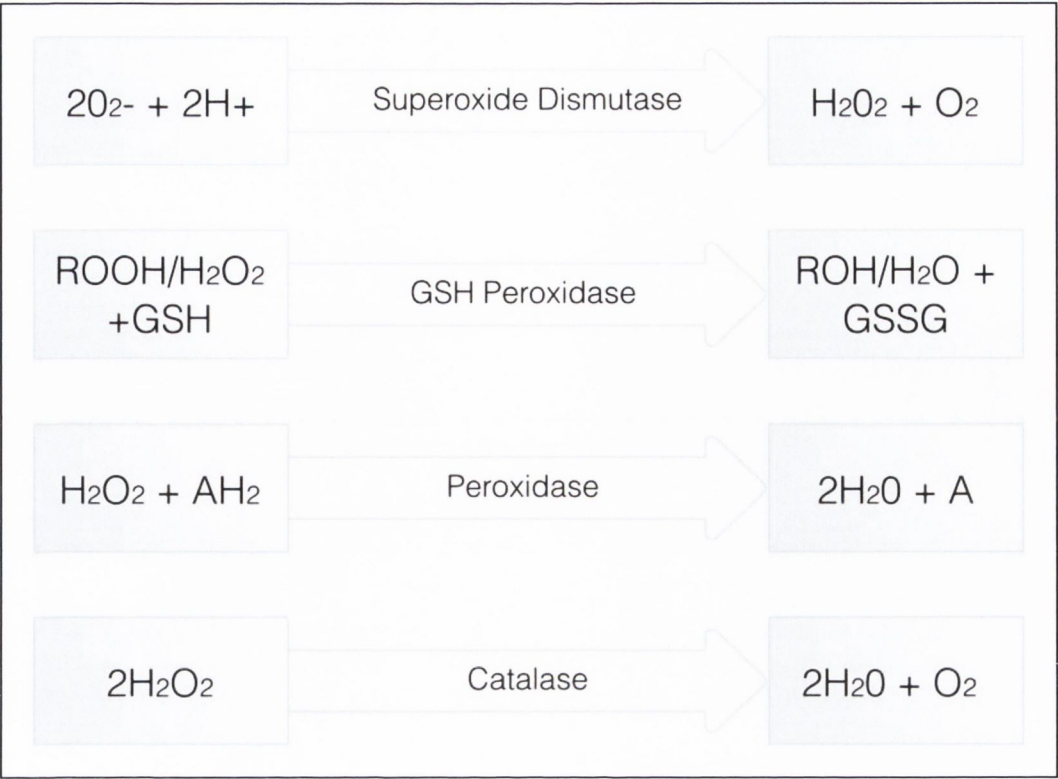


Figure 0.8: Metabolism of ROS by enzymatic antioxidants¹⁷⁷

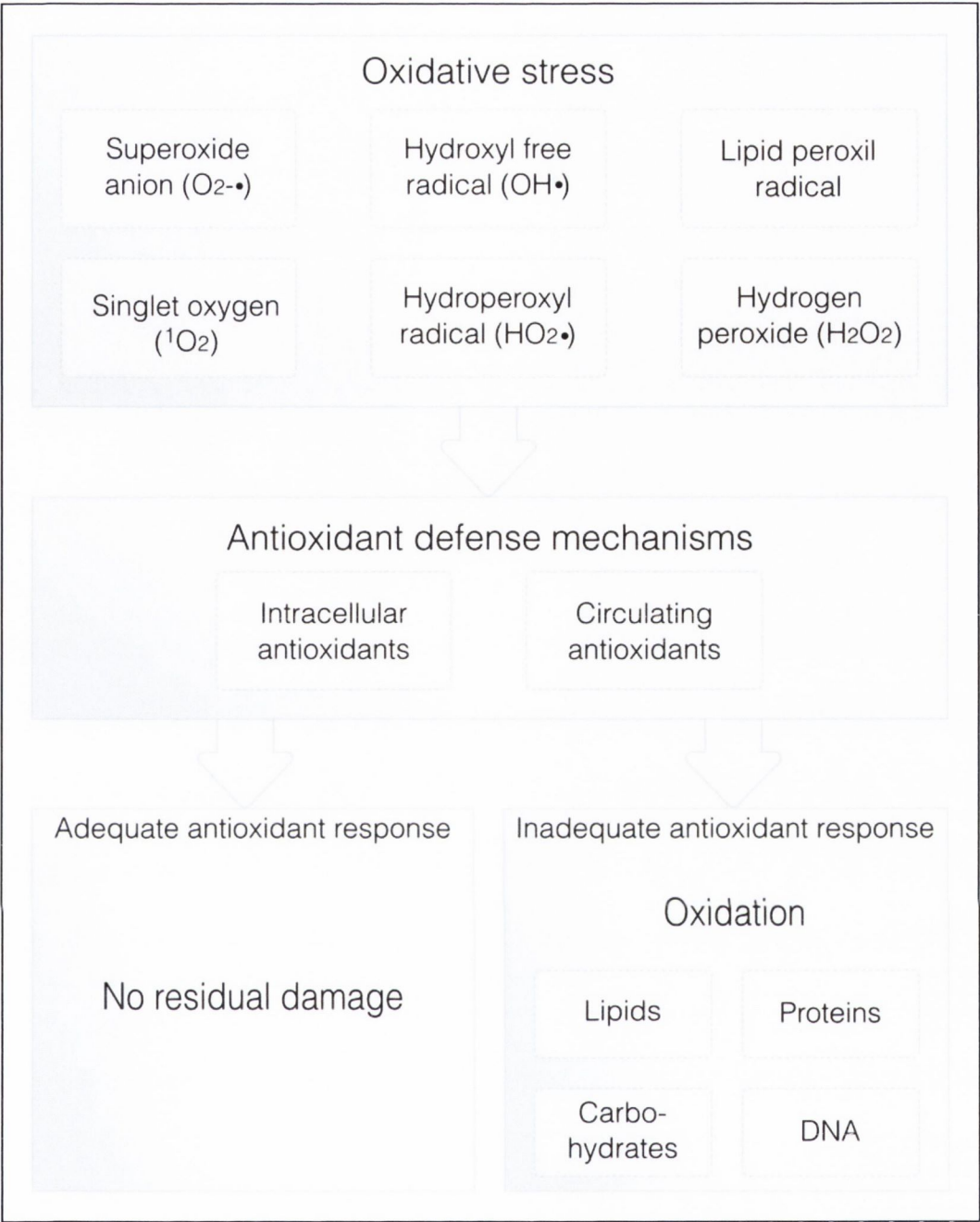


Figure 0.9: Oxidative stress model of AMD

<i>AREDS Antioxidant preparation</i>
15mg beta carotene
500mg vitamin C
400IU vitamin E
80mg zinc (oxide)
2mg copper (cupric oxide)

Table 0.6: AREDS study antioxidants⁵⁵

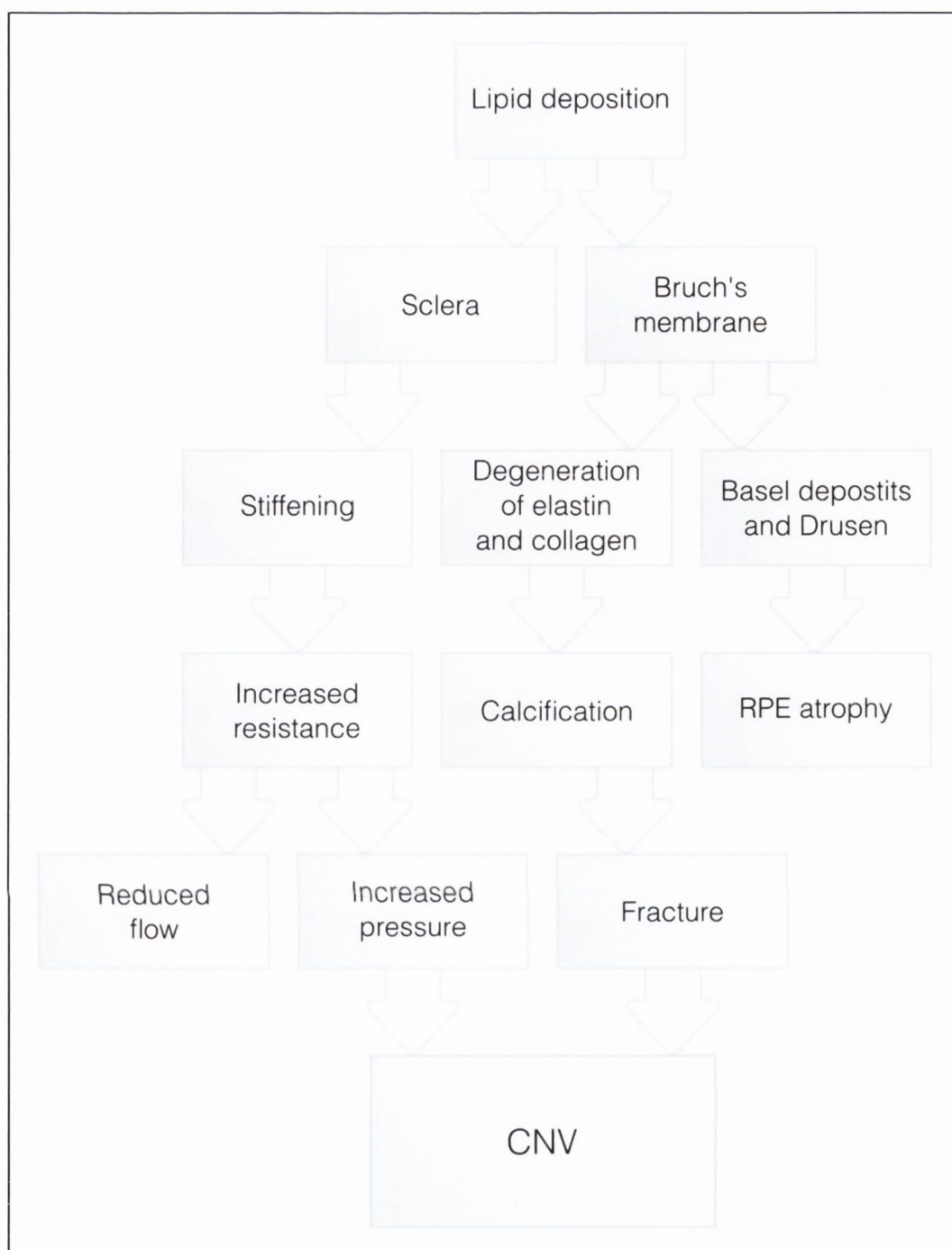


Figure 0.10: The Vascular model of AMD.⁷⁴

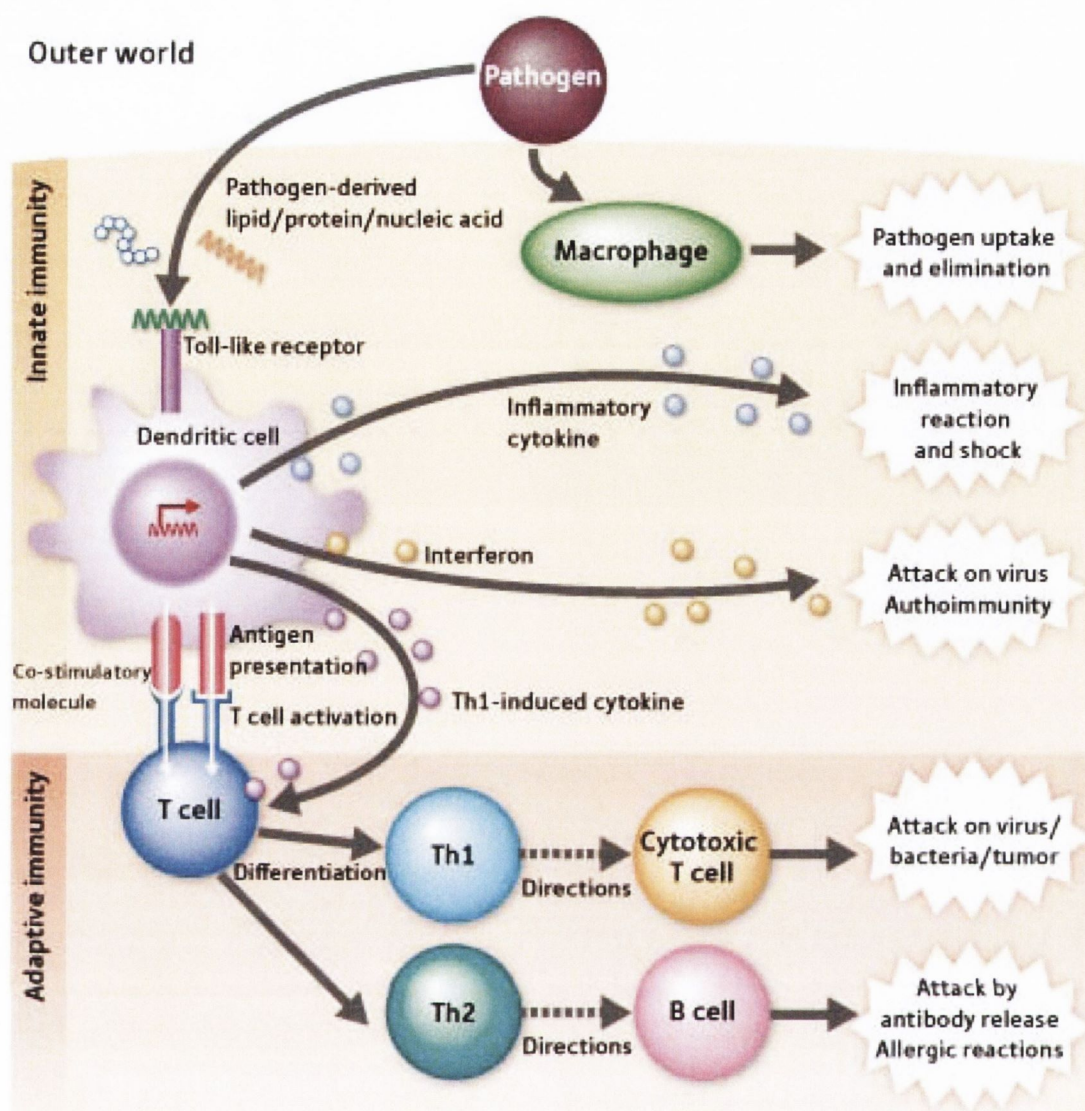


Figure 0.11: The innate and adaptive immune systems
(<http://www.rikenresearch.riken.jp/eng/frontline/5028>)

Inflammatory mediators found within drusen

Dendritic cell processes (HLA-DR, CD antigens) ^{82, 83}
Complement factors C1q ^{178, 94} ,
C3cVariable ⁹⁴ , C3 positive ⁹⁵ , C5 ^{83, 94-96} C5a, C5b-9 ^{83, 94, 96, 97}
iC3b ⁹⁵
C4
Vitronectin ^{94, 95, 97, 99-101}
Immunoglobulin lambda chains, kappa inconsistent ⁹⁴
IgG ^{83, 96, 179}

<i>Inflammatory mediators found within drusen</i>
IgM ¹⁷⁹
Apolipoprotein E
MHC Class 2 antigens ⁸³
Clusterin ^{95, 97}
Factor B ¹⁰²
Amyloid P ⁹⁴
Albumin
Amyloid
Apolipoprotein B and E
Complement receptor 1
Enolase 2
Phospholipids
TIMP metalloproteinase inhibitor 3
Retinal dehydrogenase

Table 0.7: Inflammatory mediators found within drusen.

<i>Retinal antigens associated with AMD</i>
Alpha-crystallin ¹⁸⁰
Annexin II ¹⁸¹
Carboxyethylpyrrole (CEP) adducts ⁶⁵
Enolase ¹⁸⁰
Glial fibrillary acid protein (GFAP) ¹⁸⁰

Table 0.8: Retinal antigens associated with AMD

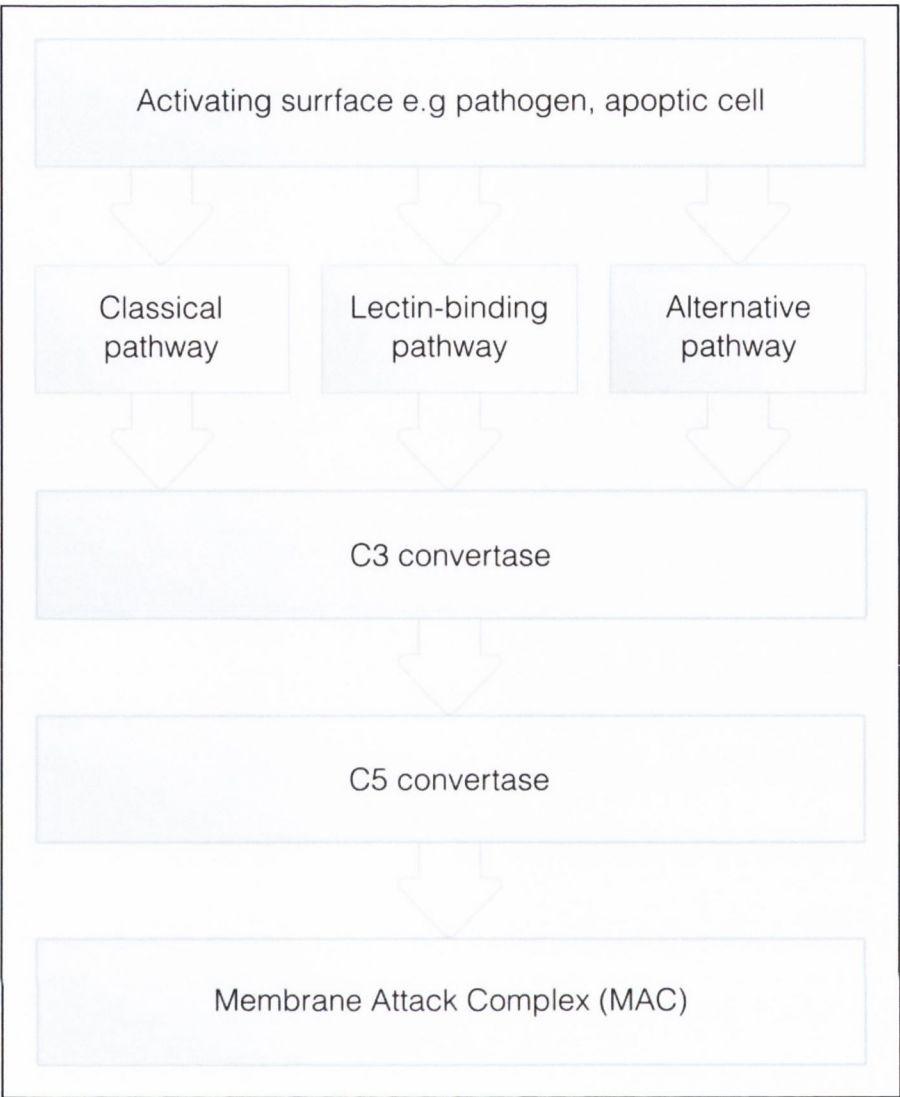


Figure 0.12: Overview of the complement system

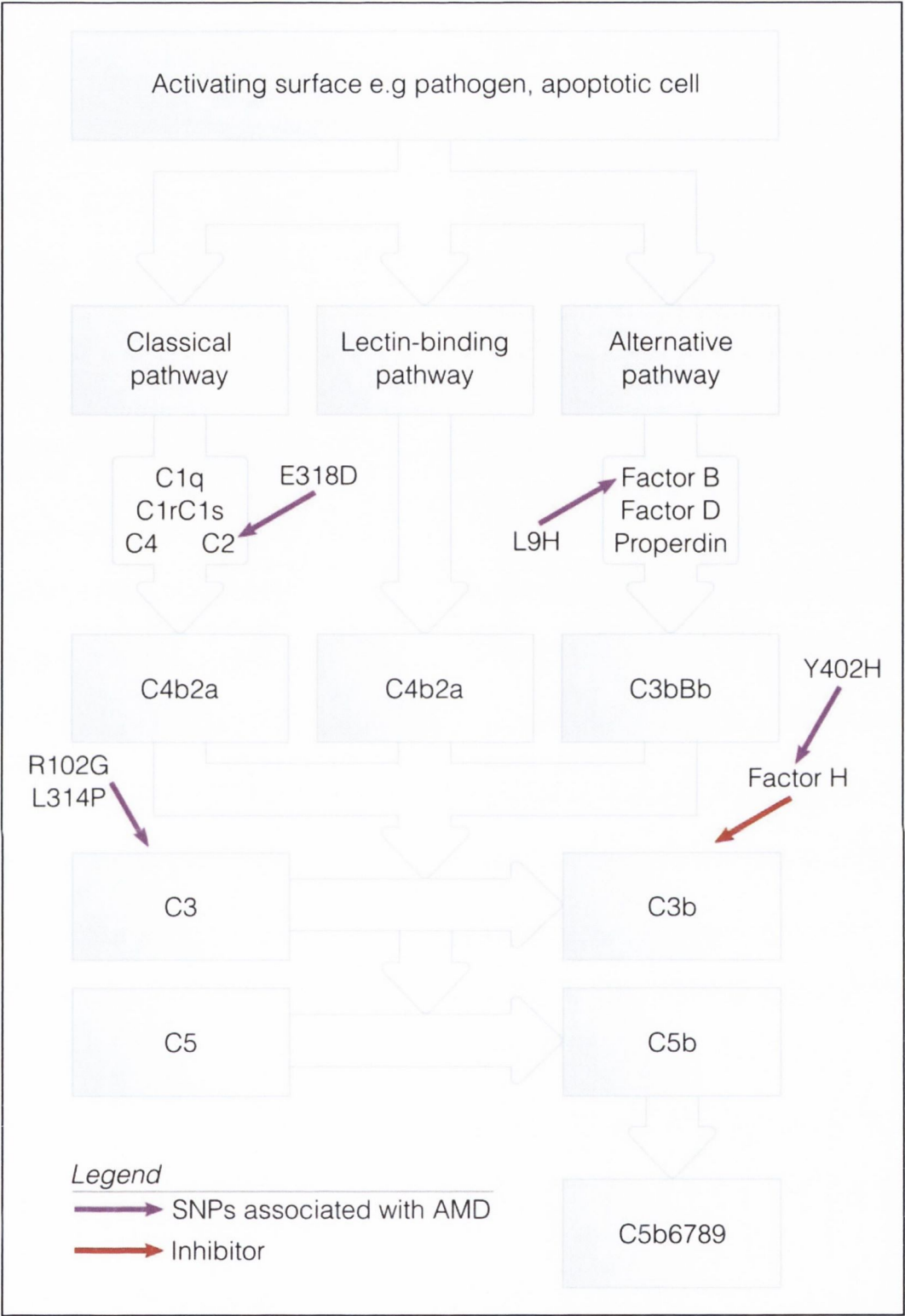


Figure 0.13: SNPs in complement factors associated with AMD

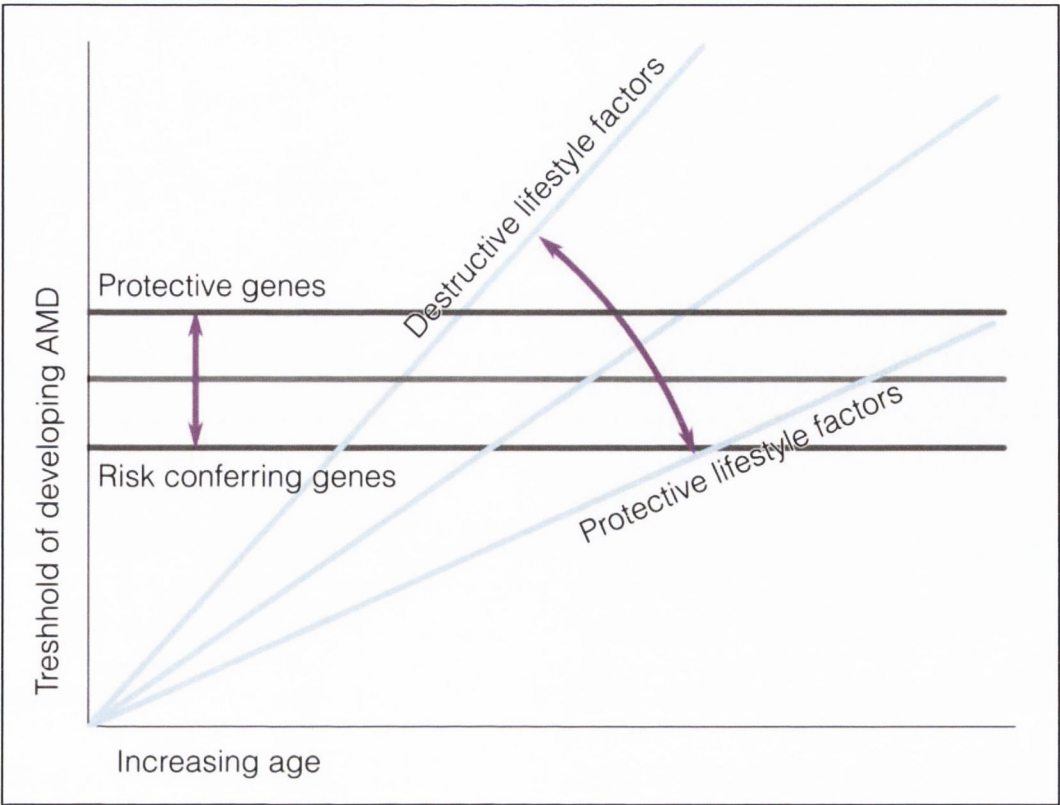


Figure 0.14: Schematic diagram of AMD risks.

Risk factors associated with AMD	
Genetic	
	Risk conferring genes
	Protective genes
Demographic	
	Age
	Race
	Gender
	Iris colour
Clinical	
	Soft Drusen
	RPE changes
	CNV in fellow eye
	Hyperopia
	CRP
	Elevated WCC
	Previous cataract surgery
Environmental	
	Cigarette smoke
	Blue/UV light
	BMI, Fat intake
	Antioxidants

Table 0.9: Risk factors associated with AMD

<i>Features of normal ageing</i>
Decreased photoreceptor density
Increased lipofuscin concentration
Accumulation of lysosomes in RPE
RPE Basal laminar deposits
Thickened Bruch's membrane
Involution of choriocapillaris
Decreased macular pigment density
Decreased photoreceptor density

Table 0.10: Changes associated with normal retinal ageing⁴

Chapter 2: Literature review

2.1. Introduction

This literature review will examine three aspects of AMD in detail. Firstly the molecular pathways that contribute to angiogenesis and the development of CNV will be described. A summary of the genetic factors that contribute to the risk of AMD will be provided and finally the influence of cigarette smoke on the pathophysiology of AMD will be discussed.

2.2. Angiogenesis and AMD

Angiogenesis is the process by which new blood vessels sprout from pre-existing vessels. It is essential to embryonic development, growth and wound repair. Neovascularisation is a stepwise process involving the degradation of the matrix surrounding the endothelial cells, proliferation, chemotaxis, migration and tube formation.⁹³ The highly specialized structure of the eye requires that visual media be kept avascular, and therefore while neovascularisation restores function in other tissues, in the eye it may result in the disruption of the visual process. CNV is therefore an inappropriate response to tissue injury and is similar in nature to the granular tissue of the generalised wound healing response.¹⁵

The molecular events that control the development of neovascular vessels are of particular clinical significance in wet AMD. In health the RPE and the choriocapillaris form a functional complex that governs flow of molecules to and from the neural retina. The choroidal capillaries are highly fenestrated and there is unimpaired passage from the vascular spaces through the Bruch's membrane as far as the RPE. RPE cells maintain the fenestrations of the choriocapillaris by constitutively secreting trophic factors.¹⁸² The tight junctional adhesions between RPE cells prevent the free passage of molecules farther through the outer retinal barrier protecting the photoreceptor layer and neural retina. CNV development occurs late in AMD and is typically preceded by RPE pathology. RPE cells associated with CNV membranes have dysmorphic, fibroblastic features indicating the active role in the pathology of CNV.¹⁸³ The position of the RPE between the neurosensory retina and CC

places it in a key position to control molecular passage between the two. In the absence of the trophic support of the RPE, the CC undergoes atrophy.¹⁸⁴

During the process of choroidal neovascularisation the proliferating endothelial cells arise from the choriocapillaris and migrate through the basement membrane and Bruch membrane⁶³. The endothelial cells are accompanied by inflammatory infiltrate and fibroblastic tissue and can infiltrate through the entire depth of the membrane and breach the RPE and retina.^{87, 185} The natural course of the invading endothelial, fibroblastic and inflammatory cells is to phagocytose damaged cells, vascularise the area and cause scar formation. The final outcome is the replacement of the highly evolved macular retina with a non-functional (disciform) scar.

The molecular control of CNV is a complex process involving an imbalance of pro and anti-angiogenic factors (Fig 2.6.1).¹⁸⁶ The RPE is the most likely source and central focus for these signals.¹⁵² In health, the ratio of anti to pro-angiogenic factors is thought to be high which maintains the quiescence of endothelial cells.¹⁸⁷ In CNV this balance is lost either from the excessive secretion of pro-angiogenic factors or the depletion of anti-angiogenic factors.¹⁸⁶ Tissue injury such as hypoxia, ischaemia or inflammation may alter the balance to promote the pro-angiogenic state. Three key molecules have been associated with this process. Basic fibroblastic growth factor (bFGF) and vascular endothelial growth factor (VEGF) are the two most potent angiogenic factors found in CNV.¹⁸⁸ In contrast pigment epithelium-derived factor (PEDF) is a potent anti-angiogenic factor and maintains the avascular Bruch's membrane.¹⁸⁹

2.2.1. Basic Fibroblastic Growth Factor

Basic fibroblastic growth factor (bFGF, FGF-2) is a pro-angiogenic growth factor originally isolated from cultured fibroblasts. There are two bFGF isoforms produced from the FGF-2 gene, one of a high molecular weight (HMW) and one of a low molecular weight (LMW)¹⁹⁰ HMW and the LMW bFGF isoforms differ by their terminal amino acid extensions. Basic FGF can be found embedded in extracellular matrixes including the Bruch's membrane.¹⁸⁹ Destruction of the matrix can release the stored factor.¹⁹¹ Basic FGF acts as a potent mitogen that promotes the budding and proliferation of choroidal endothelial cells, stimulates fibroblastic cells, epithelial cells and inflammatory cells and is a

key mediator of the wound healing response.¹⁹²⁻¹⁹⁴ It also functions as a cell survival factors and is up-regulated in response to retinal stress.¹⁹⁵

Increased bFGF secretion has been identified in excised CNV membranes in humans,^{196 197} as well as in laser-based animal models of CNV.¹⁹⁸ A cocktail of FGF-2 and lipopolysaccharide (LPS) administered intravitreally is sufficient to create a model of CNV in rabbits¹⁹⁹ and pigs.²⁰⁰ Basic FGF is secreted by the RPE and both FGF receptor 1 (FGFR-1) and FGF receptor 2 (FGFR-2) are expressed by retinal pigment epithelial cells in choroidal neovascular membranes.¹⁹⁷ Based on this data it is clear that bFGF plays a role in the process of CNV formation. Whether this is a pro-angiogenic signal or a protective response is not known.

2.2.2. Vascular endothelial growth factor

The vascular endothelial growth factors (VEGF) are of a family of related proteins which are derived from alternate splicing of the 8 exon VEGF gene on chromosome 6. There are 6 known isoforms found in humans with varying levels of solubility and heparin binding and they are among the most potent stimulants of physiological angiogenesis. VEGF displays a high affinity for endothelial cells, is controlled by regional oxygen concentrations and can readily diffuse to distant targets.²⁰¹ Hypoxia causes an increase in VEGF secretion and hyperoxia inhibits it.²⁰² The VEGF molecule contains a signal sequence, which facilitates diffusion of VEGF through the extracellular matrix. VEGF is essential for the establishment of embryonic vasculature, therefore knockout animal mutations are lethal *in utero*.²⁰³

VEGF is secreted by the RPE under both physiological^{6, 204} and pathological⁸⁷ conditions. VEGF acts as a vascular survival factor maintaining the fenestration of choroidal vessels under physiological conditions.²⁰⁵ The secretion of VEGF from the RPE is polarized, and the secretion of VEGF is 2-7 times higher from the basal side than it is from the apical side.⁶ This constitutive expression of VEGF by RPE cells is thought to be involved in the paracrine signaling between the RPE and the choriocapillaris. Fenestrations appear on choroidal capillaries only towards the RPE surface and VEGFR-2 is localised to this inner surface.²⁰⁵ It has been postulated that the increased thickness of the Bruch's membrane with age and AMD may inhibit the diffusion of VEGF from the RPE to the choriocapillaris. The

reduction of trophic VEGF support may lead to vascular atrophy and loss of the endothelial fenestrations. The resulting regions of RPE hypoperfusion lead to deposition of metabolic products in the basement and Bruch's membranes. Prolonged hypoxia may cause a surge in VEGF, prompting new vessel growth.

VEGF overexpression has been detected in the RPE cells of eyes in autopsy and in RPE cells derived from CNV membranes with AMD.¹⁹⁶ In AMD, the over-expression of VEGF is a key factor in the development of CNV.^{197, 206, 207} VEGF expression has been shown to be increased in patients with neovascular AMD¹⁰³ as well as in surgically-excised choroidal neovascular membranes, and in laser-induced animal models of neovascular AMD, localised VEGF expression has been implicated in CNV development.⁹⁷ Intra-vitreous injection of VEGF was sufficient to induce choroidal endothelial cell proliferation in non-human primates.¹⁰ Adenovirus-induced over-expression of VEGF in rat RPE resulted in choroidal neovascularisation.¹⁰⁶

VEGF secretion is influenced by a number of factors including: Hypoxia, reactive oxygen intermediates and elements such as cobalt and nickel.⁶³ RPE exposed to reactive oxygen species transiently increases VEGF mRNA and VEGF protein expression both *in vitro* cell and *in vivo*.^{108, 111} Lipid peroxidation product 4-hydroxynonenal increases the expression of VEGF at lower doses but at doses of $>5\mu\text{M}$ VEGF expression tapers off. These effects were inhibited by antioxidant treatment (GSH).¹⁰⁹ Cigarette smoke is known to contain a large quantity of radicals and may also increase VEGF secretion in RPE cells.

The strongest evidence to support the role of VEGF in AMD is the efficacy of VEGF blockade in the treatment of AMD. Anti-VEGF antibody treatments are currently a mainstay of treatment for individuals with wet AMD.¹¹ The discovery that VEGF significantly and consistently increased in eyes with neovascular AMD led to the development of anti-VEGF monoclonal antibody treatments for this condition.¹⁰⁵

2.2.3. Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF) is a secreted monomeric glycoprotein with a molecular weight of 50kDa and was originally isolated in the medium of cultured retinal pigment epithelial cells.²⁰⁸ This protein is a member of the serine protease inhibitor family of proteins. The human gene encoding the proteins, SERPINF 1, encodes a polypeptide containing 418 amino acids. The 3D structure of PEDF contains 3 β -pleated sheets and 10 β -helices. The peptide region 34-mer (Asp⁴⁴ – Asn⁷⁷) is considered critical in its role as an anti-angiogenic molecule.

PEDF is a multifunctional protein with neuroprotective and tumour-suppressing properties as well as vascular effects.¹⁸⁹ It is the most powerful anti-angiogenic protein identified to date in humans.^{118, 119} It is produced by the RPE and secreted into the basement membrane where it inhibits the formation of new vessels.¹⁸⁹ It has been hypothesized that the deposition of PEDF in the extracellular matrix presents a protective barrier to vascular ingress. If its secretion declines, as it does with age, or its function is overwhelmed then the barrier fails and neovascularisation subsequently results.¹¹⁴ It has been proposed that inhibitors of angiogenesis predominate within the avascular compartments of eye, and that vitreous, aqueous and corneal fragments all demonstrate anti-angiogenic properties *in vitro*.²⁰⁹

In vivo, the administration of PEDF has suppressed the development of CNV in ischaemia-induced,²¹⁰ laser-induced²¹¹ and VEGF-induced choroidal neovascularization.²¹² Vitreous levels of PEDF are reduced in patients with AMD.¹²⁰ PEDF, unlike VEGF, is preferentially secreted to the apical side of the RPE where it builds up in the interphotoreceptor matrix,²¹³ and expression is associated with an age-related decline.²¹⁴ From a molecular perspective, this may account for the relative resistance of the neurosensory retina to the development of new vessels in AMD. PEDF also binds to endothelial cells with considerable affinity. The anti-angiogenic properties are conveyed via an induction of endothelial cell apoptosis. PEDF demonstrates selectivity in that it stimulates apoptosis in newly forming vessels but causes no damage to fully established vessels. This selectivity has been attributed to the Fas ligand-Fas receptor system.¹⁸⁹ In established vessels, the Fas receptor proteins are not typically expressed preventing PEDF-mediated apoptosis.

The proliferation of new blood vessels in CNV is due to the loss of balance between the angiogenic effects of VEGF and the inhibitory effects of PEDF.¹⁸⁶ Oxygen levels have been shown to affect the levels of PEDF. In a hypoxic environment VEGF and interleukin-8 expression are increased and PEDF is decreased. In the reverse situation, where oxygen is abundant, PEDF is increased and angiogenic factors are decreased.²⁰⁸ and inhibits the release of VEGF in the RPE.²¹⁵ Oxidative stresses and advanced glycation end products inhibit PEDF gene expression.²¹⁰ Cigarette smoke exerts an oxidative stress on tissues and forms advanced glycation end products²¹⁶ and may therefore inhibit the expression of PEDF in the eye, leaving the effects of VEGF unchecked. Triamcinolone increases cellular expression of PEDF as it reduces VEGF in cultured RPE cells.²¹⁷ Systemic treatment with PEDF inhibits the development of retinal neovascularisation secondary to ischaemia in mice.²¹⁰

2.2.4. Other mediators with a role in choroidal neovascularisation

Angiogenesis is a complex process and it is highly unlikely that CNV is provoked purely by an increase in VEGF expression. An array of growth factors is expressed in RPE cells derived from choroidal neovascular membranes; Thrombospondin-1, FGF-5, Angiotensin II, Angiopoietin I and II have all been associated with AMD.²¹⁸ These molecules have not been as consistently replicated *in vitro* and *in vivo* as the bFGF, VEGF and PEDF.

2.2.5. Upstream regulators of VEGF induced angiogenesis

Increased expression of angiogenic factors is a late change in the molecular pathology of AMD. VEGF is regulated by mammalian target of rapamycin (mTOR). Inhibitors of mTOR include: Sirolimus as mentioned previously, Everolimus and Palomid; their efficacy in the treatment of AMD is currently being investigated.¹³⁸

2.3. Genetics and AMD

2.3.1. Family history and AMD

Family history has been recognised as a risk factor in AMD for over 30 years.²¹⁹ It has been observed that approximately 20% of patients with AMD will have a family history of the condition, and patients with a first degree relative with AMD are three to four times more likely to develop neovascular AMD.²¹⁹⁻²²¹ The OR of a sibling developing early AMD is 6.6 and neovascular AMD is 10.3.²²⁰ Family and twin studies have established the contribution of genetic predisposition to the development of

AMD. The rate of the development of AMD in monozygotic twins is approximately twice that of dizygotic twins with a concordance of up to 90%.²²⁰ Among the monozygotic twin pairs there was variable expression of the disease. This clinical heterogeneity raised the possibility of non-genetic and environmental influences on the presentation of the disease.

Determining the influence of genetic factors on the development of AMD is complicated by several factors. The late onset of the disease means that younger members of the family, though genetically susceptible, will not express the phenotype. Assessment of the older generations may underestimate the prevalence where other family members passed away at a younger age and prior to the onset of the disease. Therefore the information available from family members may be inaccurate. The high prevalence of the disease, and the wide range of phenotypes, also implies that there is more than one gene responsible and that non-genetic factors have an influence on the development of the disease.²²²

2.3.2. Techniques of genetic study

In conditions suspected of having an inherited component, the candidate genes may be isolated in a number of ways. Association studies are performed on large patient cohorts and have a greater ability to detect common variants in the population, however if the causative gene is rare then association studies may not have the power to detect this. Linkage studies are performed on cohorts of affected families. These studies have a greater ability to identify rare mutations. The “logarithm of odds”, or LOD score, represents the likelihood of a particular linkage relationship. In an unlinked observation the odds of recombination is 50:50 due to independent assortment (0.5). A LOD score of greater than 3 is considered to be significant by convention. A score of 2 is suggestive of linkage. The most consistently linked chromosome loci are on chromosomes 1 (1q25-32) and 10 (10q26).⁴³ Targeted analysis of these candidate loci has resulted in the discovery of alleles and haplotypes (collections of alleles at a locus that are inherited together) that are instrumental in the development of AMD. Genome wide association studies (GWAS) and genome wide linkage studies are a more modern approach to isolating candidate genes. The FARMS study has linked AMD to five other loci on chromosomes 5, 14, 16, 19 and 21.²²³ These methods can therefore be used to identify putative gene loci but regions identified require further study.

2.3.3. Single nucleotide polymorphisms (SNPs)

SNPs are substitutions of a single base that are commonly seen in the population (Fig 2.6.2). They are copying errors that occur frequently but may not directly affect the gene product. Thus they are considered to be normal variations rather than specific mutations. In 2005, three research teams simultaneously reported a strong genetic risk factor in the complement factor H gene.^{107-109, 224} The change in this single base pair was predicted to contribute to over half of disease presentations and has since become the most significant risk factor associated with AMD. Prior to this discovery, the contribution of the complement system to the disease process was largely unknown. It now appears that activation of the alternative complement pathway is a key step in AMD pathogenesis. Rivera et al reported a second SNP (LOC387715) later in 2005 that was also strongly associated with AMD.²²⁵

Major SNPs associated with AMD

The Complement Factor H gene (CFH gene)

The CFH gene contained 23 exons and is approximately 94kb in size. It is localised, amongst a region of genes controlling the complement factors, to chromosome 1q32. Association analyses led to the discovery of a single nucleotide polymorphism (SNP) Y402H located on chromosome 1 that may be responsible for up to half of the presentations of the disease.¹² The single nucleotide polymorphism substitutes a cytosine (C) for a thymidine (T) and consequently a tyrosine for a histidine amino acid. Therefore the homozygous variant is depicted as C/C and the heterozygous variant is C/T. Variation results in a downstream alteration in the construction of Complement Factor H (CFH), a key molecule in complement regulation. The Y402H altered CHF product is dysfunctional and is less efficient in regulating complement *in vitro*.²²⁶ A single copy of the defective CFH SNP confers an increased risk (OR) of developing AMD of 2.4 (95% confidence interval [CI], 2.2-2.7).²²⁷ The homozygous state is associated with an OR of 6.2 (95% CI, 5.4-7.2).

The ARMS2 gene locus

The ARMS2 gene locus on chromosome 10q26 has been linked to an increased susceptibility to AMD.⁴⁷ The PLEKHA1 (pleckstrin homology domain-containing, family A, member 1) variant, and a predicted gene at LOC387715 have been strongly associated with AMD within this locus.^{44, 51, 52} A SNP in the promoter region of the HTRA1 also at the same locus, only 6kB from the LOC387715 SNP exhibits extremely strong linkage disequilibrium with this SNP. Determining the correct SNP from the

HTRA1/PLEKHA1/ARMS2 regions has been complicated. All three SNPs are highly linked and the functional pathway of this haplotype has not been described. It is therefore unclear which is the true causative gene. The strong linkage disequilibrium however allows assessment of one SNP to act as a proxy marker for the genetic risk conferred by the locus.²²²

The LOC387715 SNP substitutes a guanine (G) for a thymidine (T) at position rs10490924. The protein product altered by the substitution of a serine in the place of an alanine at codon 69 (A69S). This product is thought to be involved in mitochondrial function but the predicted protein is not homologous to other known proteins. The OR associated with the heterozygous LOC387715 genotype is 2.5 (95% CI, 2.2-2.9).²²⁸ The homozygous genotype confers an OR of 7.3 (95% CI, 5.7-9.4). The HTRA1 (rs11200638) variant results in an alanine (A) for a guanine (G) such that the homozygous variant is denoted as A/A and the heterozygous variant A/G. The reported ORs for the HTRA1 mirror the LOC387715 associations due to the high degree of linkage.

Minor SNPs associated with AMD

The CFH and ARMS2 variants are the most influential genetic factors in AMD. The combined analysis of the two linked SNPs can predict disease outcome in 74% of patients with AMD and are the major genetic determinants of the disease.¹² Multiple minor SNPs have also been associated with AMD. These variants modify risk to a lesser extent. SNPs that have been consistently associated with AMD are illustrated below (Fig 2.6.3). Characterizing the minor SNP associations is valuable as it refines the models to predict disease based on genetic risk factors.²²⁹ It also isolates potential proteins for study, such as complement and angiogenic factors, which are involved in the disease pathogenesis.

PEDF

Lin et al reported an SNP in exon 3 of the gene encoding PEDF in the Taiwanese population that was associated with neovascular AMD. The variant conferred an OR of 3.9 in wet AMD but showed no association with dry AMD. The SNP is located at rs1136287 and results in a cytosine (C) to thymidine (T) base substitution. The substitution corresponds to a methionine to threonine alteration at the level of amino acid transcription. It is an important candidate for assessment as it is consistent with the biological observations linking reduced PEDF levels with neovascularisation. Variants in the PEDF gene have also been associated with poorer response to treatment of PCV in the Asian population.²³⁰

VEGFA

There is conflicting evidence on the role of mutations in the VEGF A gene in AMD. Churchill et al reported a significant association between VEGF polymorphisms and neovascular AMD.²³¹ Although this represents a plausible molecular pathway in AMD, neither a larger study in the Caucasian population²³², nor study in the Chinese population replicated the association.²³³

Complement Factor B

Based on the contribution of the CFH variant to the development of AMD, other complement factors have been investigated for associations. Gold et al identified allelic variants in Factor B that were associated with AMD. The CFB gene is 6kb and contains 18 exons. The polymorphisms are located on chromosome 6p21 within the major histocompatibility complex class III (MHC Class III).¹⁰² It is possible that mutations in CFB reduced the haemolytic activity of the enzyme thus promoting unrestricted complement activation.

Complement Factor 2

Another key factor of the complement cascade, complement factor C2 was associated with AMD. The gene is 18kb located 500bp from the gene encoding factor B on chromosome 6p21 on the MHC class III.¹⁰² It has been shown that the combined analyses of CFB/C2 loci can predict clinical outcome in 74% of patients and 56% of controls,¹⁰² however when incorporated into a model that includes CFH and ARMS2 they provided only a modest increase in specificity and sensitivity.²²⁹

Complement factor 3

The C3 gene is 41kb in size, contains 41 exons and is located on chromosome 19p13. Like most of the components of the complement pathway, the majority of circulating C3 is produced in the liver. An R102G gene variant has been associated with an increased risk of developing AMD possibly through an error in the transcription of C3.^{117, 234}

Complement Factor I

The complement factor I (CFI) is a 63kb gene with 13 exons located on chromosome 4q25. This factor cleaves and inactivates C4b and C3b suppressing the complement pathway. An SNP (rs10033900) located upstream from the CFI region has been associated with AMD.¹¹⁵ Dysfunction in a complement suppressor gene would predispose to unrestricted complement activation and inflammation.

MnSOD (SOD2)

A SNP in the gene for the intramitochondrial superoxide dismutase has been associated with neovascular AMD.²³⁵ A dysfunctional SOD pathway would compromise retinal defenses against oxidative stress and provide a link between the environment and genetics in the pathology of AMD.

Paraoxonase

Paraoxonase is calcium dependent glyco-protein associated with the protection of lipids against the effects of oxidations. Two SNPs in the gene for paroxonase have been associated with the development of AMD in the Japanese population.⁶⁹

Protective genes in AMD

Determining the variants that confer protection against the development of AMD is as important in AMD as the risk associations. Isolating these genes is more complex than finding the risk factors as these cases do not present to ophthalmic services.

Apolipoprotein E

The ApoE gene is involved in lipid metabolism and manifests three common polymorphisms E2, E3 and E4. The most common form is the E3 gene. A variant of the apolipoprotein E gene (APOE), the E4 allele at 19q13.2, was associated with a decreased risk of developing AMD.²³⁶⁻²³⁸ Souied et al noted that the APOE E4 allele is associated with a lower frequency of neovascular AMD.²³⁹ Individuals that possess the E4 allele are 2 times less likely to develop AMD.²⁴⁰ A mouse model of ApoE deficiency exhibits accumulation of material in the Bruch's membrane similar to the basal collections in AMD.²⁴¹ The ε2 variant however, has been associated with a slightly increased risk of AMD development.^{236, 237, 239}

2.3.4. Monogenic macular dystrophies with contribution to AMD

Monogenic dystrophies display typical Mendelian inheritance patterns. They display pathologic features similar to those found in AMD but at a much younger age. Analysis of the gene mutations involved has yielded information not only about the dystrophy disorders but also regarding general observations that may be applied to AMD.

Stargardt macular dystrophy (STGD)

STGD is an autosomal recessive condition with an onset in late childhood or early teenage years. Clinically it displays features that are similar to AMD. The retinas undergo an accumulation of lipofuscin at the level of the RPE. The condition has been localized to the ABCA4 gene.²⁴² The ABCR4 gene encodes a 150kb ATP-binding transmembrane protein gene, which is found exclusively in the retina. It is involved in the visual cycle in the transport of all-trans-retinaldehyde. The mutation in an animal model leads to the accumulation of lipofuscin and subsequent degradation of photoreceptors.

Heterozygous mutations in the ABCA4 gene have been associated with AMD.^{243, 244} Though the ABCA4 gene has been implicated in the development of the disease, it is likely contributory to less than 1% of cases. PROML1, a second gene implicated in the development of STGD, is located on chromosome 4p15 and encodes for a protein product called prominin.

Sorsby fundus dystrophy (SFD)

SFD is characterized by pathological changes in Bruch's membrane and by choroidal neovascularisation. It manifests initially as loss of night vision in the third decade of life followed by the deterioration of macular vision. This dystrophy displays an autosomal dominant inheritance pattern, which has been mapped to a gene on 22q13. The gene product is a tissue matrix metalloproteinase (TIMP3). Dysfunction of this MMP leads to abnormal extracellular matrix turnover, the thickening of Bruch's membrane and the deposition of abnormal material. TIMP-3 suppresses chemotaxis of endothelial cells in response to VEGF and bFGF *in vitro* and *in vivo*²⁴⁵ Disregulation of TIMP3 could facilitate the proliferation of CNV. TIMP-3 content is elevated in eyes with AMD when compared with controls.²⁴⁶ However no sequence variants have been associated with AMD yet.

Malattia leventinese, Doyme honeycomb retinal dystrophy

Both of these dominant macular dystrophies have been linked to a gene EFEMP1 at 2p16-21. These conditions show the accumulation of drusen. The gene product is an extracellular matrix protein but the EFEMP1 gene has not been implicated in the pathogenesis of AMD.

Late-onset retinal degeneration (L-ORD)

L-ORD is an autosomal dominant condition with similar clinical findings to AMD. The causative gene is CTRP5.²⁴⁷ Like AMD, the onset is in the fifth to sixth decade of life, but the main presenting complaint for this condition is night blindness. Yellow white deposits accumulate beneath the RPE and in the Bruch's membrane reminiscent of drusen and progression to CNV and chorioretinal atrophy have also been observed.²⁴⁸ L-ORD may be differentiated from AMD by its classical Mendelian inheritance pattern and more widespread distribution of sub-RPE deposits and atrophic changes. The product of the CTRP5 gene is a short chain collagen, which is secreted by the RPE. It may facilitate adhesion of the RPE to the underlying basement membrane, as well as Bruch's membrane. This suggests a role for impaired basement membrane adhesion in the pathogenesis of L-ORD and AMD.

2.3.5. Genetic and environmental synergism

Reports have also noted that the combined effects of smoking and genetic susceptibility exceed the sum of the independent effects.²²⁷ This may indicate a common biological pathway by which both entities exert their effects.²⁴⁹ Patients that are homozygous for both CFH and ARMS2 risk variants who also smoke are 144 times more likely to develop AMD.²⁵⁰ These findings are further strengthened by observations that cigarette smoke can directly activate the complement system by activating factor C3.⁵⁵ Considering the current evidence, the most likely hypothesis is that the cigarette smoke directly triggers an inflammatory process that culminates in AMD. How this occurs on a molecular level is not known.

2.4. Cigarette smoking and AMD

There are approximately 1.1 billion smokers worldwide, which will increase to an estimated 1.3 billion by 2025.²⁵¹ Cigarette smoking is the most significant environmental risk factor contributing to the development of AMD.¹²¹ A meta-analysis of the contribution of smoking estimated that the increased risk of developing AMD is two to four-fold though the majority of the data included in this analysis was derived from the Caucasian population.¹²¹ There is also a dose-response relationship, where the higher the cigarette consumption, the greater the risk.¹²¹ Passive smokers are also at an associated increased risk.²⁵² Cigarette smoke further affects AMD by promoting progression from atrophic to neovascular AMD.²⁵³ This may occur five to ten years earlier in smokers than in non-smokers.²⁵⁴ Cessation reduces the risk of developing AMD and progression to neovascular AMD²⁵⁵

and for every 1000 smokers that successfully quit, there would be 48 fewer cases of macular degeneration with 12 fewer cases of blindness.²⁵⁶ Smoking has been linked to the development of large drusen, neovascular membranes and atrophic AMD.^{23, 254} Continuing to smoke cigarettes after diagnosis has been associated with recurrent CNV post Argon treatment.²⁷ Smoke-induced damage in AMD is mediated through direct chemical damage, direct oxidation, depletion of antioxidant protection, immune system activation and atherosclerotic vascular changes.²⁵⁷

2.4.1. Cigarette smoke constituents.

Cigarette smoke is separated into two phases, a gas phase and a tar phase based on passage through a standard filter¹²⁴. With each cigarette, smokers consume over 4,000 different compounds²⁵⁸. Therefore it is unlikely that cigarette smoke exerts its pathological effects through a single biochemical pathway. Though the consequences of ocular exposure to the full range of cigarette smoke constituents are not yet known, it is thought that oxidative damage, vascular and inflammatory changes play key roles in the pathogenesis of AMD.²⁵⁹

2.4.2. Oxidative stress damage

As previously mentioned, It has been hypothesised that cumulative oxidative damage to the RPE contributes to the development and progression of atrophic AMD.^{11, 57} Cigarette smoke contains >10¹⁵ free radicals per inhalation¹²⁴ and numerous other chemicals that may be metabolized into reactive oxygen intermediates. These oxidants in cigarette smoke can pass through the alveolar walls and enter the circulation.²⁶⁰ Plasma markers of lipid peroxidation are increased after smoking, which confirms that smoke-derived radicals pass into the circulation with the potential to exert widespread systemic effects.²⁶¹ Mice exposed to chronic cigarette smoke display RPE apoptosis and Bruch's membrane alterations which can be attributed to oxidative damage.²⁶² Chronic smoke exposure in mice also yields basal laminar deposits reminiscent of drusen.²¹⁶

Acrolein-induced oxidative stress

Cigarette smoke also contains more stable components that induce oxidative damage, in addition to directly generating short-lived reactive oxygen species. Acrolein is an unsaturated aldehyde found in the gas phase of cigarette smoke in quantities of 3-220µg per cigarette.²⁶³ It is capable of exerting an oxidant-mediated damage, inducing protein modifications and promoting the formation of advanced

glycation end-products (AGEPs) and advanced lipid end products (ALEPs).²⁶⁴ RPE cells exposed to acrolein show a decrease in viability and mitochondrial membrane potential due to oxidative stress.²⁶⁵

Cadmium-induced oxidative stress

The main source of cadmium intake in humans, is through cigarette smoke.²⁶⁶ Plasma and retinal levels of cadmium are significantly higher in smokers than in non-smokers.²⁶⁷ Cadmium accumulates preferentially in the RPE and choroid ²⁶⁸ and may contribute to the development of AMD through an increase in reactive oxygen species. Cadmium exposure reduces viability and membrane integrity of cultured RPE cells and this damage was likely oxidative.²⁶⁸ Cadmium, interestingly, has been found at increased concentrations in the urine of smokers and in the urine of non-smokers with AMD supporting a role for cadmium in the pathogenesis of AMD.²⁶⁹

Cigarette smoke and depletion of antioxidant protection

Enzyme systems exist to protect against oxidant-mediated cellular damage. Diet-derived antioxidant vitamins B, C and E provide protection by reacting with radicals, thereby terminating oxidation cascades. Cigarette smoke depletes plasma concentrations of vitamin C, E and carotenoids ²⁷⁰⁻²⁷³ and supplementation of antioxidants inhibits cigarette smoke induced oxidative damage *in vivo*.²⁷⁴ Acute cigarette smoke exposure also reduces the levels of endogenous circulating anti-oxidant molecules. Glutathione, cysteine, methylumbellifere glucuronide and ferroxidase are all reduced in serum after smoke exposure.^{275, 276} Long term smoking also depletes intraplatelet stores of glutathione.²⁷⁷ Enzymes that degrade reactive molecules and generate endogenous antioxidants such as superoxide dismutase (SOD), catalase and glutathione peroxidase are also crucial in cellular defense against oxidative damage. Disruption of superoxide dismutase in mice results in drusen-like deposits in the eye, and SOD knock-out animals have been used as a model of dry-AMD.²⁷⁸

Local ocular antioxidants

Carotenoids are present in a large concentration in the macula providing protection from oxidative damage.⁹ Reduced levels of macular pigments have been associated with AMD, due to the loss of this antioxidant protective capacity.⁶⁰ Supplementary therapy with dietary macular pigments may offset this damage and reduce disease progression in macular degeneration. Cigarette smoking has been

shown to reduce macular pigment ²⁷⁹ and, by compromising local antioxidant protection, may aid and abet the promotion of AMD.

2.4.3. Non-oxidative chemical damage by cigarette smoke

The other non-oxidative stress pathways that contribute to AMD include nicotine, polycyclic aromatic hydrocarbons and inflammatory pathway mediators.

Nicotine

Of the numerous constituents of cigarette smoke, nicotine is the only known ingredient to possess addictive properties. Nicotine promotes angiogenesis *in vitro* ²⁸⁰ and *in vivo* ²⁸¹ and these biological effects can be applied to AMD. Angiogenesis is likely due to a nicotine-induced increase in expression of Vascular Endothelial Growth Factor (VEGF) in endothelial cells.²⁸² A nicotine-induced increase of VEGF could account for the expedited progression to neovascular AMD seen in smokers. Nor nicotine, a metabolite of nicotine catalyses the metabolism of retinoids to all-E-retinal which can lead to the accumulation of lipofuscin, a constituent of drusen, in RPE cells.²⁸³ Nicotine also exerts a vasoconstrictive action via α -adrenergic stimulation which may impair blood flow through the choroid.²⁸⁴

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) have been linked to the toxicity of cigarette smoke through vascular and carcinogenic effects in cardiovascular and respiratory disease.²⁸⁵ Benzo[a]pyrene is a PAH found in cigarette smoke and that damages nuclear and mitochondrial DNA in bovine RPE²⁸⁶ through the formation of a reactive epoxide. Benzo(e)pyrene is a related polycyclic aromatic hydrocarbon molecule which can cause caspase-mediated cell apoptosis of human RPE cells²⁸⁷, perhaps through the generation of similar epoxides.

2.4.4. Cigarette smoke induced inflammation

Drusen deposits in atrophic AMD contain evidence of chronic low grade inflammation supporting the hypothesis that inflammation is central in pathogenesis of AMD.^{15, 83} The complement cascade may be central to this process. A number of genetic risk factors associated with AMD are involved in the activation and regulation of the complement pathway and thus far, SNPs in complement components C3, CFH, C2 and Factor B have been implicated in the pathogenesis of AMD.^{102, 107, 117} Inflammatory

mediators also govern the progression from atrophic to neovascular AMD. Depletion of macrophages in a laser-induced animal model of CNV inhibits the immune response and the subsequent neovascularisation.⁸⁴

Cigarette smoke and complement pathway

Cigarette smoke can directly activate C3, a key component in the Alternative complement pathway¹²² as well as reduce serum levels of CFH, a circulating inhibitor of complement activation. It is possible therefore that complement-related genetic risk factors and cigarette smoke-induced complement activation act synergistically to promote inflammation in AMD.

Cigarette smoke and other inflammatory mediators

Cigarette smoke is also associated with a state of systemic chronic low-grade inflammation.²⁸⁸ Leukocyte and neutrophil concentrations are elevated in the blood of smokers.^{276, 289} Human monocytes are activated by cigarette smoke *in vitro* through oxidant-mediated pathways²⁹⁰ indicating that the oxidative stress and immunological hypotheses of AMD aetiology may not be mutually exclusive.

Vascular changes

As mentioned previously, the vascular model of AMD provides an alternative hypothesis for the aetiology of AMD.⁷⁴ This model postulates that the initial changes in AMD occur in the choroidal vasculature in a process analogous to atherosclerosis. Cigarette smoking as a risk factor for AMD fits well in this hypothesis. Cigarette smoke exacerbates atherosclerotic changes in the coronary arteries²⁸⁵, oxidizes cholesterol and low-density lipoproteins²⁹¹ and activates platelets promoting aggregation.²⁹² Smoke also induces blood vessel constriction through α -adrenergic receptor activation. *In vivo*, animals exposed to chronic cigarette smoke display significantly increased choroidal vascular resistance.²⁹³ A decrease in choroidal circulation may impair the clearance of debris from the RPE and lead to the deposits in the Bruch's membrane, as seen in AMD.⁷⁸ Cigarette smoke exposure significantly alters the branching pattern and extracellular matrices of proliferating vessels.²⁹⁴ These vessels were accompanied by a higher number of fibroblasts than unexposed controls. Thus smoke may further compromise the infiltrating vessels in CNV membranes.

2.4.5. Conclusion of the pathogenesis for cigarette smoke review

The effects of cigarette smoke influences both the onset and progression of AMD. How exactly cigarette smoke exerts its effects on the molecular pathology of AMD is not known but it is likely that the pathological effects are mediated through multiple biochemical pathways.

2.5. Aims of the project

The aims of this project are to show the potential sites of interaction between underlying genetic susceptibilities and environmental influences (cigarette smoke) in neovascular AMD. Our current hypothesis proposes that cigarette smoke exerts its toxic effects by altering the expression of vasoactive proteins at the level of the RPE, ultimately promoting angiogenesis. To test this we used *in vitro* and *in vivo* methods. We also consider the possibility that known genetic risk factors in regions coding for vasoactive proteins may contribute to this process of angiogenesis in Irish patients with AMD. To examine this, a cohort of AMD patients and appropriate controls were recruited. The findings are presented in the sections below.

2.6. Figures and tables

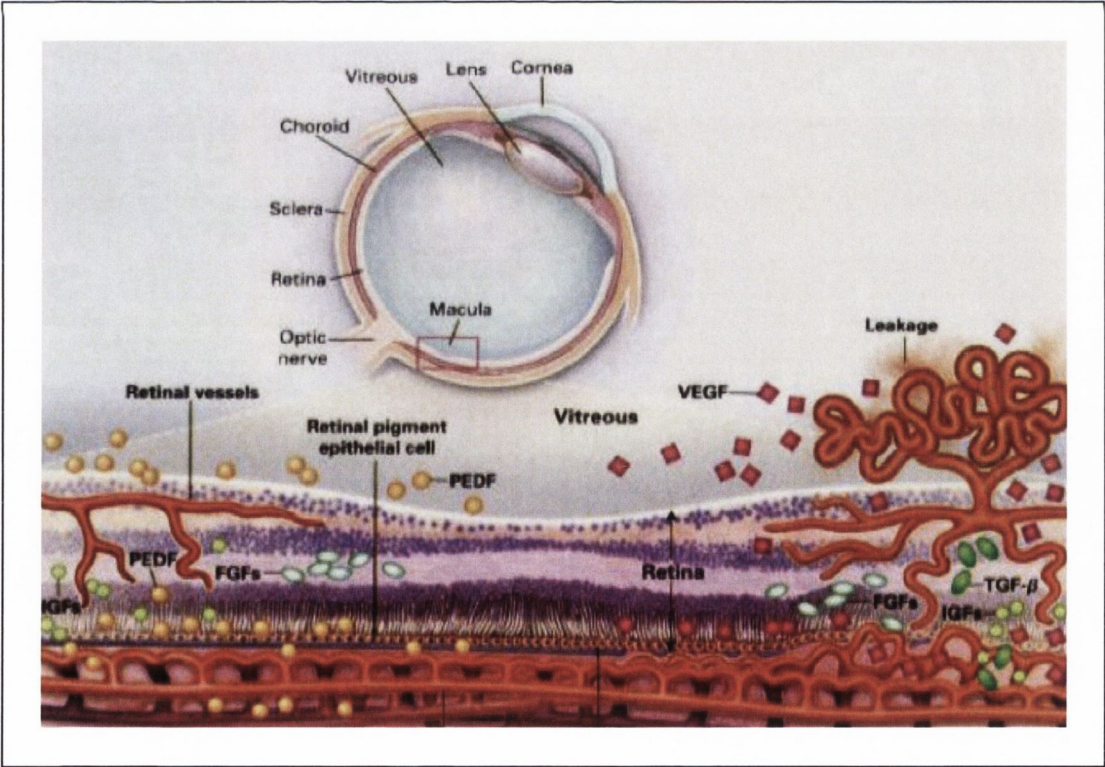


Figure 2.6.1: Angiogenic molecules implicated in neovascular AMD

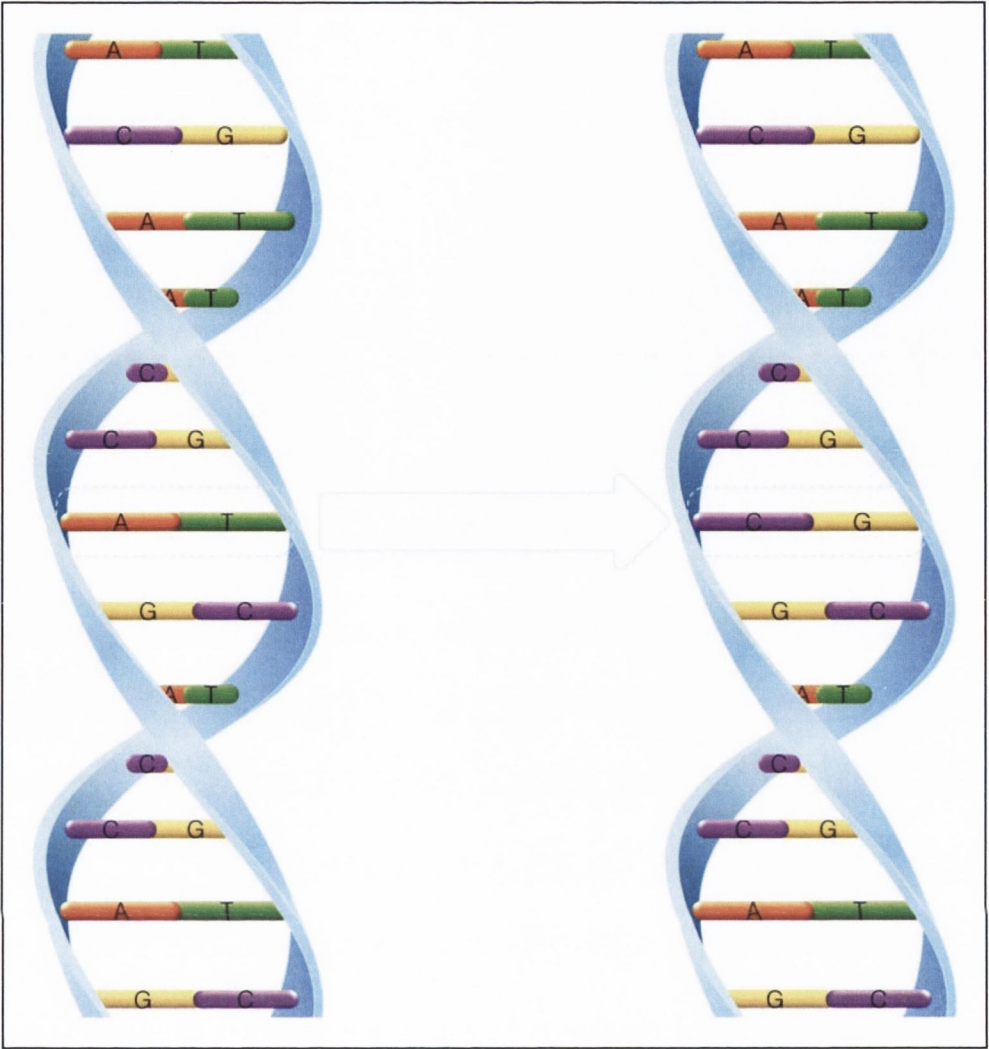


Figure 2.6.2: Single nucleotide polymorphisms

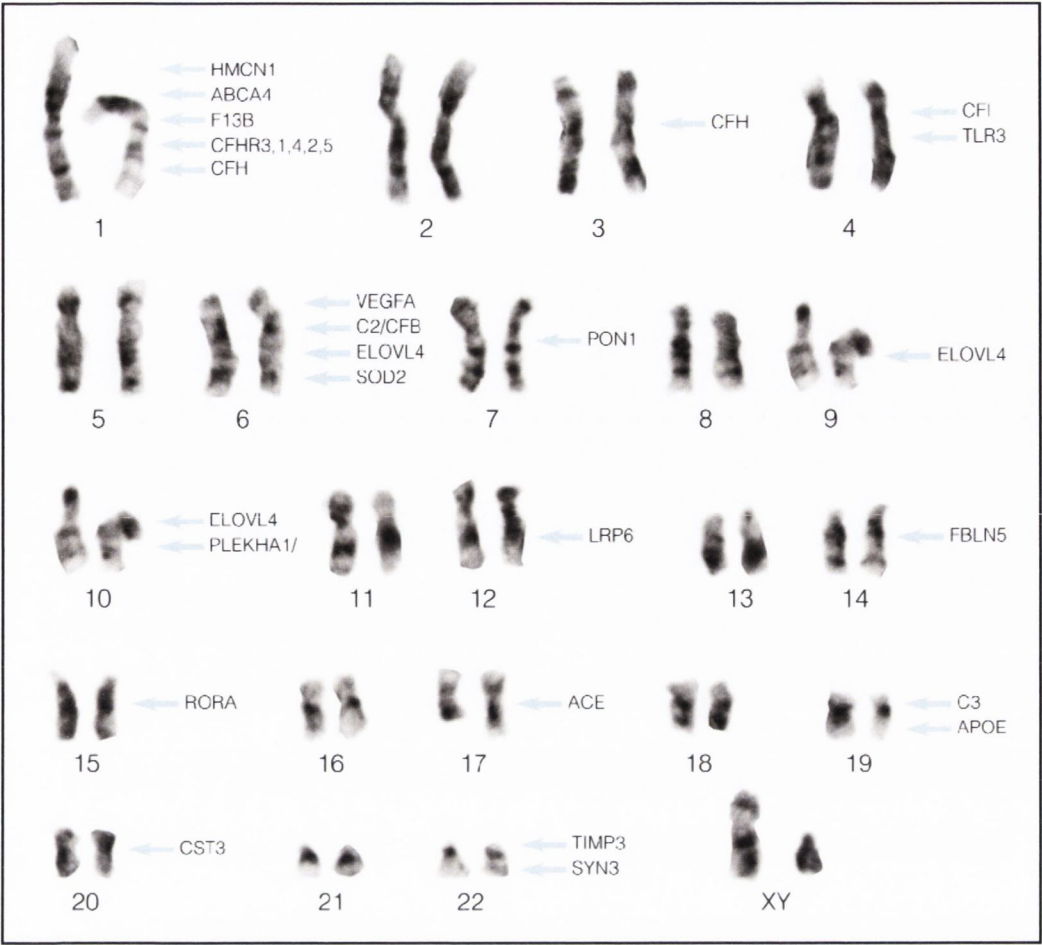


Figure 2.6.3: SNPs associated with AMD replicated in multiple studies

Putative methods of smoke induced retinal damage
Direct chemical damage
Direct oxidation
Depletion of antioxidant protection
Immune system activation
Atherosclerotic vascular changes

Table 2.6.1: Possible mechanisms of cigarette smoke toxicity in AMD

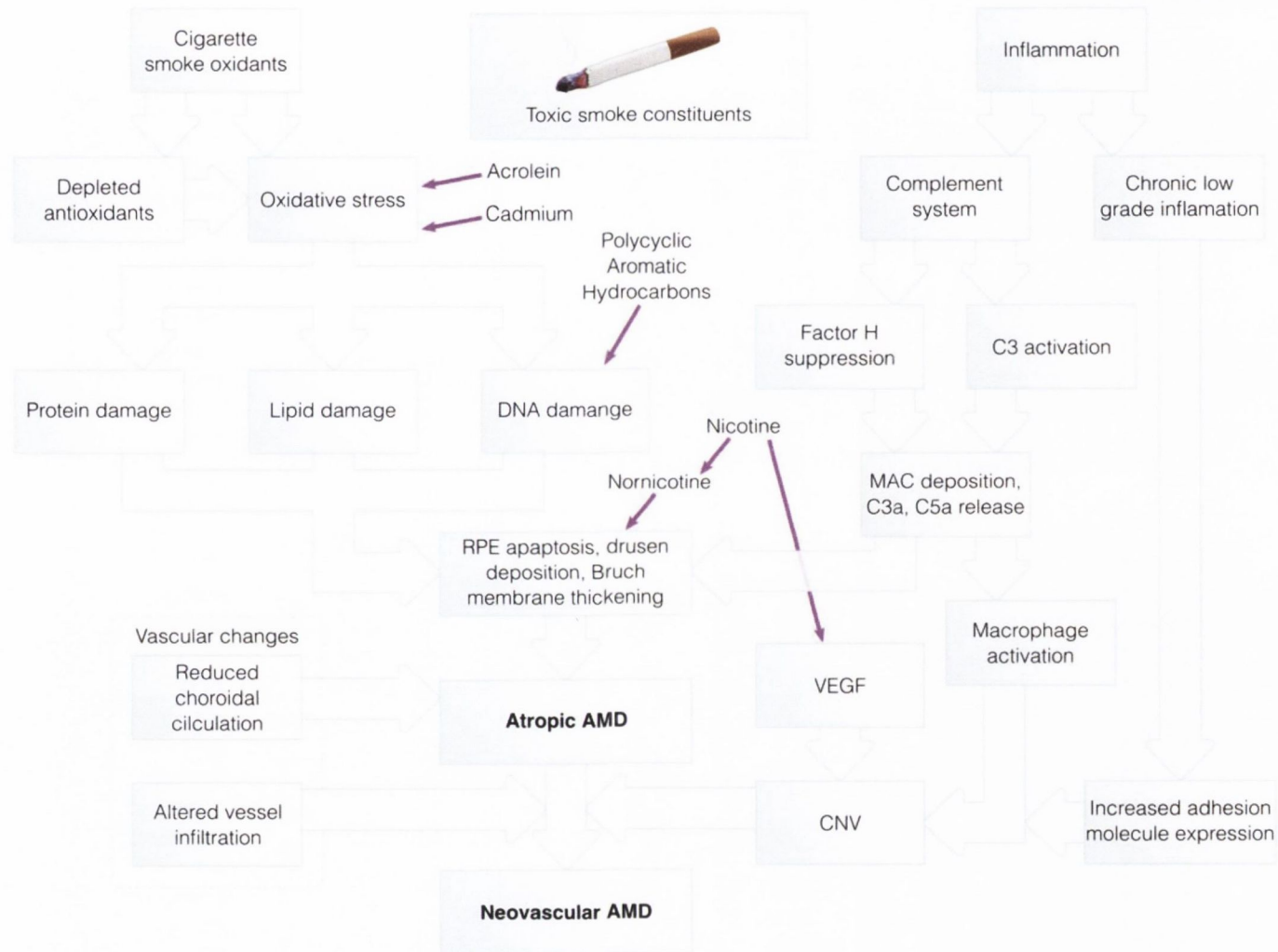


Figure 2.6.4 The pathogenesis of cigarette smoking in AMD

Chapter 3: *In vitro investigations*

3.1. Introduction

The earliest site of pathological changes in AMD is the RPE. Changes in this layer, because of its location and function as the inner blood retinal barrier, influence both the neural retina at the apical side of the RPE and the choroidal vasculature at the basal side. These cells are highly resistant to the effects of external stressors. Young smokers do not develop AMD below the age of 55, and a short history of cigarette smoking does not result in any permanent damage to the RPE. This indicates that a healthy young RPE can adequately compensate for the effects of cigarette smoke. However as smokers age, the changes of AMD begin to manifest more frequently than among non-smokers. This represents a threshold at which the cumulative damage of cigarette smoke overcomes the capacity of the RPE to eliminate it. Experiments were designed under the hypothesis that the earliest pathological effects of cigarette smoke manifest in the RPE prior to Bruch's membrane and choriocapillaris changes.

Previous *in vitro* investigations have described RPE apoptosis in response to external stressors, including oxidative stress and cigarette smoke constituents.^{265, 295, 296} Apoptosis of the RPE layer is a late event in the pathology of AMD. Changes in protein expression are likely to precede the morphological changes of atrophy and apoptosis. To test this hypothesis, an *in vitro* RPE cell line was prepared. We optimized the cells to ensure adequate differentiation by maintaining them for prolonged post confluence durations. Cigarette smoke was extracted into a growth medium for exposure. A protocol of low, moderate and high concentrations of cigarette smoke was also designed. Morphological changes, viability markers, and markers of cell proliferation were all assessed after 24, 48 and 72 hours of exposure. The changes in angiogenic growth factors bFGF, VEGF and anti-angiogenic growth PEDF in response to cigarette smoke were quantified and compared with control samples. The aims of the *in vitro* experiments were as follows:

3.2. Summary of aims

- 3.1.1 To examine growth curve and morphology of ARPE-19 cell line

- 3.1.2 To assess markers of differentiation in the ARPE-19 cell line
- 3.1.3 To determine the genetic risk factors in the ARPE-19 cell line
- 3.1.4 Preparation of a smoke exposure protocol
- 3.1.5 The effects of CSE on morphology and viability *in vitro*
- 3.1.6 The effects of cigarette smoke on VEGF, bFGF and PEDF *in vitro*

3.2.1. Growth curve and features of the ARPE-19 cell line

Prior to experimentation, it was necessary to establish the growth curve of the ARPE-19 cell line. This basic tissue culture technique allows the calculation of a lag time, doubling time and a saturation density at confluence. This may be compared to the original reported growth curve of the ARPE-19 cell line to ensure the quality of the line. Calculation of the growth curve also dictates the cell splitting protocol and seeding density for all experiments using the cell line. The cells were examined at sub-confluence, confluence and after periods of post confluence to ensure that morphological features were consistent with the reported cell line.

3.2.2. Differentiation in the ARPE-19 cell line

The ARPE-19 cell line (ATCC CRL 2302) is a spontaneously arising retinal pigment epithelial cell line established from a 19 year old male donor which is of near normal karyotype.¹²⁷ This cell line has the potential to form highly differentiated monolayers in culture that closely resemble RPE cells *in vivo* in both morphology and function. Like RPE cells *in vivo*, ARPE-19 cells have the potential to form tight junctions¹²⁷, phagocytose rod outer segments²⁹⁷ and form polarized layers.¹²⁵ The ARPE-19 cell line selected for experimentation was used at a late passage (passage 20-28). Cell lines propagated through multiple passages may lose many of their original characteristic features in a process known as de-differentiation. Additional measures are required with later passage cells to optimize differentiation and accurately model the *in vivo* state.

The morphology and functional profile of newly plated cells is considerably different to those that have been permitted to differentiate. When originally isolated, the ARPE-19 cell line was maintained for up to 10 months⁴⁶ and prolonged culture has an effect on differentiation and protein expression in this cell line.^{47, 48} The reported durations allowed for differentiation in other experiments vary from 2 days to 10 months.²⁹⁷⁻³⁰⁴ In these experiments, cellular differentiation

was promoted by plating cells on uncoated plastic and maintaining for post confluent periods of up to six weeks.³⁰¹ It was possible to block groups of T25 flasks at the same passage and period post confluence for exposure of each one of the four protocols, as cells were split in 1:4 ratio. The effects of any difference in culture time are reduced by comparing the intra group differences in each block. Although prolonged cell culture without the use of antibiotic treated medium predisposes it to infection, careful aseptic maintenance and regular mycoplasma assessment can maintain viable cultures. Omission of antibiotics removes a confounding factor that may alter the expression of proteins to be examined from the cell. To confirm that the line maintained differentiated properties, cells were examined for the presence of Cellular Retinaldehyde-Binding Protein (CRALBP), a known marker of differentiation. CRALBP is a protein characteristically found within fully differentiated RPE cells.¹⁴⁷ Cells were examined using both immuno-histochemical and Western blot techniques for the presence of CRALBP.

3.2.3. Genetic risk factors in the ARPE-19 cell line

Over 200 publications have reported *in vitro* results based on the use of the ARPE-19 cell line. Many have discussed the findings of *in vitro* experiments and related them to the potential disease mechanisms in AMD. Knowledge of the cell line's genotype is pertinent because the interaction between genetics and the environment is central to the aetiology of AMD. The ARPE-19 was originally derived from a 19-year-old male. These experiments aimed to characterize three SNPs associated with the development of AMD and report the odds ratio (OR) associated with the baseline genetic risk. The cell line was therefore assessed for the presence of the two major genetic determinants of disease, CFH (Y402H) SNP and the LOC387715 (ARMS2) SNP. The presence of the minor SNP PEDF (Met72Thr) was also determined in the cell line.

3.2.4. Preparation of a smoke exposure protocol

As mentioned previously, cigarette smoke is a complex chemical compound. Isolated analysis of all 4,000 constituents of cigarette smoke would be excessively time consuming and inaccurate, omitting all potential toxic interactions. It is therefore preferable to obtain an extract of the cigarette smoke containing as large a fraction of smoke constituents as possible. After a review of the current literature, a consistent reproducible method of generating cigarette smoke extract for use *in vitro* was selected an exposure protocol prepared. The choice of cigarette for

experimentation was based upon industry marketing reports.¹²⁸ Globally Marlboro is the number one selling cigarette brand and as such, Marlboro Red Cigarettes Class A (Tar 10mg, nicotine 0.8mg and carbon monoxide 10mg) were selected for experimentation. The use of standardized experimental cigarettes was rejected, as it is less consistent with human smoking habits. Though this presents a greater degree of pack-to-pack variability, it more closely models human smoking. Several methods of cigarette smoke extract (CSE) generation were considered in preparing the smoking protocol. The ideal protocol was determined to be one that most accurately mimicked the patterns of smoking *in vivo*¹²⁹ whilst providing adequate quantities of CSE for experimentation. It was also considered beneficial to obtain an automated system to ensure accurate reproducibility of the extract. Earlier methods of CSE generation involved drawing the cigarette smoke continuously through a culture medium using a pump system, thus burning through the entire cigarette in a single puff.¹³⁰⁻¹³² This pattern of smoking does not accurately reflect human habits. Moreover it is known that the changes in airflow through the cigarette as it is smoked affects the burning process and may subsequently affect the products of combustion.¹²⁴ Thus a method that considered human habits was preferred. More recent experiments have attempted to account for smoking patterns by setting a pump to “inhale” 35mls of air over a 2 second period followed by a pause of 28 seconds (based on the patterns of the average smoker).¹³³⁻¹³⁶ The method designed by Bernhard et al is a validated pump system that most accurately emulates these patterns.³⁰⁵

Schedules of smoke exposure were constructed by considering published results of experiments utilizing CSE or smoke constituents on other cell lines.^{142-145,306} Periods of exposure from 1 to 72hrs have been reported. A 24, 48 and 72hr exposure protocol was selected. This allows for sufficient time for the possible transcription of mRNA and protein assembly in response to the exposure. Twenty-four hours prior to cigarette smoke extract exposure the ARPE-19 cells were washed twice with PBS and the medium was replaced with a serum-free substitute. Cells were exposed to solutions that correspond to light (1.25%), moderate (6.25%) and heavy (12.5%) cigarette consumption for periods of 24hrs, 48hrs and 72hrs.

3.2.5. The effects of CSE on morphology and viability in vitro

The effects of acute cigarette smoke on the morphology and viability of RPE cells were assessed. Cells were maintained postconfluence in culture for at minimum of 6 weeks. Previous studies have described the effects of individual cigarette smoke constituents on the RPE line. However the effects of total cigarette smoke exposure on cells maintained in this manner has not yet been reported. Cellular membrane morphology and integrity were assessed by staining cell monolayers for occludin, a characteristic tight junction protein present in RPE. Occludin is localized to the apical cell membrane in confluent retinal epithelial cells.¹⁴⁶

Two methods were used to detect the effects of CSE on the proliferation of RPE cells, the Bromodeoxyuridine (BrdU) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays. Both assays have been used as markers of cell viability though each represents a different biological pathway. The BrdU assay measures DNA replication by incorporation of the reagent into DNA in place of thymidine. The reagent is then quantified giving an objective measurement of cellular replication. In contrast, the MTS assay measures cytoplasmic enzyme activity. The MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (CellTiter 96 Assay Promega) is based on a previous colorimetric assay the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is a tetrazole) assay. In the presence of active bio-reductive enzymes, MTT or MTS are reduced from yellow compounds to purple compounds due to the production of formazan. In the MTT assay the formazan compound is a precipitate and must be solubilised using a solubilisation solution which may either be dimethyl sulfoxide, an acidified ethanol solution or a solution of sodium dodecyl sulphate (SDS) in diluted HCl. The peak absorbance of the purple formazan compound produced is usually between 500-600nm. The solvent used to promote solubilisation also determines maximum absorption. The MTS assay is a more refined technique that uses MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) with the addition of phenazine, methosulfate (PMS). This confers advantages over the standard MTT assay. Firstly, the MTS reagents are reduced more efficiently than MTT. Secondly the resulting compound is water-soluble which decreases the direct toxicity to the cells. Reduction of the MTS can only occur when cellular reductive enzymes are active, and therefore it is most

often used as a proxy measurement of cellular viability. It is important to note that changes in metabolic activity can increase the MTS results.

3.2.6. The effects of cigarette smoke on bFGF, VEGF and PEDF *in vitro*

The area of active vascular growth in CNV is the choriocapillaris. To examine the potential contribution of CSE to the development of CNV, the secreted fraction of growth factors was analyzed. The proteins must be actively secreted into the extracellular space or released after cell death to diffuse across the Bruch's membrane and to affect the choriocapillaris. In both cases it is preferable to assay the supernatant rather than the cellular fraction. This project aims to demonstrate the effects of acute cigarette smoke exposure on the concentrations of bFGF, VEGF and PEDF in the supernatant of post confluent ARPE-19 cells.

3.3. Materials and methods

3.3.1. ARPE-19 cell culture

Cells were obtained from colleagues (kindly supplied by Matthew Campbell Conway Institute/TCD Genetics) at passage 19 and directly from the ATCC cell bank (LGC promochem) at passage 20. All cells used for experimentation were used between passage 24 and 28. The subculture protocol of these cells was obtained from the original paper¹²⁷ and from the ATCC data sheet (ATCC, 2007). The cells are grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 1.2g/L sodium bicarbonate, 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate (Sigma Aldrich) with 10% foetal calf serum (Fig 3.6.1) Cells are subcultivated in T25 flasks (Corning) at a ratio of 1:4 and 0.05% trypsin was used to break up cell monolayers. Medium was renewed twice weekly until the cells reached confluence and once weekly thereafter for six weeks. Cell cultures for SNP analysis were used at confluence, as the *in vivo* state of differentiation has no bearing on the genotype. Monolayers were maintained for a minimum of 6 weeks post confluence for all other experiments.

3.3.2. Growth curve and morphology assessments

To establish the growth curve of the ARPE-19 cell line, a confluent T75 flask of ARPE-19 (CRL 2302) cells was washed twice in phosphate buffered saline (PBS). The cells were trypsinised with 3mls of 0.05% trypsin (Gibco) and the flask was incubated for 5 minutes. The flask was gently

agitated, then inspected under the microscope to ensure that all cells had been lifted. Trypsin was deactivated by the addition of 5mls DMEM:F12 with 10% fetal calf serum. The suspension was spun to form a cell pellet which was re-suspended twice in PBS and finally in 5mls of culture medium in order to be counted. A 10 μ l of cell suspension was loaded on to each side of the haemocytometer and the cells were counted. The 5ml cell solution contained 1.2 x 10⁶ cells. To produce a 1 x 10⁶ cell suspension, 1ml of cell culture medium was added and the test tube was inverted to mix. Ten T25 flasks were pre-warmed with 5mls of ARPE-19 cell culture medium. Each of these flasks were seeded with 100 μ l of the 1 x 10⁶ cell suspension which equated to 100,000 cells total or 20,000 cells per ml in the T25 flask. Thus all 10 flasks were seeded with 2-x 10⁴/mL medium. All flasks were incubated under the same conditions, namely 37 C and 5% CO₂. Flasks were obtained for cell counts at the following time intervals; 24 hrs, 48hrs, 72hrs, 120hrs and 168hrs. Cells counts at each time interval were graphed to display the growth curve. The general morphology of the sub-confluent and post confluent ARPE-19 cells was determined by plating ARPE-19 on to T25 flasks. Cells were maintained as described and photographed using phase contrast microscopy (Zeiss Axioplan 2, Oberkochen, Germany). Images were collected at sub-confluent densities, at three weeks post confluence and again at five weeks post confluence (Fig. 3.6.3).

3.3.3. Expression of markers of differentiation in ARPE-19 cells

Immunohistochemical and western blotting techniques were used to determine the expression of CRALBP in cultured monolayers of ARPE-19 cells. Cells were seeded onto glass coverslips in 24-well dishes and maintained post confluence as described. Coverslips were washed twice with ice-cold PBS and fixed with ice-cold methanol for 15 minutes, permeabilised for 30 minutes with 0.5% Triton in PBS and blocked using a 5% goat serum solution. Slides were incubated overnight at 4°C with a 1:200 dilution of rabbit derived polyclonal anti-CRALBP antibody (donated by Ross Collery UCD), washed thoroughly and incubated with a secondary antibody (goat anti-rabbit IgG-CY3 or Alexa Fluor⁵⁶⁸) for 4 hours at 37°C. Cell were counterstained with FITC/Phalloidin stain for the presence of actin and nuclei stained with 1:10,000 dilution of 4'-6-Diamidino-2-phenylindole (DAPI). Slides were mounted in mounting medium and visualized at room temperature using a fluorescent microscope (Zeiss Axioplan 2, Oberkochen, Germany) with integrated software.

CRALBP detection using SDS PAGE and Western blot

ARPE-19 cells were seeded onto plastic T25 flasks and maintained post confluence as previously described. Medium was removed from flasks and the monolayers were washed twice in PBS. 300µl of RIPA lysis buffer was pipetted into the flasks and the cell layer was scraped. The lysates were homogenized mechanically by passing through a syringe 20 times. Samples were left on ice for 20 minutes and cellular debris was removed by centrifugation (10 minutes at 14,000rpm). The supernatant was retained and the pellet was discarded. The protein lysate was separated using a 12% polyacrylamide gel and transferred overnight to PVDF membrane. Blocking was performed using a 5% solution of non-fat powdered milk. Membranes were incubated at 4°C overnight with a rabbit derived polyclonal anti-CRALBP antibody (donated by Ross Collery UCD) in a solution of 5% milk. Blots were subsequently washed and incubated for three hours at room temperature with an anti-rabbit IgG antibody conjugated to horseradish-peroxidase. Images were developed using a chemi-luminescent detection solution and x-ray films. Blots were stripped and re-probed using a mouse monoclonal anti-β-actin antibody to standardize protein load concentrations.

3.3.4. Genetic risk factors in the ARPE-19 cell line

Three T-75 flasks of ARPE-19 cells were examined for the presence of SNP risk factors; CFH variant Y402H (rs1061170), ARMS2 variant A69S (rs10490924) and the PEDF variant Met72Thr (rs1136287). Cells were grown to confluence, trypsinised using a 0.05% trypsin solution and centrifuged for 10 minutes at 1200rpm. The supernatant was removed, cells were re-suspended in PBS centrifuged and washed twice in PBS. DNA was isolated using a phenol extraction protocol and precipitation in 100% ethanol. Samples were dried, re-suspended in buffer and frozen at -20°C prior to polymerase chain reaction (PCR). DNA samples were amplified by PCR with the described primers (table 3.2.1) and all samples were tested in triplicate. Control samples were obtained from volunteer human DNA samples of known genotype.

The CFH SNP was amplified and produced a fragment that is 272 base pairs in length. Successful amplification was confirmed by combining 5ul of product with 1ul of bromophenol blue. Samples were loaded onto a 2% agarose gel containing 1ul of ethidium bromide and electrophoresis was

performed. Fluorescent photography was performed to illuminate the fragments. All PCR fragments were confirmed against a 100bp ladder. Samples that did not show successful amplification were discarded and PCR was performed again. Samples were digested overnight using the NlaIII restriction endonuclease (New England Biolabs). The results of the digests were imaged by electrophoresis on 2% agarose. Non-variant fragments were cleaved by the endonuclease into 187bp and 85bp fragments. DNA containing the C variant was not cleaved by the enzyme resulting in the complete 272bp fragment. Samples heterozygous for the SNP resolved all three fragments.

The LOC 387715 SNP was amplified and produced a fragment of 728 base pairs which were confirmed on a 2% agarose gel as previously described. The successful products were digested overnight by the PvuII restriction endonuclease (New England Biolabs). The results of the digests were imaged by electrophoresis on 2% agarose. Samples that did not include the variant SNP were digested into fragments that were 487bp and 241bp in length. The enzyme did not cleave samples containing the T to G variant SNP. Again, samples that were heterozygous for the SNP resolved all three fragments.

The PEDF SNP was detected in a similar manner. Samples were amplified and confirmed on 2% agarose gel to yield a 250bp product. Successful products were 250bp in length. Samples were digested using the BssSI enzyme (New England Biolabs). Amplified DNA that did not contain the C to T substitution was cleaved to fragments that were 170bp and 80bp in length. The variant was not cleaved. Heterozygous samples resolved all three fragments.

3.3.5. Preparation of cigarette smoke extract (CSE)

The cigarette smoke extraction method described by Bernhard et al³⁰⁵ was emulated with some modifications (Fig 3.6.1). In this system, when a lit cigarette is attached (1), the pump [Automatic Ismatec Peristaltic pump, microprocessor controlled] (7) is activated and draws 35mls of water over 2 sec from the 1litre flask. This vacuum draws an equivalent volume of cigarette smoke through the inert tubing [Ismatec] (2). The smoke is subsequently bubbled through a Pasteur pipette (4) into a glass filter tube containing a 30ml 1:1 mix of Dulbecco's modified Eagle Medium/ Ham's F12 medium pre-warmed to 37°C. After each 2 sec "puff", the machine pauses for 28

seconds. The cycles repeat ten times and result in a total smoking time of 5 minutes. This burns approximately 75% of the cigarette. To produce a suitable control, the system is set up without a cigarette in position and 350mls of air is bubbled through 30mls of medium. All samples are pH balanced and sterilized by passing through a filter of 0.22 μ l pore diameter prior to their use in cellular experiments. A considerable number of the active radicals generated during the combustion process are not stable so the CSE must be used immediately in culture after preparation.¹³⁷ Freezing to -20°C is insufficient to preserve the radical activity and freezing to -80°C has not yet been validated. Our apparatus differs slightly from the one designed by Bernhard. The smoke from 4 cigarettes was used in place of 2 cigarettes and this was bubbled through 30mls of pre-warmed medium instead of 8mls. The glass filter tube (5) is larger accordingly. In this generation system, every milliliter of medium contains the residue 0.133 (4/30) cigarettes. Bernhard's volumetric calculations^{133, 138} have been extrapolated from assumptions that a smoker, on average, inhales 350ml of smoke per cigarette and has a blood volume of 6 litres. A habit of 20 cigarettes/day would correspond to 20 cigarettes' worth of smoke constituents distributed over 6 litres of blood.^{139, 140} This is equal to 0.0033 smoke constituents per milliliter of blood. CSE, by these calculations, contains 40 (1.33/0.0033) times the amount of smoke constituents as the blood of a person who smokes 20 cigarettes a day. Diluting the CSE to 2.5% yields a solution comparable to the blood of a 20-a-day smoker. Using solutions from 1.25% to 12.5% smoking patterns of 10-a-day to 100-a-day may be emulated.

3.3.6. Cellular morphology assessment

Immunohistochemical techniques similar to those described for the CRALBP were used to determine the expression of occludin, a tight junction protein, and localized to viable cell membranes in cultured monolayers of ARPE-19 cells. Cells were seeded onto glass coverslips in 24-well dishes and maintained post confluence. Slides were treated with one of the three concentrations of CSE or control solution as described above. Coverslips were washed twice with ice-cold PBS and fixed with ice-cold methanol for 15 minutes, permeabilised for 30 minutes and blocked using a 5% goat serum solution. Slides were incubated overnight at 4°C rabbit derived polyclonal anti-occludin antibody (ZYMED), washed thoroughly and incubated with a secondary

antibody (goat anti-rabbit IgG-CY3 or Alexa Fluor⁵⁶⁸) for 4 hours at 37°C. Cell nuclei were counterstained with 1:10,000 concentration of 4'-6-Diamidino-2-phenylindole (DAPI). Slides were mounted in mounting medium and visualized at room temperature using a fluorescent microscope (Zeiss Axioplan 2, Oberkochen, Germany).

3.3.7. Cellular viability assessments

BrdU

ARPE-19 cells were prepared by seeding 96-well flat-bottomed plates with 100µl of a 1.2×10^5 /mL cell suspension. These cells were brought to confluence and maintained for at least 6 weeks post confluence in an incubator at 37°C and 5% CO₂. Two forms of control were used, blank wells with no cells to provide negatives for absorbance readings and an untreated cell group to establish background cellular absorbance. 24 hours prior to smoke exposure, medium was removed and plates were washed twice with PBS. Medium was then replaced with serum free DMEM:F12. After exposure to the described CSE protocol, cellular DNA replication was assessed by BrdU incorporation, a commercially available assay (Calbiochem). This technique labels replicating DNA by integration of bromodeoxyuridine (BrdU) within the newly synthesized strands. BrdU working stock (1:2000) was prepared in fresh serum free culture medium. Medium was aspirated from the 96-well plates and a 20µl of the working stock added to each well. Plates were then incubated for 4-6 hours at 37°C, emptied and 200µl of fixative was added to each well. The fixative functions to both fix the cells to the plates and to denature the protein to facilitate BrdU antibody binding. The plates were fixed for a period of 30 minutes at room temperature and then washed. The anti-BrdU antibody is prepared as a 1:100 solution and 100µl of this is added to the wells and samples for 1 hour at room temperature. Three additional washes are performed and 100µl of the reconstituted peroxide goat anti-mouse IgG horseradish-peroxidase (HRP) conjugated antibody is added for 30 minutes. Further washes (x 3) with wash buffer containing surfactant were performed with the final wash consisting of flooding the plates with distilled water (dH₂O). Horseradish peroxidase catalyses the conversion of tetra-methylbenzidine (TMB) causing it to change from a colourless solution to a blue solution. 100µls of this TMB substrate is added to each well in the dark. After 15 minutes, 100µl of stop solution (2.5N sulphuric acid) is added to the wells in the same order as the TMB. The colourimetric reaction was detected using a

96-well spectrophotometric plate reader at an absorbance of 450nm and 595nm. Samples were analyzed in triplicate and all experiments were repeated three times and on separate days.

MTS

The MTS assay (CellTiter 96 Assay Promega) was performed as follows: ARPE-19 cells were seeded to 96-well plates similar to the BrdU protocol described above. Cells were maintained for at least five weeks post confluence. In preparation for smoke exposure, culture medium was withdrawn and replaced with serum free medium 24 hours prior to experimentation. Plates were exposed to varying concentrations of CSE as described above. 20µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (CellTiter 96 Assay Promega) was pipetted into each well after exposure. Plates were incubated at 37°C for 2 hours and were read using a 96-well spectrophotometric plate reader at an absorbance of 490nm. Cell free control wells were treated in a similar fashion to account for direct effects of CSE on the MTS reagent and mean effects were deducted from final experimental results. Samples were analyzed in triplicate and all experiments were repeated three times on separate days.

3.3.8. Enzyme-linked Immunosorbant Assays (ELISAs)

Cells were cultured in 6-well plates and maintained 5 weeks post confluence as previously described. Cultures were assessed in blocks of 4 to ensure all samples were of comparable age. Culture medium was removed, plates washed and serum free medium replaced 24 hours prior to all experiments. Wells were exposed to 1 of the 3 concentrations of CSE randomly allocated for the control solution for periods of 24, 48 or 72 hours reaching a total supernatant volume of 3mls per well. After exposure, the supernatant was removed, centrifuged at 1000 RPM to remove non-adherent cells, and aliquoted. These cells must therefore be removed otherwise the lysis of non-adherent cells would release the intracellular contents and alter the protein content of the supernatant. Samples were either analyzed immediately or frozen to -80°C. Commercially available quantitative sandwich ELISA kits for VEGF, bFGF and PEDF were used (R&D systems and MD Bioproducts).

The VEGF ELISA assay (R&D system DVE00)

The plates provided in this assay are pre-coated with a mouse-derived monoclonal antibody (IgG) generated against human VEGF, which detects all isoforms of VEGF with an affinity for VEGF₁₆₅. Wells were prepared with 50µl of assay diluent and 200µl of either cell substrate or VEGF protein standard was added to each well. Recombinant protein standards for calibration and quantification were assessed in duplicate and supernatant samples for experimentation in triplicate. Plates were sealed with an adhesive strip and incubated for two hours at room temperature. After this incubation interval, 100µl of the detection antibody was added. This secondary antibody was a goat anti-human IgG conjugated to horseradish-peroxidase, which forms the upper layer of the “sandwich” ELISA. After a two-hour incubation period at room temperature the plates were washed carefully 4 times using Tween buffer. After this, 100µl of the colourimetric substrate solution (1:1 H₂O₂:TMB) was added to each well, and the plate was wrapped in aluminium foil to maintain darkness. The reaction was halted after 20 minutes with a stop solution (2N sulfuric acid) and plates were assessed using a 96-well spectrophotometric plate reader at an absorbance of 450nm and 595nm. The experiment was repeated five times on different days.

The bFGF ELISA assay (R&D systems DFB50)

Similar to the described VEGF ELISA assay, the commercially available bFGF sandwich ELISA contains 96 flat-bottomed well plates pre-coated with mouse-derived monoclonal antibody (IgG) generated against human bFGF. Plates were prepared with 100µL assay diluent followed by the addition of 100µL aliquots of either recombinant bFGF protein standards or experimental supernatant. As above, protein standards were assessed in duplicate and supernatant samples in triplicate. The plates were sealed with an adhesive strip and incubated for 2 hours at room temperature. Plates were washed four times in the wash buffers provided and blotted to remove all residual liquid. 200µL of bFGF conjugate was added to each well and incubated for 2 hours at room temperature. The wash step was repeated and 200µL of the colourimetric substrate (1:1 H₂O₂:TMB) solution was added to each well. The plate was incubated for 30 minutes in the dark. This reaction was stopped through the addition of stop solution (2N sulfuric acid). The density of the colour change was assessed using a 96-well spectrophotometric plate reader at an absorbance

of 450nm and 595nm. Again, the experiment was repeated in triplicate, five times on different days.

The PEDF ELISA assay (MD Bioproducts BPM #PED613)

Initially a PEDF ELISA kit was obtained from Millipore/Chemicon that consisted of mouse-derived anti human PEDF pre-coated wells to be used in a manner similar to the VEGF and bFGF ELISAs described previously. It was discovered however that this ELISA plate system could not detect PEDF from any of the samples tested. There were two likely explanations for this failure in detection. Samples to be assessed required predilution with urea where all samples to be assayed were treated with 8M-concentration urea, incubated on ice and diluted to 1:100 prior to ELISA. Any small quantities of PEDF secreted by the cells would be further diluted to below the detectable range. The range of detection was too narrow to assess cell culture supernatant samples. These difficulties were addressed by obtaining direct PEDF ELISA kits from an alternative source (MD Bioproducts) that had a higher sensitivity (x50) for PEDF detection and did not require pretreatment of samples with urea. The 96 well plate provided was coated with polyclonal antibody specific for human PEDF antigen. 100 μ Ls of either recombinant protein standard or experimental supernatant were pipetted into each well. Again, protein standards were assessed in duplicate and supernatant samples in triplicate. The plate was sealed, incubated for 1 hour at 37°C and washed four times as with previous ELISA assays. The streptavidin peroxide conjugate was added (100 μ Ls to each well) and incubated for 30 minutes at 37°C. The plate-washing step was repeated and 100 μ Ls of TMB substrate pipetted into each well. The plate was then wrapped in aluminium foil to ensure darkness for 20 minutes. A stop solution (2N hydrochloric acid) was added and the plates analysed using a 96-well spectrophotometric plate reader at an absorbance of 450nm with a reference absorbance of 650nm. The experiments were performed with all samples in triplicate, six times on different days.

The Bichinichoninic Assay (BCA)

The concentrations of the relative proteins in the supernatants were calculated for each ELISA as above. All medium samples were simultaneously measured for total protein concentrations using a Bicinichoninic assay (BCA) (Thermo Scientific Pierce BCA) to correct for alterations in total protein concentrations. This permitted comparison of concentration changes relative to control

samples rather than total supernatant concentrations. Each BCA assay was performed and analysed at the same time as the ELISA assessments. The BCA assay is also a colourimetric assay based on the Biuret reaction. Protein reduces Cu^{2+} to Cu^{1+} in an alkaline solution. When the BCA reagent is added, it is reduced by the copper cation to form a coloured water-soluble compound. The BCA assay was performed on flat bottomed 96 well plates. Each well contained 25 μL s of cell substrate or protein standard. Protein standards were prepared from serial dilutions of bovine serum albumin (BSA). 200 μL s of BCA working reagent (1:8) was added to each well, Plates were incubated at 37°C and then read using a 96-well spectrophotometric plate reader at an absorbance closest to 562nm (595nm).

3.4. Results

3.4.1. Growth and morphology of the ARPE-19 cell line

The growth of the ARPE-19 cell line was recorded at regular intervals over the period of one week. Cells reached a confluent density of 8.28×10^5 after 160 hours of growth. The growth curve is illustrated below (Fig. 3.4.2). From this, the optimal seeding density was determined as 1.5×10^5 , and the ratio of splitting for each flask was 1:4. The passages of ARPE-19 obtained and cultured in this project displayed a growth curve consistent with reports from both the original isolation of the line and the ATCC data. Phase contrast microscopy images obtained from the ARPE-19 cell line are shown (Fig. 3.6.3). Cells maintained post confluence displayed contact inhibition and did not continue to overgrow in the flask. The hexagonal morphology was not fully achieved, however, and cellular melanin granules did increase in density indicative of the differentiating RPE line.

3.4.2. Markers of differentiation in the ARPE-19 cell line

To establish the presence of CRALBP in ARPE-19 cell cultures, samples were cultured as previously described and analysed using immunofluorescent and Western techniques. A positive pattern of staining of both actin (FITC/Phalloidin stain) and CRALBP was seen in post-confluent APRE-19 monolayers (Fig. 3.6.4A and B). Lysates of post confluent cells similarly showed the presence of CRALBP detected by 12%SDS PAGE and Western Blot confirming the presence of this specific RPE marker of differentiation (Fig. 3.6.5).

3.4.3. Genetic risk factors in the ARPE-19 cell line

Restriction endonuclease digest results were compared to human samples of known SNP status (Fig 3.6.6). Restriction endonuclease digests were stained with ethidium bromide and separated on 2 or 3% agarose gels. All gels feature a 100bp ladder. **A:** ARPE-19 compared to two known heterozygous (T/C), one homozygous human control for the Y402H SNP (T/T) and one non-affected human control (C/C). **B:** ARPE-19 compared to two heterozygous (G/T) and two homozygous human controls for the ARMS2 SNP (T/T). **C:** ARPE-19 compared to two known heterozygous (C/T), two known human controls homozygous for the PEDF Met72Thr SNP (C/C), and two homozygous unaffected (T/T) human controls. The ARPE-19 line is heterozygous in respect to both the CFH (T/C alleles), and the ARMS2 (G/T) SNPs. The line is homozygous for the variant PEDF Met72Thr SNP (T/T) (Table 3.6.7).

3.4.4. Cellular morphology in response to CSE exposure

There was no significant morphological difference in response to CSE detected on phase contract microscopy. Cellular membrane morphology and integrity was assessed qualitatively by staining cell monolayers for occludin, a tight junction protein found localised to the cell membrane in confluent cells¹⁴⁶. When compared with controls, the distribution of occludin staining illustrated a consistent pattern across all exposure concentrations confirming that cellular morphology and tight junction integrity was preserved even at high concentrations of CSE (Fig 3.6.7).

3.4.5. Cellular viability in response to CSE exposure

The results of the BrdU assays are shown (Fig 3.6.8) At the 24hour time point, cells assayed using the BrdU protocol did not display any significant difference in proliferation in response to CSE exposure. There were no significant differences noted at the 48 or 72hour exposure times either, when compared with control samples. Cells were subsequently assessed using the MTS assay (Fig 3.6.9). When compared with controls, samples exposed to highest concentration of cigarette smoke (12.5%) displayed a 40.91% increase in bio-reductive capacity after 24 hours ($p<0.001$). This persisted up to the 72-hour time point. Samples exposed to moderate (6.25%) CSE exposure induced a transient increase in reductive capacity of 32.34% ($p<0.001$) at 24 hours, which returned to baseline at 72 hours. There was no effect on the lowest concentration of CSE on the

metabolic activity of ARPE-19 cells at any duration of CSE exposure. Most notably cells did not lose viability during any of the exposure protocols.

3.4.6. The effects of cigarette smoke on bFGF VEGF and PEDF

bFGF

Acute CSE exposure over 24 hours induced significant increases in the release of bFGF (Fig 3.6.11). Cells exposed to 12.5% CSE showed an increase in bFGF secretion of 192.1% ($p < 0.001$). At the moderate 6.25% CSE concentration, there was an increase of 56.82% detected. There was no significant effect of 1.25% CSE concentration on bFGF secretion. After 48 hours cells exposed to the 1.25% CSE and the 6.25% CSE showed no significant change in bFGF. Cells exposed to the highest concentrations of CSE displayed an increase in bFGF secretion of 1540% ($p < 0.01$). At the 72-hour time point, no change was detected either at the 1.25% or the 6.25% concentrations (Table 3.6.8). Cells exposed to 12.5% CSE for 72 hours showed an increase of 3061% when compared to control samples ($p < 0.01$).

VEGF

When compared to control samples, cells exposed to the highest concentration of CSE (12.5%) displayed a decrease in VEGF secretion of 65.65% after 24 hours ($p < 0.001$) (Fig3.6.13). Cells exposed to 6.25% CSE also experienced a significant reduction in VEGF secretion of 46.1% after 24 hours ($p < 0.001$). Exposure to the lowest concentration of CSE had no effect on the secretion of VEGF. After 48 hours of 12.5% CSE exposure, VEGF secretion was reduced by 61.69%. The 48-hour 6.25% CSE exposure protocol reduced VEGF secretion by 38.64% ($p < 0.01$). Exposure to 1.25% CSE had no effect on secretion after 48 hours of exposure. After the 72 hour exposure protocol, the 12.5% CSE resulted in a decrease of 58.09% ($p < 0.001$). The 6.25% CSE concentration resulted in a decrease of 35.08% ($p < 0.01$) after 72 hours. There was no effect observed from the 1.25% concentration after 72 hours (Table 3.6.9).

PEDF

There was no significant difference in PEDF expression detected between the supernatants of the cells exposed to CSE and control medium at any of the tested CSE concentrations or exposure

times (Fig 3.6.15). The experiments were repeated with each assessment well in triplicate, six times on separate days to confirm the negative findings (Table 3.6.10).

3.5. Discussion

Initial cell line experimentation determined the growth curve and the morphological features of the ARPE-19 cell line. Establishing a growth curve for a cellular line allows accurate prediction of confluence and allows planning for subculture protocols. The ARPE-19 line used in these experiments was obtained from two sources, as a gift and purchased from the American Type Culture Collection (ATCC) via LGC promochem. Assessment of the line showed growth and cellular features consistent with published use in both the gifted and purchased lines. The use of a cell line is not as optimal a model of the RPE as primary cultures. However the capacity for multiple subculture passages provides the quantity of RPE cells required for multiple repetitions of the experimental exposure protocols.

The cells retained functional attributes consistent with native RPE, as shown by the presence of CRALBP in the RPE line. Cultured ARPE-19 cells stained positive for the presence of CRALBP and intracellular CRALBP was confirmed on the blot. Therefore ARPE-19 cells maintained in the prolonged culture manner described, retained the potential to express a known marker of differentiation in RPE. Morphological appearance was consistent with other reports of this later passage. Though they did not achieve regular hexagonal alignment, cells exhibited contact inhibition and tight junctional complexes. Over time post confluence, cellular melanin granules became denser, which is consistent with a more differentiated state. It was concluded that to promote differentiation, cellular experiments would not be performed at sub-confluence or early confluence. Cells were maintained in an antibiotic-free medium for a minimum of 6 weeks prior to use to avoid additional confounding variables.

The only exception after the introduction of this prolonged culture protocol was for the characterization of SNPs in the ARPE-19 cell line. The DNA obtained at sub confluence is the same as that obtained after prolonged culture to the protocol was not necessary. In these experiments we report the first results characterizing the genetic risk factors predisposing to AMD inherent in

the ARPE-19 cell line. ARPE-19 is heterozygous for both the CFH and the ARMS2 SNPs. The possession of the heterozygous genotype confers a theoretical odds ratio (OR) of 2.31 with respect to the CFH SNP. The ARMS2 heterozygous state confers an OR of 2.89 respectively and when combined, both genotypes confer an OR of 8.48.⁴² In addition, this line was found to be homozygous with respect to the Met72Thr SNP in the gene encoding for PEDF conferring an OR of 3.90 of developing neovascular AMD.⁸ These baseline genetic risk factors could be directly relevant to the biological pathways governing AMD and should be considered when utilizing this cell line. Ideally, the availability of cell cultures featuring all combinations of genotypes for experimentation would yield greater information on the interactions between genetic risk factors and the environment, but this is currently unfeasible.

As mentioned previously with each cigarette, smokers consume over 4,000 compounds that undergo further interaction during combustion. With this significant volume of toxins, it is unlikely that cigarette smoke exerts its pathological effects through a single biochemical pathway. Isolating the singular pathological effects of each individual constituent would be a complex large-scale task and would still not determine the effects of the burning process on the interactions between the constituents. In these experiments, we elected to use the soluble constituents of cigarette smoke that are analogous to a fraction of smoke constituents that enter the blood of a smoker. The choice of cigarette for experimentation was based on Industry marketing reports.⁵⁰ and cigarettes were burned in a manner that mimicked the behavior of a smoker as closely as possible.⁴⁰

Previous investigations into the effects of acute cigarette smoke and cigarette smoke constituents on RPE cells have demonstrated significant changes in cellular morphology and viability. Apoptosis has been observed in response to cigarette smoke extract³⁰⁷ and individual cigarette smoke constituents.²⁸⁶ These studies maintained ARPE-19 cells in culture for periods of 48hrs to 1-week post confluence. Early use of the line post confluence is very convenient but not consistent with the cells *in vivo*.⁴⁸ Shorter culture times alter protein expression and increase the susceptibility of ARPE-19 to the effects of oxidative stress.⁴⁹ We therefore conclude that the acute apoptosis observed previously *in vitro* is a product of shorter culture times and higher cellular

susceptibility to toxic injury. In this study, this limitation is addressed by prolonging post confluent cell culture to over 6 weeks. In the original isolation report the ARPE-19 cell line was maintained for up to 10 months⁴⁶ and prolonged culture has an effect on differentiation and protein expression in this cell line.⁴⁷

The effects of acute cigarette smoke exposure on morphology were qualitatively observed by assessing the expression of a cellular membrane protein, occludin. Occludin is expressed on the cell membranes of epithelial cells and in conjunction with claudin proteins, forms the intercellular tight junctions that maintain the oBRB. By staining cells with immunofluorescent antibodies to occludin, the membranes of viable cells may be visualized. Acute CSE exposure did not alter the morphology of the cells, nor was there any evidence of widespread apoptosis, which is in contrast to previous studies. The cells remained adherent to the underlying matrix.

The effects of acute cigarette smoke exposure on cellular proliferation were examined using the BrdU assay and MTS assays. At six weeks post confluence, the ARPE-19 cells had very little active cellular turnover. On examination, there was no significant difference in cellular proliferation between the control samples and any of the CSE concentrations. This indicates that acute cigarette smoke exposure in post-confluent cells has no effect on the rate of DNA synthesis.

The MTS assay was used to provide an alternative means of viability assessment. Though typically used as a proxy measure of cell viability, the MTS assay is an assessment of metabolic activation, specifically the bioreductive capacity of cells.³⁰⁸ An increase in the conversion of MTS reagent to formazan is indicative of increased activity of mitochondrial reductase enzymes. The cellular morphology assessment and the BrdU analysis confirmed that the ARPE-19 cells were not actively dividing. The MTS assay however showed an increase in bioreductive capacity secondary to smoke. It may be concluded that cigarette smoke directly increases cellular metabolic activity. This is likely to be due to an increase in NADH and NADPH secondary to a generalized non-lethal stress response. Evidence from the morphological and viability observations indicate that the concentrations of cigarette smoke exposure used in these experiments does not cause cellular apoptosis seen in other reports. As the concentrations used in these experiments were consistent

with those of other studies, it is likely that the prolonged culture times reduced the susceptibility of the cells to stress. This is more consistent with *in vivo* findings. It was also noted that these effects were reduced at the 72 hour time period, indicating a potential normalization post exposure.

Acute CSE exposure caused a significant increase in the expression of bFGF in the ARPE-19 cell line, which was related both to CSE concentration and exposure time. This is the first report of an increase in bFGF secretion by ARPE-19 cells in response to acute CSE exposure. The cells exposed to the highest concentration of CSE exhibited an increase in bFGF after all three exposure intervals. The effect increased over time from almost double after 24 hours to a 30-fold increase in secretion after 72 hours. At the moderate concentration (6.25%) of cigarette smoke, an increase in bFGF secretion of 58.82% was observed at 24 hours. This was not observed at the later time point. There were no effects observed with the low level (1.25%) of CSE exposure. Taken with the observations from the MTS assay, it appears that the ARPE is stressed by the CSE. At a low level of cellular stress (1.25%), the intracellular defenses accommodate with no effect on reductive capacity or bFGF expression. As the concentration rises to 6.25%, changes are detected on the MTS assay that persisted for 48 hours. An increase in bFGF is observed for only 24 hours. It is likely that the rise in bFGF indicates an acutely stressed cell. Over time however, the cell has the capacity to overcome these stressors and to return to the baseline condition by 72 hours. No return to baseline was observed at the highest concentration of CSE. The increase in bFGF was matched by a persisting increase in cellular reductase activity seen on the MTS. This raises two possibilities; either the damage induced by the cigarette smoke has exceeded the cell's capacity for toxic insult, or a greater time period post exposure is required to allow the cell to recover. Further research will be required to determine the capacity of differentiated RPE to recover from toxic insults and at what threshold the damage becomes too great and the cell begins the process of apoptosis.

The expression of bFGF by ARPE-19 has been shown previously, to be up-regulated by a number of stressors including neurotrophic factors, cytokines, and oxidative stress.^{49, 62, 63} Nicotine has been shown to increase the expression of bFGF by endothelial cells.⁶⁴ Functionally, bFGF activates

migration of mesodermal cells, increases DNA synthesis⁶⁵ and acts synergistically with VEGF to promote cellular proliferation.⁶⁶ RPE cells exposed to elevated concentrations of bFGF have previously been shown to increase VEGF secretion mediated through the FGFR-2 receptor,¹⁸ and cultured endothelial cells exposed to bFGF respond by an increased expression of VEGF.⁶⁷ One other study reported an increase in bFGF expression without a corresponding increase in VEGF expression in response to oxidative stress.⁶³ It has therefore been hypothesised that bFGF plays a role as an early priming event prior to increases in other angiogenic factors. This is supported by these current findings that illustrate an early bFGF response was in response to cigarette smoke. It is unclear whether this bFGF response is beneficial to cell survival or whether it is the instigator of the angiogenic response, but there is evidence that combined bFGF and VEGF suppression may be superior to anti-VEGF therapy alone in the treatment of CNV.¹⁸³

In these experiments, ARPE-19 cells exhibited a suppression of secretion rather than a release of VEGF in response to acute CSE. Cells exposed to the highest concentration of smoke exhibited the greatest suppression with a reduction of 65.65% after the 24-hour exposure period. The 6.25% CSE concentration also significantly suppressed VEGF by 46.1%. There was no effect at the lowest level of cigarette smoke. The effects of the CSE were dose-dependent, with the higher concentrations of CSE causing greater suppression. The response lessened over time for both CSE concentrations indicating a potential recovery from cellular insult.

These results are in contrast to previous studies that described an acute up-regulation of VEGF in response to cigarette smoke or constituents.^{43, 51} Cells used in the reported studies were maintained for short periods prior to experimentation and showed acute apoptosis in response to toxic insult. The increased VEGF expression may be a response to extreme cellular crisis and the release of intracellular VEGF as apoptotic cells lyse. Our findings report a cellular environment at a point significantly earlier in the pathway responding to exogenous toxins. Though the cause of the reduced secretion of VEGF is not clear, it may be mediated through the effect of nicotine. Nicotine has been shown to decrease VEGF secretion in a porcine model³⁰⁹ but increase secretion in a primary rat RPE line.³¹⁰ VEGF is constitutively expressed by healthy RPE cells⁶⁸ and it has been shown that this baseline secretion maintains the fenestrations of the choriocapillaris supplying the RPE.⁶⁹ Reduction in trophic support of the choriocapillaris may lead to relative hypoxia of the

RPE. The development of CNV occurs late in the course of the disease and is preceded by RPE changes; it is therefore possible that the suppression of VEGF leading to RPE damage may precede cellular apoptosis, which may then be accompanied by large increases in VEGF.

We reported no change in the concentrations of PEDF in response to acute CSE exposure. It has previously been observed that PEDF secretion by RPE cells is decreased by oxidative stresses.³⁷ It is possible that the prolonged periods in culture permitted the ARPE-19 cells a greater resistance to the effects of oxidative stress. The point at which the reported responses were observed preceded severe cellular damage. PEDF may on become involved the later stages of cellular injury. It has also been hypothesised that PEDF responds to elevated local levels of VEGF. In these experiments suppression rather than elevation of VEGF was observed which might not elicit any response in PEDF. Both reduced oxidative stress and low VEGF expression may therefore account for the lack of change in PEDF.¹⁸⁶

It is possible that relative rates of diffusion through the Bruch's membrane play a significant role in the pathology of AMD. AMD is associated with increase membrane thickness. If the initial response of RPE is a chronic reduction in VEGF secretion, as the Bruch's membrane thickens the diffusion of this reduced concentration is even impaired further. The trophic maintenance of the choriocapillaris may be compromised resulting reduced fenestration and reduced provision of oxygen and glucose that in turn is diminished by the impaired diffusion. The resulting relative hypoxia could potential lead to RPE cells committing to the apoptotic pathway. The cell death and hypoxic drive could create a high regional concentration of VEGF. The surge in VEGF may therefore promote the development of CNV in this initial VEGF suppression model.

The findings in our current study are the first to show that ARPE-19 cells in prolonged culture may tolerate acute cigarette smoke exposure without suffering a loss of morphology or viability. Cigarette smoke extract exposure had a significant impact on the levels of expression of both bFGF and VEGF. These results may represent the first description of the molecular events that may precede the development of CNV in individuals heterozygous for the known genetic variants associated with AMD. Investigation into the methods by which cigarette smoke exerts its toxic

effects may shed light on the biological pathways that culminate in the changes of Age-Related Macular Degeneration.

3.6. Figures and tables

Dulbecco's Modified Eagle's Medium)/ Ham's Nutrient Mixture F-12	
Component	g/L
Inorganic salts	
CaCl ₂ • 2H ₂ O	0.1166
CuSO ₄ • 5H ₂ O	0.0000013
Fe(NO ₃) ₃ • 9H ₂ O	0.00005
FeSO ₄ • 7H ₂ O	0.000417
MgCl ₂ • 6H ₂ O	0.0612
MgSO ₄	0.04884
KCl	0.3118
NaHCO ₃	1.2
NaCl	6.996
Na ₂ HPO ₄	0.07102
NaH ₂ PO ₄	0.0543
ZnSO ₄ • 7H ₂ O	0.000432
Amino acids	
L-Alanine	00445
L-Arginine • HCl	0.1475
L-Asparagine • H ₂ O	0.0075
L-Aspartic Acid	0.00665
L-Cystine • 2HCl	0.01756
L-Cysteine • HCl • H ₂ O	0.03129
L-Glutamic Acid	0.00735
Glycine	0.01875
L-Histidine • HCl • H ₂ O	0.03148
L-Isoleucine	0.05447
L-Leucine	0.05905
L-Lysine • HCl	0.09125
L-Methionine	0.01724

<i>Dulbecco's Modified Eagle's Medium)/ Ham's Nutrient Mixture F-12</i>	
Component	g/L
L-Phenylalanine	0.03548
L-Proline	0.01725
L-Serine	0.02625
L-Threonine	0.05345
L-Tryptophan	0.00902
L-Tyrosine • 2Na • 2H ₂ O	0.05579
L-Valine	0.05285
Vitamins	
D-Biotin	0.0000035
Choline Chloride	0.00898
Folic Acid	0.00265
myo-Inositol	0.0126
Niacinamide	0.00202
D-Pantothenic Acid • 1/2Ca	0.00224
Pyridoxine • HCl	0.002031
Riboflavin	0.000219
Thiamine • HCl	0.00217
Vitamin B12	0.00068
Other	
D-Glucose	3.15
HEPES	3.5745
Hypoxanthine	0.0021
Linoleic Acid	0.000042
Phenol Red • Na	0.00863
Putrescine • HCl	0.000081
Pyruvic Acid • Na	0.055
DL-Thioctic Acid	0.000105
Thymidine	0.000365
ADD	
L-Glutamine	0.365

Table 3.6.1: Constituents of DMEM:F12 obtained from Sigma-Aldrich

Preparation of cell culture medium
1:1 Dulbecco's Modified Eagle Medium: Ham's F12 medium
1.2g/L Sodium Bicarbonate
15mM HEPES Buffer
0.5mM Sodium Pyruvate
2.5mM L-glutamine
10% Fetal Calf Serum

Table 3.6.2: Constituents of the ARPE-19 cell medium

Gene	Forward Primer 5'-3'	Reverse Primer 3'-5'	Endo-nuclease
CFH	GTTCGTCTTCAGTATACAT	AGACCAGCCATTCTCCAT	NlaIII
ARMS2	CAGCACCTTGTCACCACA	CTAACAACCCGGACGAGAAG	PvuII
PEDF	CAGCACCTTGTCACCACA	CCTCGCTGAACTGAAATGT	BssSI

Table 3.6.3: Primers used for Polymerase Chain Reaction (PCR)

PCR Mix	
DNA	5 µL
Buffer mix	2.5µL
Forward Primer	0.25µL
Reverse Primer	0.25µL
dNTP	0.5µL
Tac Enzyme	0.125µL
DNase free H ₂ O	16.375µL
Total Volume	25µL

Table 3.6.4: PCR constituents for cellular amplification

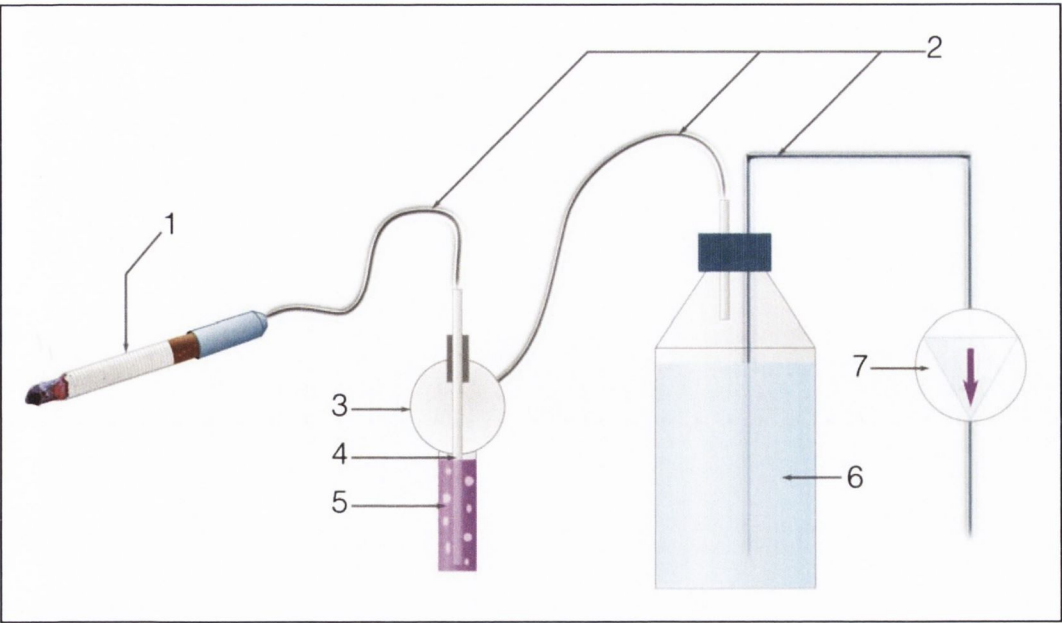


Figure 3.6.1: CSE generation apparatus (1 = Cigarette, 2 = Inert tubing, 3 = Two-port bung, 4 = Pasteur pipette, 5 = Test tube with side arm, 6 = Flask capacity over 1L, 7 = Microprocessor controlled peristaltic pump)

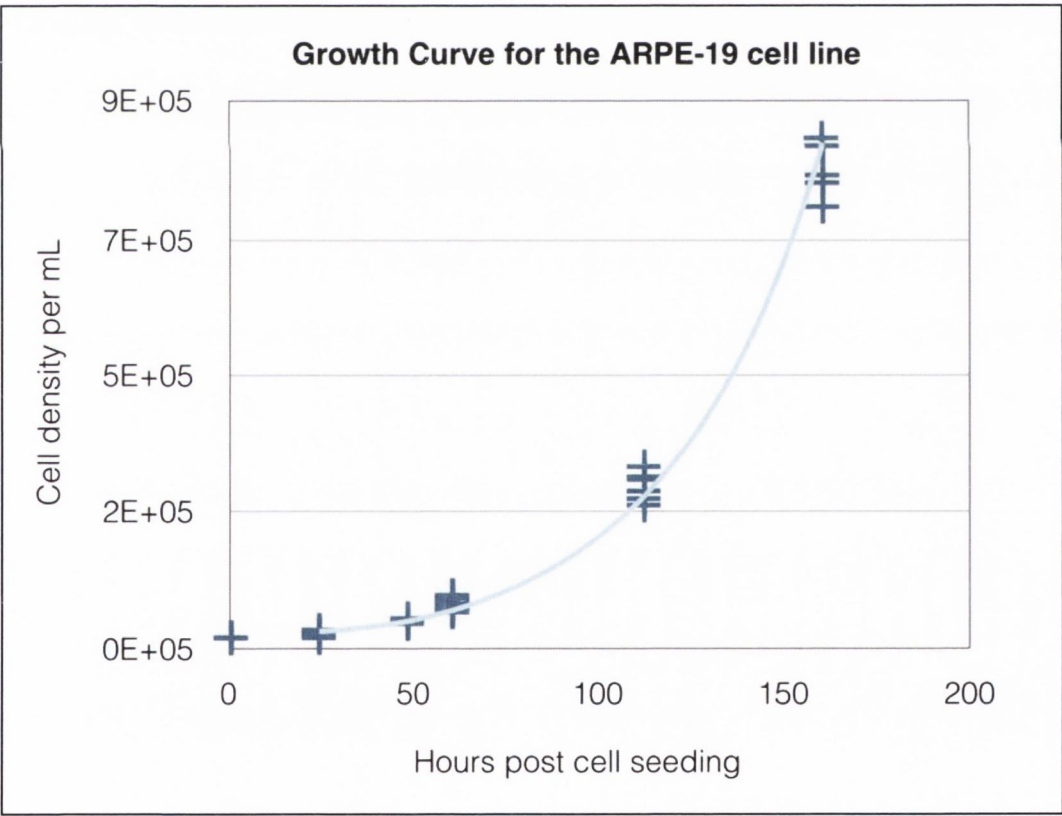


Figure 3.6.2: Growth curve of the ARPE-19 cell line

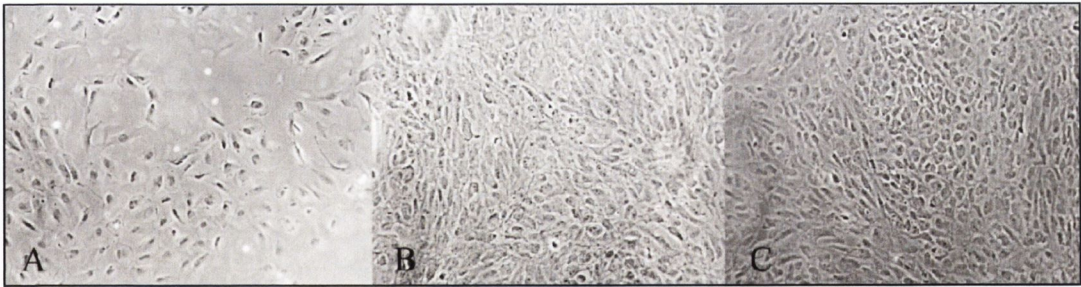


Figure 3.6.3: Phase contrast ARPE-19 images. A: Sub-confluent B: 3 weeks post-confluence C: 5 weeks post-confluence

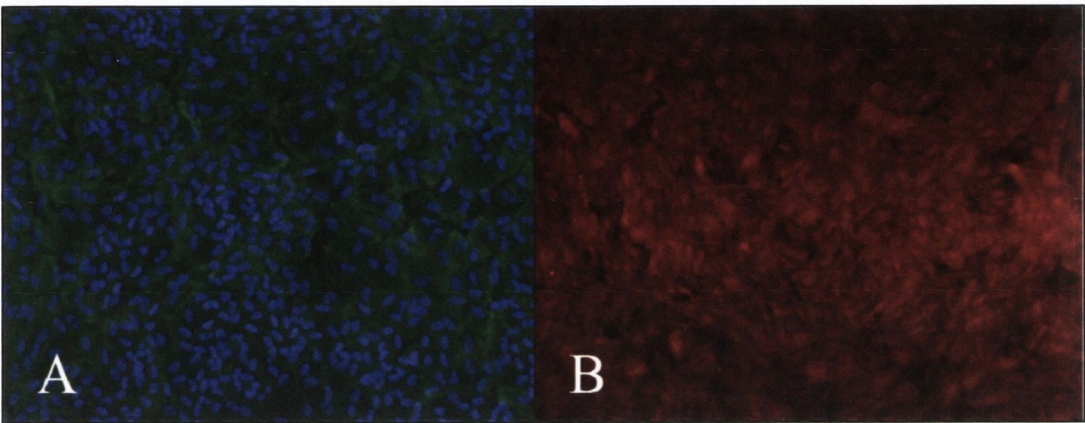


Figure 3.6.4: Immunocytochemistry A: ARPE-19 Phalloidin/FITC and DAPI B: ARPE-19 cells stained with Anti-CRALBP/CY3

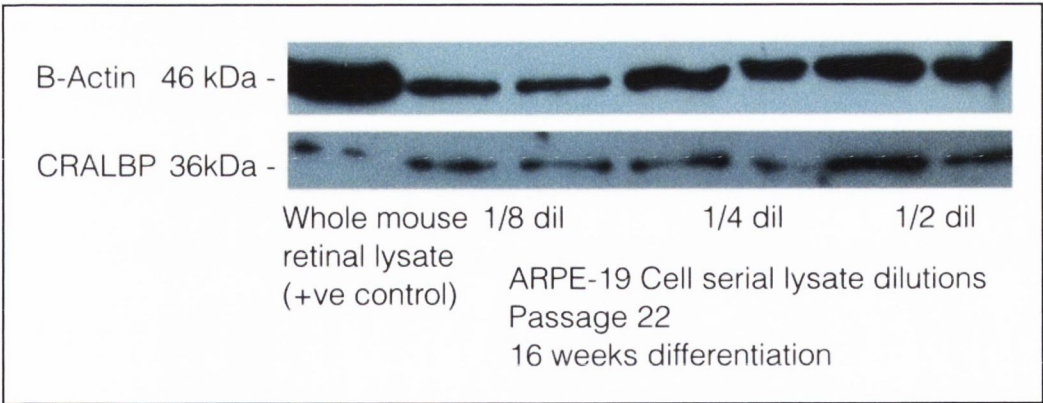


Figure 3.6.5: Western blot of CRALBP in the ARPE-19 cell line.

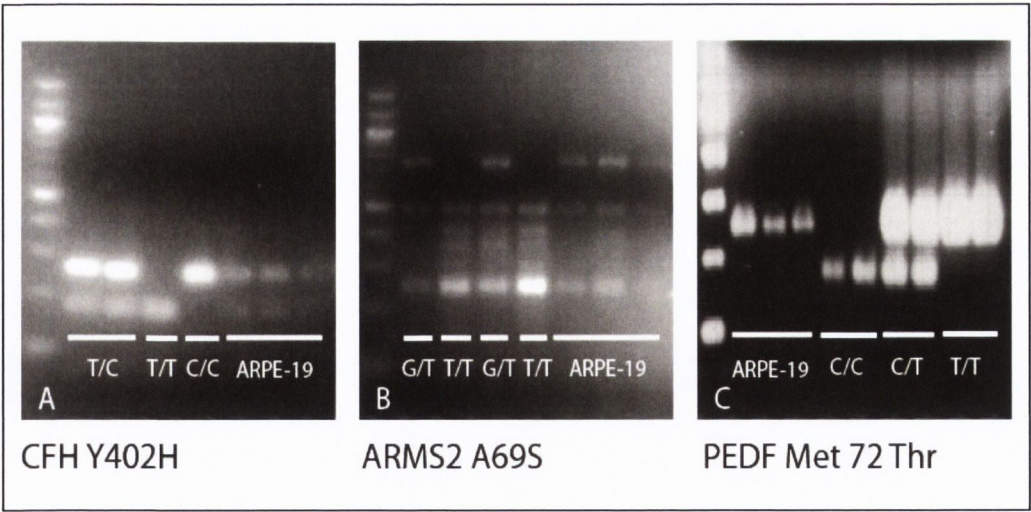


Figure 3.6.6: Restriction endonuclease digest

ARPE-19 SNP results	
CFH Y402H	T/C
ARMS2 A69S	G/T
PEDF Met72Thr	T/T

Table 3.6.5: ARPE-19 SNP digest results

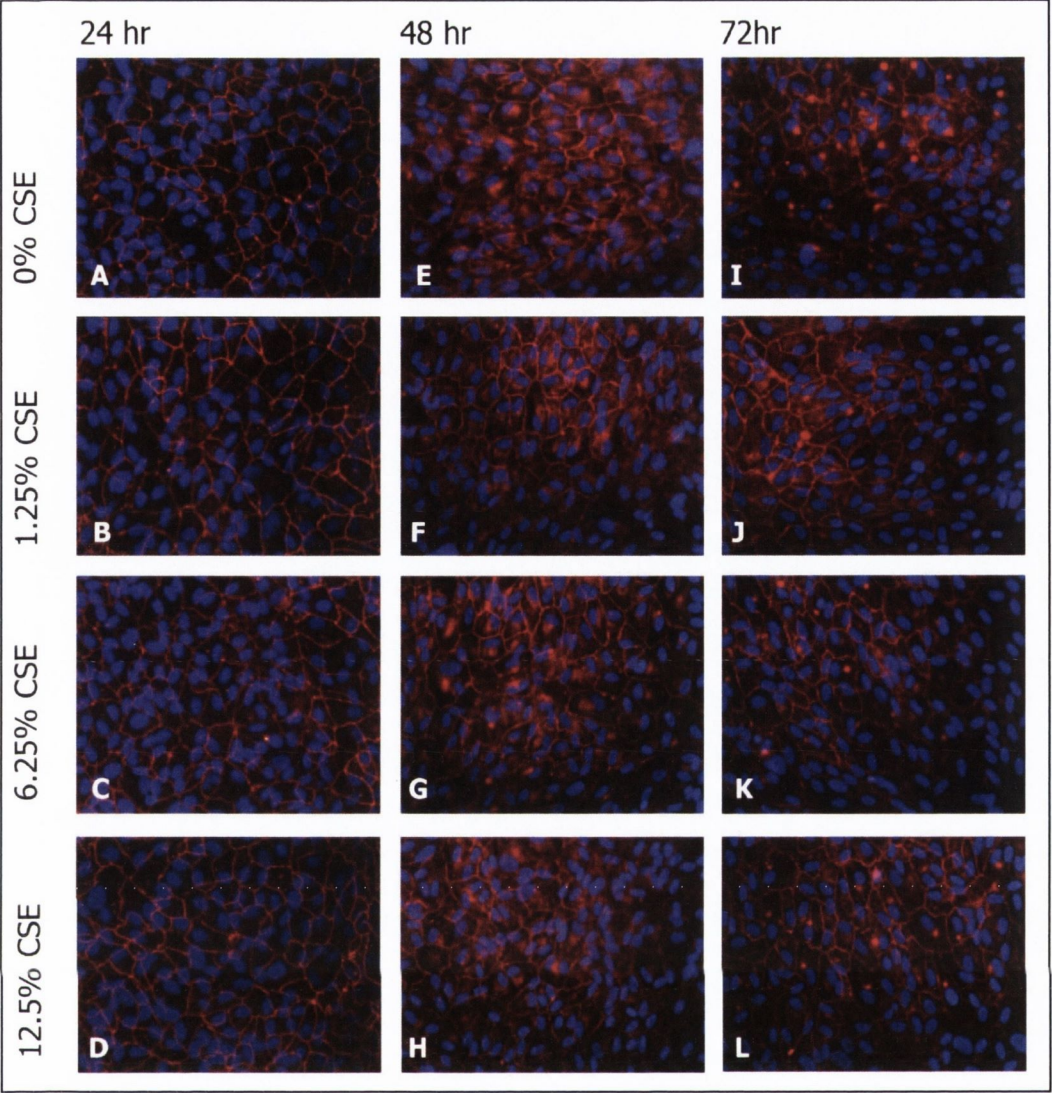


Figure 3.6.7: Expression of occludin in ARPE-19 cells exposed to CSE **A-D**: 24 hours **E-H**: 48 hours **I-L**: 72 hours.

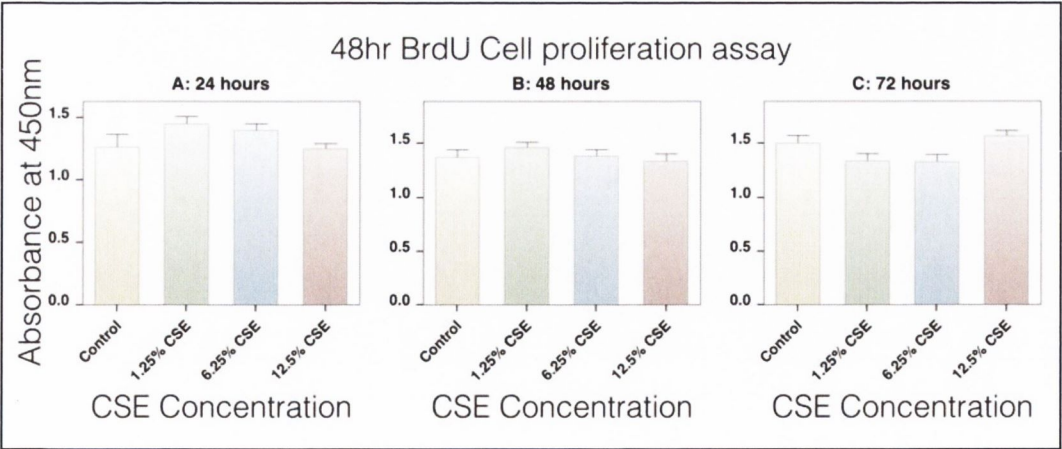


Figure 3.6.8: BrdU assay of ARPE-19 cells exposed to CSE **A**: 24 hours **B**: 48 hours **C**: 72 hours.

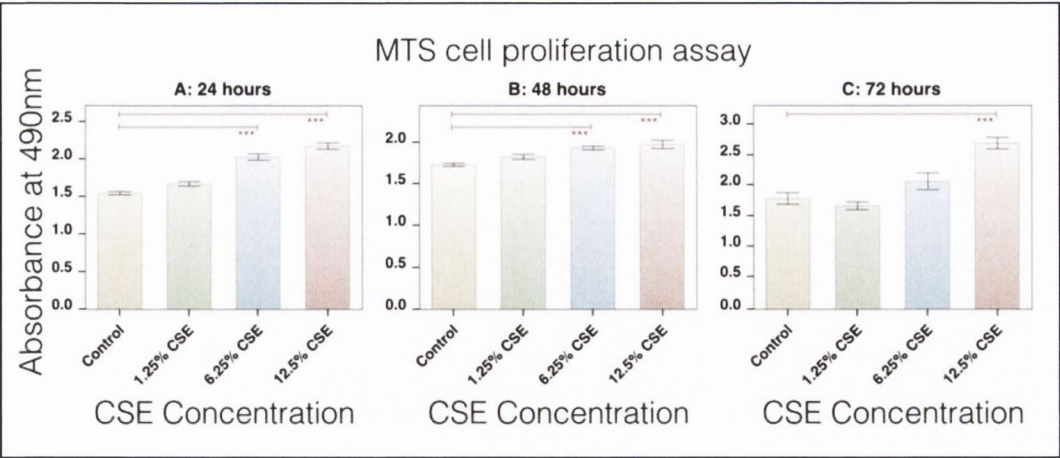


Figure 3.6.9: MTS assay of ARPE-19 cells exposed CSE **A**: 24 hours **B**: 48 hours **C**: 72 hours.

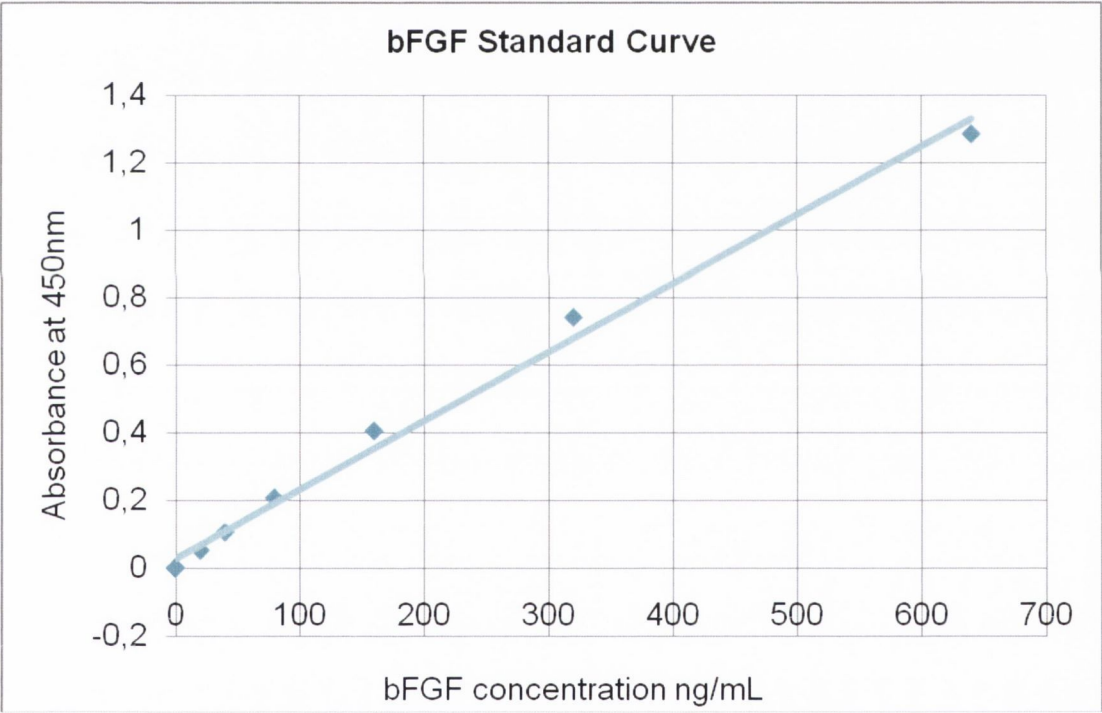


Figure 3.6.10: Standard curve for bFGF ELISA

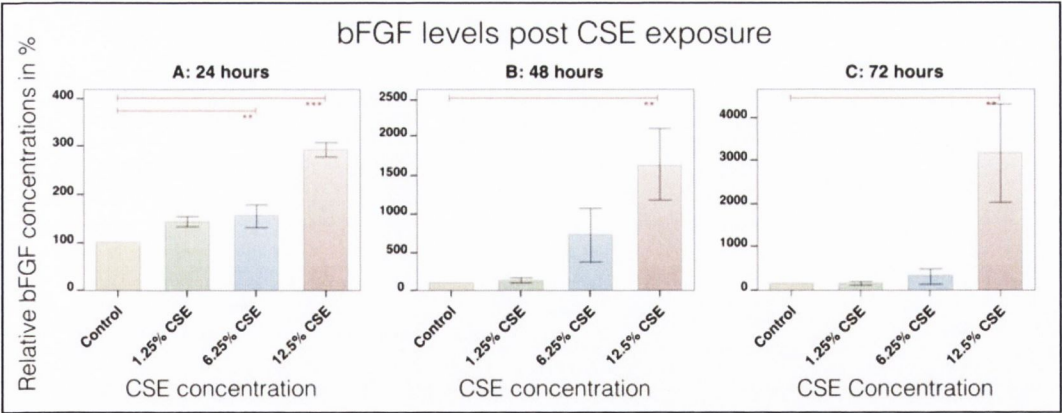


Figure 3.6.11: ARPE-19 supernatant FGF-2 concentrations **A:** 24 hours **B:** 48 hours **C:** 72 hours.

Duration	CSE concentration	Change	95% CI of difference	P - Value
24h				
	1.25%	41.40%	-0.98 - 83.79	> 0.05
	6.25%	56.82%	11.43 - 99.21	< 0.01
	12.50%	192.10%	149.7 - 234.5	< 0.001
48h				
	1.25%	32.58%	-10.21 - 108.6	> 0.05
	6.25%	624.10%	-429.4 - 1678	> 0.05
	12.50%	1,540%	486.3 - 2593	< 0.01
72h				
	1.25%	27.52%	-2288 - 2326	> 0.05
	6.25%	210.80%	-2104 - 2526	> 0.05
	12.50%	3,061%	746 - 5376	< 0.01

Table 3.6.6: Changes in bFGF secretion in response to CSE

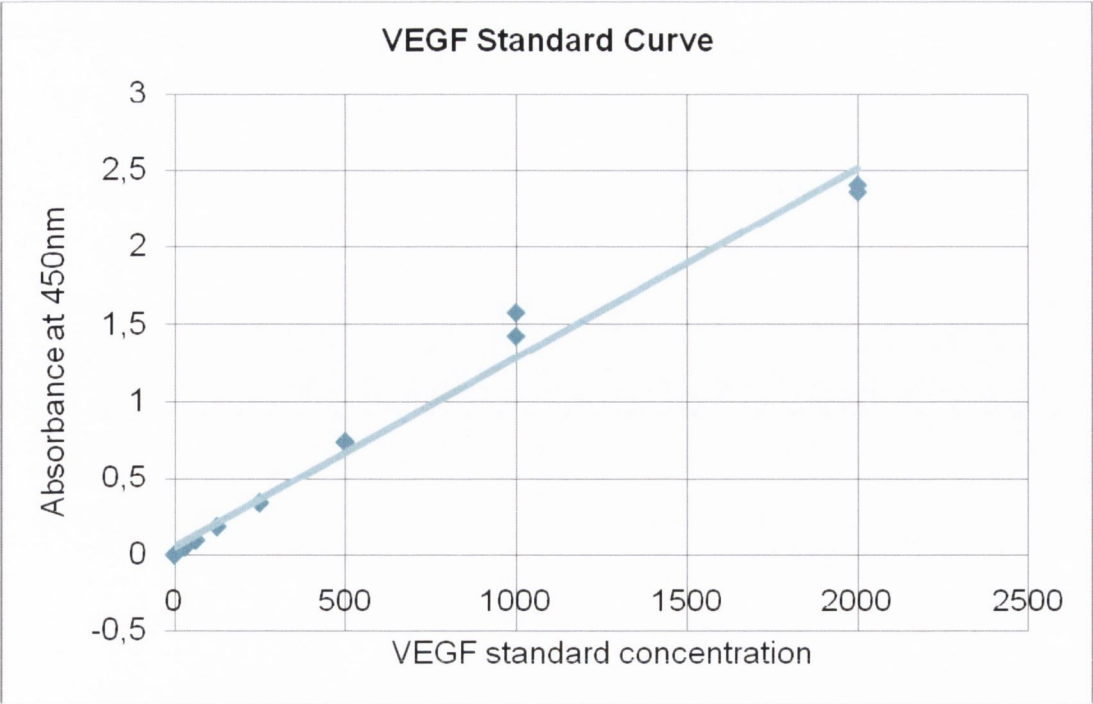


Figure 3.6.12: Standard curve for VEGF ELISA

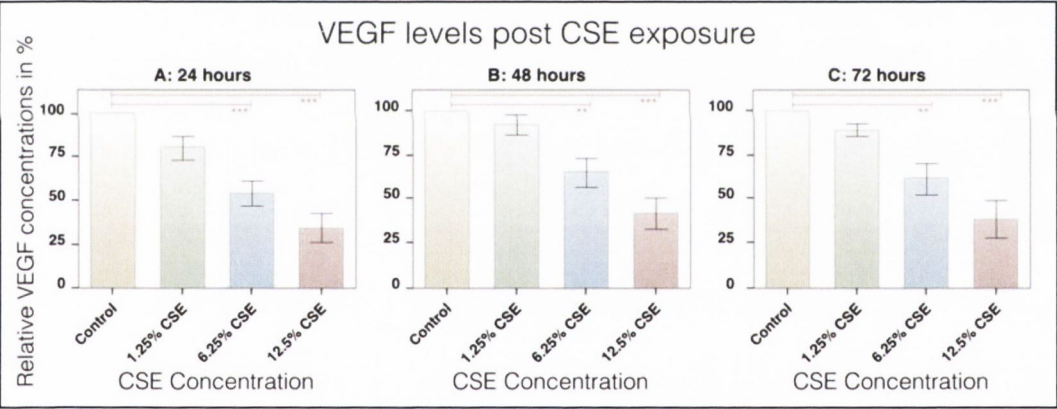


Figure 3.6.13: ARPE-19 supernatant VEGF concentrations **A**: 24 hours **B**: 48 hours **C**: 72 hours.

Duration	CSE concentration	Change	95% CI of difference	P - Value
24h				
	1.25%	-19.83%	-40.85 - 1.193	> 0.05
	6.25%	-46.10%	-67.12 - -25.08	< 0.001
	12.50%	-65.65%	-86.67 - -44.63	< 0.001
48h				
	1.25%	-8.45%	-34.28 - 17.38	> 0.05
	6.25%	-35.68%	-60.92 - -9.25	< 0.01

Duration	CSE concentration	Change	95% CI of difference	P - Value
72h	12.50%	-58.09%	-83.92 - -32.26	< 0.001
	1.25%	-11.16%	-36.85 - 14.53	> 0.05
	6.25%	-38.64%	-64.33 - -12.94	< 0.01
	12.50%	-61.92%	-87.61 - -36.23	< 0.001

Table 3.6.7: Changes in VEGF secretion in response to CSE

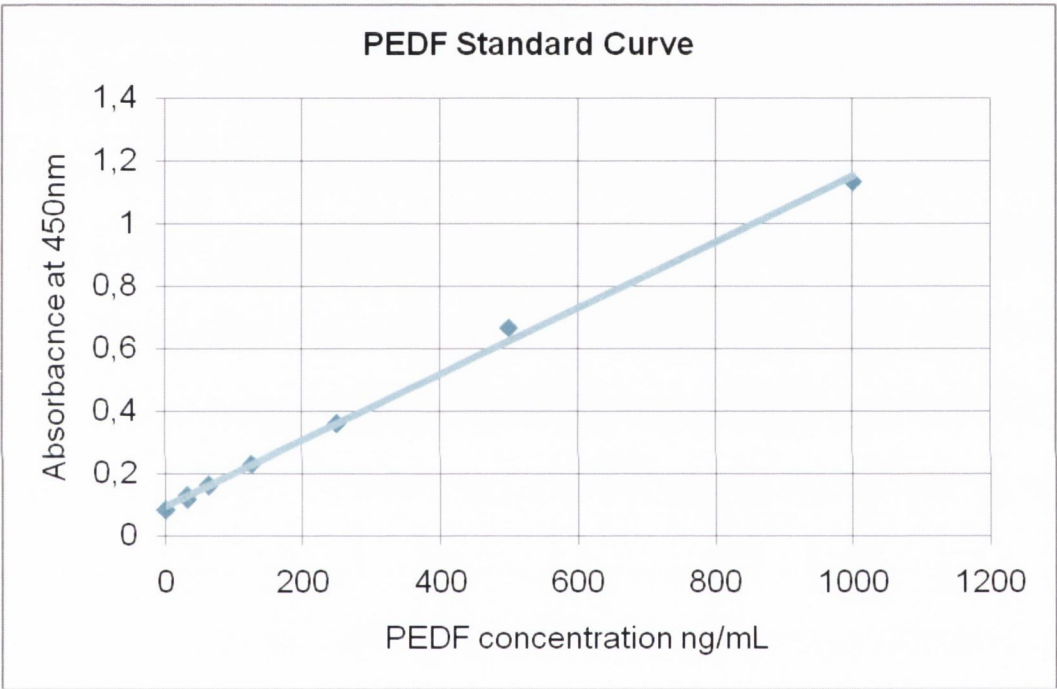


Figure 3.6.14: Standard curve for PEDF ELISA

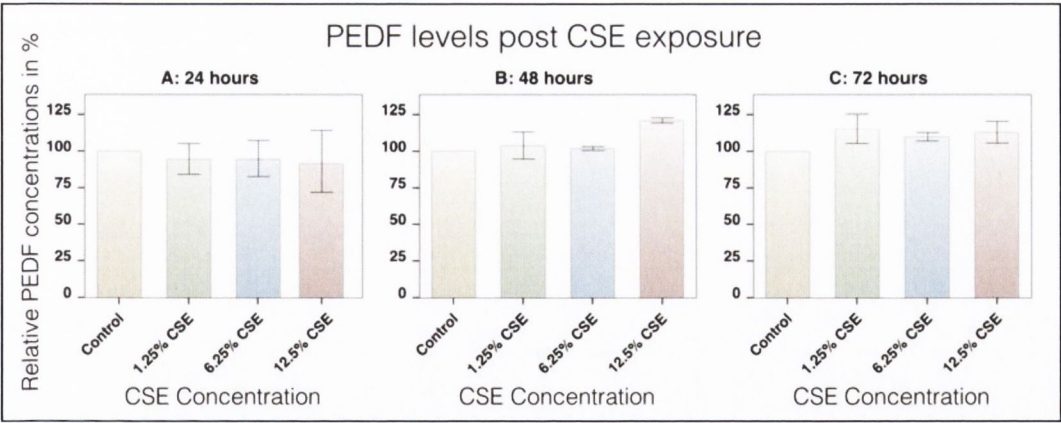


Figure 3.6.15: ARPE-19 supernatant PEDF concentrations **A**: 24 hours **B**: 48 hours **C**: 72 hours.

Duration	CSE concentration	Change	95% CI of difference	P - Value
24h				
	1.25%	-5.87%	-55.99 - 67.74	> 0.05
	6.25%	-5.38%	-56.48 - 67.25	> 0.05
	12.50%	-6.44%	-55.43 - 68.3	> 0.05
48h				
	1.25%	-3.73%	-25.9 - 18.44	> 0.05
	6.25%	-1.70%	-23.87 - 20.47	> 0.05
	12.50%	-20.88%	-43.06 - 1.28	> 0.05
72h				
	1.25%	-15.50%	-45.57 - 14.56	> 0.05
	6.25%	-7.81%	-37.87 - 22.26	> 0.05
	12.50%	-11.34%	-41.4 - 18.72	> 0.05

Table 3.6.8: Changes in PEDF secretion in response to CSE

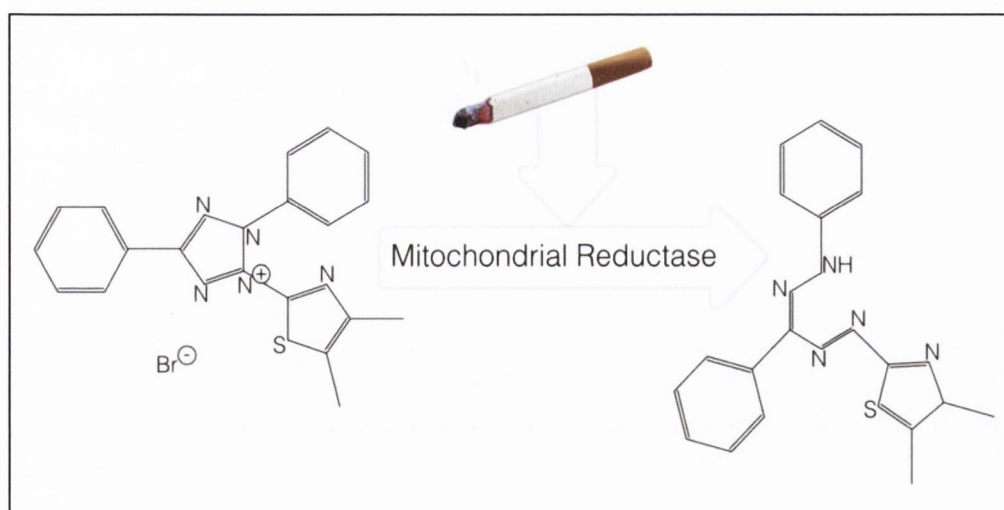


Figure 3.6.16: Cigarette smoke conversion of MTS reagent to formazan

4.1. Introduction and rationale for the use of animals

Pathological changes associated with AMD are restricted to the level of the retinal pigment epithelium and Bruch's membrane in 90% of cases. These patients are categorized as atrophic or dry AMD. Approximately 15% of patients will progress to the neovascular form of AMD and 10% will progress to severe widespread atrophy (GA). The factors that govern progression to late AMD are unknown and it is not clear what determines the development of CNV in some patients and GA in others. Cigarette smokers are at a greater risk of developing AMD and when the disease has become established smokers progress to neovascular complications five to ten years sooner than non-smokers.

Although the RPE has been described as the control centre for the molecular messages governing angiogenesis, the RPE, Bruch's membrane and the choriocapillaris function as a unit and are all affected during angiogenesis. We hypothesize that cigarette smoke influences, not only the early development of AMD at the level of the RPE, but also directly promotes the vascular proliferation at the choriocapillaris seen in CNV. The *in vitro* cell culture investigations describe the earliest responses to the insult of CSE at the level of the RPE. *In vitro* cell culture techniques are limited however and cannot accurately model more complex systems. Co-culture of RPE and endothelial cells can be used to examine simultaneous responses to stress in both cell types but these models do not form a basement membrane equivalent to the Bruch's membrane. It is therefore currently not possible to model the pathology of CNV *in vitro*. An animal model was required to determine the effects of CSE on the development of CNV. A murine model is particularly useful as it allows sufficient animal numbers to be gathered to illustrate the effects of a given intervention without the complications and costs of using higher order animals. Through the use of *in vivo* experimentation we aim to describe the influence of cigarette smoke on angiogenesis, electrical function and general oxidative stress in the eye.

4.2. Aims

4.2.1 Effects of acute (IVT) administration of CSE on retinal vasculature

- 4.2.2 Establish novel method to permit chronic cigarette smoke exposure in a mouse model.
- 4.2.3 Determine the effect of chronic cigarette smoke exposure on the general physiology, and electrophysiology of SOD1 ^{-/-} mice
- 4.2.4 Determine the effect of chronic CSE exposure in the SOD1 ^{-/-} mouse on oxidative stress marker: Acrolein.
- 4.2.5 Determine the effect of chronic CSE exposure in the SOD1 ^{-/-} mouse on the volume of CNV in a laser-induced model.

4.2.1. The effects of acute intravitreal CSE on retinal vasculature

Cigarette smoke alters angiogenesis and branching patterns of newly developing embryonic vessels²⁹⁴ but the effects of acute smoke exposure on well established retinal vessels has not yet been described. Various methods of administration were considered to achieve a significant retinal concentration of CSE in a single dose. These included intravenous (IV), intraperitoneal (IP) and intravitreal injection (IVT). In a healthy animal, a single dose of cigarette smoke extract administered through the systemic circulations would be absorbed by circulating antioxidants and hepatic mechanisms. This is not likely to have sufficient penetration to the eye to elicit a response so in order to achieve a more potent dose, CSE was administered through IVT. IVT injection achieves a high concentration of CSE in the vitreous with direct exposure to the retinal vasculature. CSE was prepared by adapting the *in vitro* extraction system to prepare CSE in PBS solution. This solution was compatible with use in mice. Animals were treated and euthanised 72 hours after exposure. The acute effects of CSE on the morphology of retinal vessels treated in this manner were observed.

4.2.2. Establishing a method of chronic CSE exposure in vivo

There are a number of problems encountered when considering an *in vivo* investigation on the effects of chronic cigarette smoke exposure. Firstly the standard animal smoke exposure chamber is costly and requires a large dedicated storage area.²¹⁶ Animals exposed to chronic cigarette smoke must be maintained separately from other experimental animals, to prevent the effects of passive smoke, for periods of up to six months. Secondly, despite containment these units are associated with high degree of operator exposure to cigarette smoke during use. It is unacceptable

both from an ethical and legal standpoint to permit investigators to be exposed to cigarette smoke during experimentation. To counter these difficulties we have proposed, and validated, an alternative method of chronic smoke exposure based on the previously described *in vitro* CSE generation technique.

The *in vitro* cigarette smoke extraction system produces a cell culture medium that is analogous to the absorbed smoke extract in the blood of a smoker. By extracting the cigarette smoke into phosphate buffered saline (PBS) solution it is possible to prepare an extract that can be scaled down and administered to animals. The doses administered to mice are calculated by weight and estimated intravascular volume, to equate it to the levels circulating in the plasma of smokers (Table 4.6.1). This novel system requires validation to ensure that when the CSE is administered via intraperitoneal injection (IP) it is absorbed systemically. We aimed to determine the effects of IP CSE injection on the serum and urinary levels of cotinine, a biomarker of nicotine metabolism.

Although the proposed *in vivo* method of cigarette smoke administration is significantly cheaper and more self-contained than large exposure chambers, there is a limit to the number exposures that may be administered. While intraperitoneal injections are tolerated well by animals, every injection carries the risk of visceral puncture and animal death. The maximum number of injections recommended by the Bioresources department from an animal welfare and ethical standpoint was thirty. We therefore selected a breed of mouse that was most likely to manifest a response to cigarette smoke, if one were present, within that exposure time. The SOD1^{-/-} mouse is a knockout mouse bred on a C57BL6 background. These animals are genetically deficient in superoxide dismutase 1 (SOD1), a key enzyme in systemic protection from oxidative stress. We hypothesize that the reduced capacity of this mouse to compensate for oxidative stress will promote penetration of the CSE to the eye. This would permit the oxidative stress in the CSE to accumulate in the RPE more rapidly than in wild type mice. Therefore if chronic CSE exposure were to effect the volume of CNV, it would be observed in this breed of mice earlier than in the wild type.

4.2.3. The effects of chronic CSE exposure on physiology and electrophysiology of the SOD1-/- mouse

We characterized the effects of chronic CSE exposure on a number of parameters including behaviour, body weight and electrophysiology in the SOD1-/- mouse. Electroretinography (ERG) is a commonly used electrophysiological technique that produces an objective measurement of retinal photoreceptor function. The subject is exposed to a flash of light that activates an electrical potential within the retina. The resulting patterns produced distinctive waveforms, which may be changed in some disease states. The light stimulus may be manipulated to isolate either rod function or cone function, providing ERG patterns with individual characteristics (Fig. 4.6.1). The initial negative deflection on the ERG tracing represents photoreceptor and Muller cell hyperpolarization; this is described as the a-wave. A pure rod response may be recognized by the absence of an a-wave. The second deflection in the tracing is known as the b-wave and is generated in second order neurons, typically the on-bipolar cells. The recorded parameters include the amplitude of each wave and the latency to maximal response. The dark-adapted response maximizes the contribution of rod photoreceptors (Fig 4.6.1). The single flash stimulus in the light adapted state maximizes the contribution of the cone response (Fig 4.6.2).

This test has been successfully adapted for use in mice.³¹¹ The main advantage to the use of the ERG is the possibility of repetition after a treatment intervention. Tracings were obtained prior to, and following, the cigarette smoke exposure protocols to determine any generalized electrophysiological response to chronic cigarette smoke exposure in a susceptible model. ERG tracings for both isolated rod and isolated cone parameters from SOD1-/- were compared.

4.2.4. The effects of chronic CSE exposure in the SOD1 -/- mouse on oxidative stress marker – Acrolein

Acrolein (propenal) is an unsaturated aldehyde byproduct of lipid peroxidation. Increased concentrations of acrolein have been associated with oxidative stress and cigarette smoke exposure.²⁶⁵ The SOD1-/- knockout mouse is more susceptible to oxidative stress. To assess the effects of smoke exposure on the SOD1-/- animal model, animals were exposed to a randomly allocated protocol of CSE injection or control injection. Injections were performed daily for thirty

days as previously described. The effects on the retina were assessed by antibody staining of frozen sections through the mouse eye.

4.2.5. The effect of chronic CSE exposure in the SOD1 -/- mouse on the volume of laser-induced CNV

There is no animal model of AMD available that consistently produces the retinal pigment epithelial or neovascular changes seen in AMD. There are a number of techniques available to induce blood vessel growth including hypoxia and injection of bFGF with lipopolysaccharide. These techniques have variable results and do not consistently provide comparable neovascular complexes. In contrast, the laser-induced mouse model of choroidal neovascularisation is a well-established technique of generating CNV.³¹² An argon-diode laser uses light in the green spectrum to create a hole at the level of Bruch's membrane through which blood vessels proliferate from the choroidal vasculature under the retina. This technique has been widely adopted since the first reported use³¹² and the roles of numerous cytokines and effects of treatment modalities have been demonstrated using this method.^{103-105, 313} Laser-induction methods provide CNV more consistently than the available genetic animal models.²⁷⁸ If the same laser settings are used and burns of consistent size are formed, the vascular membranes produced are of a consistent volume. The influence of an external factor may therefore be determined by the direct comparisons in the volumes of CNV.

4.3. Material and methods

4.3.1. Animals used for experimentation

All animal experiment protocols were assessed and approved by an internal ethics committee in Trinity College Dublin (TCD) prior to commencement. All investigations carried out adhered to the ARVO statement for the use of Animals in Ophthalmic and Vision Research. The researcher completed the Laboratory Animals in Science and Teaching (LAST) training modules prior to any work undertaken that involved animals. A B1 certificate was obtained from the Irish government and was allocated to the moderate severity banding. Two breeds of mice were used over the course of these experiments and are outlined in the following subsections.

C57 Black 6 mouse (C57BL/6)

This strain of commonly used inbred mice was originally obtained from Jackson laboratories but is routinely bred in the Trinity College Dublin bioresources department. C57Bl6 mice were used to establish and optimize the cigarette smoke exposure protocol and the laser CNV protocol.

SOD1 knockout mouse (B6.129S7-Sod1^{tm1Leb}/DnJ)

Superoxide dismutase 1 knockout (SOD1^{-/-}) animals were bred on a C57 Black 6 background. Over time they develop drusen-like deposits on the retina and 10-15% will spontaneously develop CNV. Animals were purchased from the Jackson laboratories and bred on-site in the bioresources department of the Ocular Genetics Unit in Trinity College Dublin. These animals were housed and aged for at least one year prior to their use in the chronic cigarette smoke exposure protocols.

4.3.2. General animal handling techniques

Animal welfare was a particular concern at every stage of experimentation. Established welfare protocols were adhered to. Animals were given habitat enhancers and additional bedding material where required. Animals that showed illness or stressed behaviour were removed from the experimental protocol. Animals in severe distress were euthanized.

Anaesthesia protocol in mice

Animals were anaesthetised with weight based intraperitoneal injections of xylocaine (7mg/g) and ketamine (15mg/g) diluted in normal saline. Depth of anaesthesia was confirmed by application of limb pressure to confirm the absence of a pain response. Mice were administered a weight based IP injection of atipimazole (0.01mg/g) to reverse the anaesthesia post operatively. Animals were placed over a heated pad and under a heat lamp and observed until a diagnosis of full recovery could be made.

Intraperitoneal injection procedure

Wearing gloves, the mouse was lifted by the tail and placed on a flat surface or the cage lid. The loose skin at the back of the neck was grasped firmly to immobilize the head and front paws. The mouse was lifted and the hind legs and tail were held with the ring finger and little finger. Tilting the head backwards allowed the intra-abdominal contents to slide cranially and reduced the risk of organ perforation. The skin was held taut and the needle was inserted at an angle of

approximately 20 degrees in the lower right quadrant to avoid the caecum and bladder. The mouse was observed for 3-4 minutes after the procedure.

Urine and blood collection

Urine was collected from the mice by lifting them out of the cage and holding them over a collection dish. As part of the startle reflex mice often release some urine upon being lifted up. Gentle stroking of the lower abdomen also promoted the release of urine. Mice tolerated the handling well, and there is very little discomfort associated with this procedure. Serum was obtained from the mouse post mortem by dividing the sternum, isolating the heart and pipetting blood from the ventricles and the thoracic cavity.

Mice perfusion protocol for vessel staining

Animals were anaesthetised as described. A midline incision followed by a sternotomy was performed to expose the heart while it was still perfusing under the anaesthetic. A perfusion pump was inserted into the left ventricle and used to fix the vasculature with 4% freshly prepared paraformaldehyde. The paraformaldehyde perfusion process was complete when pallor was detected upon observation of the liver. The perfusion pump is then filled with a solution of 10mg/mL FITC-Dextran. The animal is perfused for 5-10 minutes and then euthanised by cervical dislocation.

Euthanising animals

All animals were sacrificed using either CO₂ asphyxiation or terminal anaesthesia. Cervical dislocation was employed to confirm death, which is in keeping with Trinity College Dublin's bioresources departmental guidelines.

4.3.3. Intravitreal injection of CSE

Eight C57BL/6 mice were used to determine whether direct high concentration intravitreal exposure to CSE would have a morphological effect on the retinal vasculature. Animals were randomly allocated one of the following treatments:

- Low concentration: 20cigs/day
- Medium concentration: 50cigs/day
- High concentration: 100cigs/day

- Control: Sham PBS injections

All intravitreal experiments were performed with the assistance and supervision of Mr. Paul Kenna (Trinity College Dublin). Two animals were selected for each treatment group. Once under anesthesia, pupils were dilated using 2.5% phenylephrine and 1% tropicamide and a contact lens was placed over the eye. 3 μ l of either CSE or control PBS were injected into the posterior chamber of both eyes of all 8 mice. The pars plana was identified and 3 μ l of either CSE or control PBS was injected into the posterior chamber of both eyes of all 8 mice using a Hamilton syringe. The murine lens occupies a significantly greater proportion of the eye than the human lens, and therefore care was required to avoid a traumatic cataract. The vitreous cavity in the mouse contains approximately 7 μ l.³¹⁴ The injection therefore represents an increase of 42% in volume. The equivalent intravitreal injection volume in the human eye would be an injection of 2.1ml based on an average human vitreous volume of 5ml.

Mice were maintained postoperatively on a heat plate while the anaesthesia was reversed. Animals were observed for between 10 to 15 minutes post revival to confirm successful reversal of anaesthesia prior to returning them to the cages. Animals were terminally anaesthetized using xylazine and ketamine 72 hours post exposure to the allocated protocol. The eyes were removed by proptosing the globe and using a forceps to exert traction behind the equator, pulling out the globe and a portion of the optic nerve. Care was required to prevent expulsion of the globe's contents.

Eyes were dissected and their lenses were removed immediately. Eyecups were washed three times in TBS and stained overnight with FITC Isolectin B4 (*Griffonia simplicifolia*) which has a specific affinity for the sugar residues of endothelial cells.³¹⁵ Retinal flatmounts were prepared by performing a Maltese cross incision under magnification, and setting in mounting medium (vectashield) and covered with a coverslip. Fluorescent photographs of the stained vasculature were obtained at multiple magnifications, 10x, 20x and 40x.

4.3.4. Generation of a CSE exposure protocol

Cigarette smoke extract was prepared in the manner previously described for *in vitro* assessments but with some modifications. CSE was prepared in pre-warmed phosphate buffered saline in place of tissue culture medium. The volumes for injection were prepared based on the Bernhard et al volumetric calculations. A total of 4 cigarettes are extracted into 30mls of PBS. This is equivalent to 0.133 cigarette constituents per milliliter of extract. As mentioned previously, a smoker consuming 20 cigarettes per day will distribute this over approximately 6 litres of blood, which is equal to 0.0033 cigarette constituents per milliliter. The extract prepared is therefore 40 times the concentration of smoke constituents found in the circulation of a 20cig/day smoker. The intravascular volume of a mouse is estimated as 0.065mls/g weight. Thus a 25g mouse would have an estimated intravascular volume of 1.625mls. The total dose of CSE to achieve a concentration of 0.0033 (20cigs/day) CSE equivalents per ml is calculated as total intravascular volume multiplied by 0.0033 e.g. $1.625 \times 0.0033 = 0.0053625$ CSE equivalents. Using the 1.33 CSE/ml working stock, the volume required for administration may be calculated from the equation:

$$\text{CSE dose required} \times 1000\mu\text{l} / \text{Stock concentration}$$

In the case of the 25g mouse:

$$0.0053625 \times 1000\mu\text{l} / 1.33 = 4.031954887\mu\text{l}$$

Thus the dose required to achieve the equivalent of 20cigs/day for a 25g mouse is 4.031954887µl. A table was constructed to provide the weight based dosing volumes for mice from 20g-40g for 20, 50 and 100cigs/day. The calculated volume was added to 200µl of PBS to permit sufficient volumes for injection. For all chronic CSE exposure protocols, animals were treated with a weight based intraperitoneal injection of a randomly allocated CSE concentration. These exposures were performed daily for a course of thirty days.

4.3.5. Validation of the CSE exposure protocol by cotinine assessment

The efficacy of the CSE administration was assessed through the use of a biomarker. Nicotine has a relatively short half-life in serum, making it a poor marker of smoke exposure. Cotinine, however, is a stable metabolic product of nicotine and has a significantly longer half-life. Nicotine is metabolized to cotinine in the liver through the cytochrome P450 system¹⁷³ and can be assessed in humans in both serum and urine. A pilot project involving a single C57BL/6 animal was

constructed to determine whether cotinine assessment to confirm CSE exposure is better performed on plasma samples or urinary samples.

Cotinine assessment: urine vs. serum analysis

One 24g C57BL/6 mouse was administered a high concentration CSE equivalent of 500cigs/day concentration (121.5µl in 200µl PBS) via intraperitoneal injection. The animal was sacrificed 12 hours post exposure. 400µls of urine and 200µls of blood were obtained for analysis. The serum fraction of the blood was obtained by centrifuging for 10 minutes at 1000rpm. The supernatant was removed and used for analysis. Three samples of urine and three samples of serum were assayed using a commercially available cotinine ELISA kit. Results were graphed and compared for statistical significance.

Cotinine ELISA protocol (Calbiotech C0096D).

The ELISA used for the detection of cotinine differs from the *in vitro* ELISA methods previously described. The kit is a solid phase competitive ELISA where the cotinine in the samples to be assayed competes with the horseradish-peroxidase (HRP) conjugated cotinine for binding sites. It should be noted that the fewer the sites available for the HRP conjugate to bind, the higher the concentration of cotinine. HRP catalyses the colour change and therefore the intensity of colour is inversely proportional to the concentration of cotinine in the sample. The ELISA was performed as follows: A standard curve was prepared using standards concentrations provided with the kit. 100µl of urine or serum samples were plated in a row of the 96 well plate in triplicate, while cotinine standards were plated in duplicate. 100µl of enzyme conjugate was added to each well and shaken for 30 seconds. The plate was incubated for 60 minutes at room temperature. Wells were washed six times with 300µl of distilled water at each cycle. Plates were dried and 100µl of substrate reagent was added to each well. The plates were incubated at room temperature for 30 minutes and the colourimetric reaction was stopped by the addition of stop solution. Plates were read at 450nm within 15 minutes of terminating the colour reaction.

Serial urinary cotinine assessments

The effects of CSE on the urinary excretion of cotinine were assessed by serial measurements of urinary cotinine over a period of 24 hours after CSE administration. C57BL/6 mice (n = 9) were randomly allocated to one of three levels of cigarette smoke exposure;

- Low concentration: 20cigs/day
- Medium concentration: 50cigs/day
- High concentration: 100cigs/day

Animals were weighed and the CSE dose was calculated. A single intraperitoneal injection was administered in the manner described previously. Urine samples were collected at 0, 6, 12 and 24hr time periods. Urinary concentrations of cotinine were calculated and plotted for statistical analysis.

4.3.6. Evaluation of general behaviour and weights in response to CSE

The effect of serial intraperitoneal CSE injections on general behaviour was assessed by isolating each mouse for 10 minutes daily, and after each injection. The mice were observed for signs of pain, respiratory distress, abnormal mobility and abnormal grooming habits. On their return to the cages the interactions with other mice were observed for aggressive or bullying behaviours. Mice were weighed weekly for the four week exposure period to observe any effect on their general health that appeared.

4.3.7. ERG assessment of the SOD1^{-/-} CSE treated mice

SOD1 ^{-/-} mice were selected for chronic CSE exposure. Animals were randomly assigned to smoke exposure or control groups. Mice for exposure were dark adapted overnight. ERG assessments were recorded with Mr. Paul Kenna. Animals were placed under anaesthesia and pupils dilated as previously described. A rubber band was used to proptose the eye to reduce movement and interference from the eyelids. The ground needle electrode was placed in the tail. Vidisic (Cetrimide carbmer) was dropped on the cornea and the gold wire electrode was placed at the limbus. The reference electrode was placed in the animals' mouth. Isolated rod responses (scotopic) were obtained with 24dB white stimulus. Isolated cone responses (photopic) were obtained in the light-adapted state. Tracings were recorded using RETIport32 system V4.1.10 (Roland consult). Each animal received one month of daily intraperitoneal (IP) injections of a

randomly allocated concentration of CSE. Mice were observed for ten minutes after each injection and weighed weekly, to monitor for any adverse responses to the protocol. This was repeated daily at the same time for a period of 28 days. Tracings were obtained on day 0 and repeated on day 28. Animals were sacrificed using terminal anaesthesia after the second ERG was obtained.

4.3.8. Analysis of oxidative stress marker – Acrolein

SOD1 ^{-/-} mice were selected for chronic CSE exposure. Animals were randomly assigned to smoke exposure or control groups in a manner similar to the ERG protocol. After exposure, animals were sacrificed and their eyes removed. Globes were incised, the lens removed and the eyecups were preserved in 4% PFA for 2 hours, were then washed thoroughly three times in PBS. Eyecups were cryoprotected by immersion in a solution of 10% followed by 20% then 30% sucrose solution. Protected eyes were embedded in OCT and snap frozen with liquid nitrogen. The frozen tissue was mounted on a chuck at -20°C and sections were obtained on microscope slides using a cryostat.

Slides were demarcated using a PAP wax pen. Sections were permeabilised with 0.5% Triton 100x for 30 minutes. Proteins were blocked with a solution of 5% goat serum for 30 minutes. The primary antibody, a rabbit polyclonal anti-Acrolein antibody (AbCam ab37110) was prepared as a 1:500 dilution in 1% goat serum in 0.5% Triton in PBS. Sections were incubated with the primary antibody in a humidified chamber overnight. Sections were washed six times in PBS and incubated with a green CY2 conjugated mouse anti-rabbit secondary antibody for three hours at room temperature. Slides were washed again with PBS and counterstained with dapi for 15 seconds. Sections were mounted in vectashield and covered with a coverslip for fluorescent photography.

4.3.9. Laser-induced CNV protocol

Mice were anaesthetized using a combination of ketamine and xylazine as described. Once under anesthesia, pupils were dilated with 2.5% phenylephrine and 1% tropicamide and a contact lens was placed over the eye. A Zeiss operating microscope was used to directly visualize the murine retina. Four separate burns were applied to the retina approximately two disc diameters from the optic disc. Three or four regions of the retina within 3mm of the optic disc are laser-photocoagulated (532nm IRIDEX laser) and both of the animals' eyes were treated.

There is no macula in the mouse eye to target, and so burns were applied to areas of the retina that avoided the retinal arcades. Burns were applied using the following Argon laser (IRIDEX) settings: 50um diameter, 100ms duration and 150mW intensity. The blanching of the retina and the formation of a bubble at the laser site confirmed Bruch's membrane rupture. Previous studies have reported choroidal neovascular membranes in 90% of the affected regions in as early as seven days after laser treatment provided that this bubble at time of laser was observed. Lesions that do not display a bubble upon application by the laser do not form CNV. Two weeks after laser treatment animals were euthanised, and both eyes were enucleated. When combined with the chronic CSE exposure protocol, animals were exposed to CSE for two weeks prior to laser treatment and for the following two weeks prior to their sacrifice.

The enucleated globes were dissected and the corneas and lenses were removed. The remaining eyecups were fixed in 4% paraformaldehyde (PFA) overnight. The eyecups were washed twice in PBS and the neural retina gently peeled from the RPE. This step was crucial for clear visualization of the sub retinal membranes. Four radial cuts were made to the eyecups to form a Maltese cross and the samples were washed again in PBS and incubated. Infiltrating choroidal endothelial cells were stained with Isolectin B4 that fluorescently labels infiltrating vessels projecting from the choroidal surface. Samples were mounted on glass slides using mounting medium for viewing and photography.

4.3.10 CNV volume assessment

The volumes of CNV membranes were assessed by two methods. Two-dimensional volumes were calculated by assessment of images obtained using standard fluorescent photography. The pixel densities on the fluorescent images were calculated and compared using Photoshop image software. Confocal laser scanning microscopy performed three-dimensional digital quantification. Flat mounted retinal tissue was scanned in sections and a three-dimensional z-stack image was generated. The integrated IMARIS software produces a volumetric measure of the fluorescence in a given sample. In the case of the CNV membranes, the fluorescence is provided by the Isolectin B4 staining of the infiltrating endothelial cells. The volumes in the CSE treated and control groups were compared using the Student's T-test.

4.4. Results

4.4.1. Intravitreal injection of CSE

Retinal vascular sections were assessed using fluorescent photography. Representative images of the retinal vascular staining in response to acute intravitreal smoke exposure are shown (Fig. 4.6.2A-D). Images were assessed at 20x and 40x magnifications. Vessels were assessed on calibre, width and length and total pixel volume on graphical analysis software (Adobe Photoshop). Particular attention was paid to the sites of vascular bifurcation for signs of budding and potential new vessel formation. Medium and small vessels did not show any structural changes in response to acute CSE exposure. There was no effect of intravitreal CSE exposure on neuroretinal vasculature.

4.4.2. Cotinine analysis in CSE exposure

Serum and urinary cotinine results

The standard curve from the cotinine ELISA shows an exponential rather than a linear correlation. A logarithmic transformation must be performed, which results in the linear relationship to calculate concentrations from this form of standard curve. The equation $y = -1.3553x + 1.4278$ was used to calculate the log of the concentration from the log of the absorbance (Fig. 4.6.3). Twelve hours after IP injection of CSE, concentrations were compared and are results are displayed (Fig. 4.6.4). The mean concentration of cotinine in serum was 0.8736ng/mL. The concentration in urine was 2.204ng/mL represents an increase of 252.3%. The difference between the two was significant ($p = 0.0029$).

Serial urinary cotinine assays

Experiments were designed to determine the effects of CSE administration on urinary cotinine (Fig. 4.6.4). Urinary concentrations of cotinine were assessed at the 0, 6, 12 and 24-hour time points post exposure ($n = 9$). The average of the three CSE concentrations at each time interval was taken (Fig. 4.6.5). When compared with the pre exposure samples, there was a significant increase in urinary cotinine detected at both 6 and 12 hours. There was no significant difference in urinary cotinine after 24 hours. The highest concentrations were detected at the 6-hour time period ($p < 0.0022$). The effects of the three concentrations of CSE are illustrated ($p < 0.021$) (Fig.

4.6.6). The 20-cig/day concentrations resulted in a mean increase in 33.8ng/mL. The 50-cig/day concentrations resulted in a mean increase in 133.6ng/mL. The highest concentration of 100 cig/day resulted in an increase in 370.2ng/mL. These differences were statistically significant ($p<0.02$).

4.4.3. SOD1 -/- general morphological and physiological features

SOD 1 -/- mice bred in the clean unit of the Trinity College Dublin bioresources department for three generations and were maintained for up to 30 months. Prior to one year of age there were no phenotypic differences detected among these mice (Fig. 4.6.10A) Mice displayed pathologies that accumulated with time after one year (Fig. 4.6.10B-D). Large areas of ulceration were detected around the oral mucosa, eyelids and ears. The resulting granulation tissue was aggressive and prone to bleeding and re-ulceration. Lesions grew to occlude the eyes completely rendering them unfit for ERG or laser treatment. Mice maintained for over two years displayed ear granulation so severe that the entire pinna was shed. Mice were treated with topical fuscithalmic to remove any infectious stimuli for these lesions.

A single animal was sacrificed for pathological and histological assessment. Samples were submitted fixed whole in 10% formalin submitted to the University College Dublin (UCD Belfield) veterinary pathology department. The pathology report is included in the additional materials section (appendix one). Upon external examination multifocal large areas of ulceration with granulation tissue were evident on the head, around the periorbital areas and on the dorsal aspect of the neck. Histopathologically, the epidermis exhibited multifocal areas of acanthosis and spongiosis with variable orthokeratotic hyperkeratosis. There were multifocal, to coalescing, areas of ulceration covered by cellular debris, dead and dying neutrophils and bacterial cocci occasionally. Subjacent to ulcerated areas the superficial dermis was thickened by densely cellular neutrophil rich infiltrate within areas of fibroplasia and angiogenesis (granulation tissue). There were multifocal dilated hair follicles with no hair shafts, and which often contained keratin, throughout all sections. There were also multifocal microabscesses within the dermis in sebaceous glands and hair follicles. There was diffuse thickening of the superficial dermis subjacent to the intact epidermis by fibrous tissue (dermal fibroplasia). Superficial dermis also had mast cells infiltrates, occasional multinucleated giant cells and diffuse melanin incontinence. The lesions

outlined above involved all areas and elements of the skin, and are chronic persistent changes of an inflammatory nature. Given the overall picture it is unlikely that the changes are primarily due to the occasional colonies of cocci seen. Neuromuscular changes were also noted following an examination of animal mobility and behaviour. Mobility became impaired and spastic while the tails adopted a rigid appearance. Animals were still able to groom and feed and there was no change in weight assessments. The SOD1 gene has been associated with motor neurone disorders such as amyelotic lateral sclerosis (ALS). The development of muscular abnormalities in this breed may relate to this disease pathology in humans.

Despite the morphological changes seen, these animals were otherwise stable, and chronic CSE experimentation was therefore possible. Chronic CSE treatment was associated with minor pain and some urination at the time of injection. This is considered a normal mouse pain response. During post injection observation there were no stereotypical movements or signs of distress observed as a result of injection of the CSE in the treated or the control groups. Mice returned to baseline activity rapidly. On return to occupied cages there were no aggressive patterns of behaviour observed. No mouse was lost as a result of the IP injection procedure.

4.4.4. SOD1 -/- ERG results

The ERG protocol required proptosis of the globe to permit the contact of the gold wire electrode. Some mice developed significant epithelial pathology and periorbital scarring. This impaired the globe proptosis required for ERG assessment. In these cases, tracings from a single eye were obtained where possible. Fourteen animals were allocated to this experiment. Two of the original cohort of SOD1-/- animals did not survive the anaesthesia. One animal developed severe bilateral scarring which prevented ERGs from being obtained post treatment. ERG tracings were obtained prior to, and following, the treatment in all other animals. (CSE exposure n= 6, sham injections n= 4) In a paired T-test analysis of CSE treatments prior to and following ERGs there was no significant difference in the sham injection group (0.1651). In contrast, the CSE treated group displayed a significant decrease (495mV) in B wave amplitude after CSE treatment ($p = 0.0141$) (Fig. 4.6.9). In a similar paired T-test analysis there was no significant difference found between the cone-adapted B wave ERG amplitudes in the sham group ($p = 0.1464$). An assessment of the

CSE treated mice, before and after treatment, indicated that there was no statistically significant difference in cone optimized ERGs. ($p = 0.0834$) (Fig. 4.6.10).

4.4.5. Acrolein staining in the SOD1^{-/-} mouse

Representative images of the retinal sections are shown (Fig. 4.6.13). Images A-C depict the retinal section of an untreated SOD1^{-/-} mouse, incubated without acrolein antibody, but with secondary CY2 antibody. This is provided to illustrate non-specific fluorescence in the retina as a negative control. Animals treated with 30 days of CSE showed a denser pattern of acrolein staining (Fig. 4.6.13 D-F) when compared with sham injections (Fig. 4.6.13 G-I). The staining protocol was performed on eight eyes from eight different mice, four allocated to the CSE treatment, four allocated to the control group.

4.4.6. SOD1^{-/-} laser-induced CNVs

Intraoperative complications observed included a failure to achieve rupture of Bruch's membrane, intravitreal haemorrhage, subretinal haemorrhage and anaesthetic complications. Inducing consistent laser burns in the retina required an initial period of optimization prior to CSE exposure. Trial laser burns were performed on C57BL6 mice. A number of staining procedures were trialed to achieve optimal staining of CNV. Perfusion with FITC and Isolectin was also attempted. Post mortem staining of the endothelial cells with isolectin B4 was also trialed. Representative images from these optimization experiments are shown (Fig 4.6.14). Post mortem incubation with Isolectin B4 and CY3 secondary produced the most optimal images on the basis of these results.

CNVs were analysed from the sham injection ($n=15$) and the CSE injection treatments ($n=19$). Representative images from this treatment protocol are shown in (Fig.4.6.15). The two-dimensional and three-dimensional volumetric analysis of the laser-induced CNV membranes illustrated a significant difference between the volume in control animals and those treated with AMD. Two-dimensional assessment showed a mean increase in CNV of 85.88% ($p=0.033$) (Fig. 4.6.17). Confocal microscopy was performed in order to confirm this. CNV membranes were scanned throughout their entire thickness to provide a z-stack analysis. The z-axis provides the third dimension which yields a more specific volumetric analysis. Results of the z-stack volumes were compared (IMARIS software). Three-dimensional volumetric analysis showed an increase in

volume of 53.5% between the CSE treatment and the sham injections protocol (Fig. 4.6.17). This was statistically significant ($p = 0.0171$).

4.5. Discussion

A significant limitation of the use of mice in the investigation of AMD is the lack of an evolved macula. Maculae are present in primate species alone. From an ethical point of view, clinical research in primates should not be performed where alternatives may provide equivalent information. In addition primate research is not currently performed in Ireland. Mice are a suitable alternative with an otherwise highly developed neural retina, RPE and choriocapillaris, however all findings must consider the limitations of the mouse model. Using a mouse allows for increased animal numbers which permits optimization and reproducibility that would not be possible in a primate model.

The administration of a single high concentration intravitreal injection permitted the assessment of an acute direct high dose CSE injury to the retinal vasculature. The administration of CSE to the intravitreal space directly exposes the retinal vasculature to high concentrations of toxins. On morphological assessment however, there was no effect of acute intravitreal smoke exposure on the retinal vasculature. Acute exposure had no effect on the caliber or branching pattern of these established quiescent vessels. There was no evidence of endothelial budding or precursors to frank neovascularisation. It may be concluded that acute high concentration intravitreal smoke exposure does not induce angiogenesis in the retinal vasculature *de novo*. It was therefore necessary to use the laser-induced model to create new vessels to determine the effects of cigarette smoke extract.

Intravitreal injection may only be performed once on a single animal. This method is therefore not suitable for chronic interventions. An alternative approach to chronic cigarette smoke exposure was required to determine the effects of cigarette smoke on the retina. Legal and ethical constraints required the design of a novel method of cigarette smoke administration to achieve chronic exposure. *In vitro*, cigarette smoke extract was generated which contained the equivalent soluble fraction of cigarette smoke constituents. Dilution of the extract allowed the preparation of

CSE which approximated the circulating smoke constituents in smokers. We proposed that by scaling the extract to murine proportions, administration of this extract would result in systemic absorption. Intravascular injection of liquids in mice requires tail vein injections. Repeated injections injure the vein, and therefore this technique did not suit the chronic exposure protocol proposed. The intraperitoneal (IP) route of injection is simple and repeatable, and was therefore selected as the method of administration. In addition, the low level of trauma associated with IP injection makes it the preferable exposure method for chronic experimentation. The absorption of drugs administered through the IP route is variable, and therefore not appropriate for acute smoke exposure experimentation. With chronic exposure, however, a steady state is reached over time, reducing potential variability overall.

This proposed IP exposure method is a novel approach and it was necessary to confirm absorption of the CSE from the abdominal cavity into the systemic circulation. Nicotine is a specific constituent of cigarette smoke which has no other dietary source. The presence of intravascular nicotine therefore confirms absorption. Cotinine was detected both in serum and urine after CSE exposure; however it was detected in significantly higher concentrations in serum than in urine. Serum sampling also required a large volume of serum that required sacrificing the animal, making serial measurements impossible. Urine samples were easy to obtain without injuring the animal. Therefore urinary assessment was selected as the means of monitoring CSE exposure in animals. Serial measurements of urinary cotinine excretion indicated a peak in excretion after 6 hours, with no residual detection of cotinine at 24 hours.

Animals were also exposed to concentrations equivalent to 20, 50 and 100 cigarettes a day and cotinine concentrations at this peak 6-hour time point were compared. The concentration of cotinine in the urine samples displayed a dose-response relationship indicating a difference between the allocated CSE protocols. The detection of urinary cotinine confirmed that intraperitoneal administration of CSE is absorbed into the intravascular space, metabolized and eliminated through the kidneys. The relationship between increasing CSE concentrations and urinary cotinine levels confirms that intraperitoneal administration of CSE is a valid, safe method

of introducing smoke toxins to an animal model. These findings therefore support the use of this alternative CSE administration system.

The SOD1-/- knockout mouse was selected for the chronic cigarette smoke exposure for a number of reasons. The use of the intraperitoneal (IP) method of cigarette smoke administration was limited to approximately 30 days for animal welfare reasons. The oxidative stress hypothesis of AMD suggests that cumulative oxidative stress at the level of the RPE is the cause of AMD. In this experimental model we selected an animal with a genetically modified susceptibility to oxidative stress, aged the animals and then exposed them to an extract that is high in oxidants. Given the constraints imposed by the reduced exposure time, this protocol optimizes the possibility of finding a contribution of CSE to the volume of laser-induced CNV.

Despite the advantages, the susceptibilities of the SOD1-/- breed also caused significant difficulties. Animals were similar to the wild type mice before the age of 12 months, but as the animals aged, they became increasingly affected by granulomatous skin lesions and mobility problems. This had not been previously reported in the literature. The eyes became unusable in a number of cases due to excessive granulation. Although the administration of CSE by IP injection did not induce significant pain, animals that had significant dorsal neck involvement were clearly distressed on handling. In response to the degree of pathology and disability noted in this breed, it was concluded that this was an issue of animal welfare. Based on our findings, the ethics and bioresources department of Trinity College Dublin resolved to cease breeding the SOD1-/- mouse on animal cruelty and ethical grounds upon completion of these experiments (Cruelty to animals act, 1876).

Chronic CSE administration elicited only a brief pain response. Persisting behavioural changes or mobility impairments, above what had been observed at baseline, were not observed. Mice returned to normal activity immediately upon returning to the cage. Serial weight measurements over the course of the exposure protocol did not show any fluctuations in response to the treatment indicating little effect of CSE on the general health of the animal.

ERG assessment of the SOD1^{-/-} animals indicated a significant decrease in rod ERG (dark adapted B wave) amplitude. The dark-adapted b waves are optimized to detect rod responses. This indicates that CSE exposure has a significant effect on the rod photoreceptors and bipolar cells in the SOD1^{-/-} mouse. Similar analysis of the cone b wave activity in response to CSE was non-significant. Specific impairment of rod photoreceptor function is not a characteristic feature of AMD. This reduction in function observed is more likely due to the reduced defense against oxidant damage inherent in this breed rather than being a specific feature of cigarette smoke based pathology. In normal eyes, the magnitude of cone amplitude responses is less than rod responses because the higher numbers of rods generate a stronger electrical signal when compared to cones. It is possible that oxidative stress affects both the rod and cone photoreceptors in the SOD1^{-/-} mouse equally however since there are significantly greater numbers of rods in the mouse retina than cones, any effects would manifest initially in the rod responses as seen. A smaller effect of CSE would require greater animal numbers to attain significance. This was not possible in the context of these experiments.

Qualitative analysis of the effects of CSE on oxidative stress was detected by staining retinal sections for Acrolein. Acrolein staining was detected in both control and CSE sections indicative of increased susceptibility to oxidation in the SOD1^{-/-} line. An increased density of staining was detected in the CSE treated animals when compared with controls. This indicated that intraperitoneal treatment with CSE exerts an additional oxidative stress on the retina, resulting in increased accumulation of acrolein. This observation provides support for the role of oxidative stress in the pathogenesis of cigarette smoke in AMD.

Previous studies have described the effects of individual cigarette smoke constituents on the growth of CNV²⁸¹ however this is the first report on the effects of the soluble fraction of cigarette smoke on the volume of laser-induced model. A two dimensional CNV analysis was based on the staining intensities of the CY3 CNV membranes. Photographs obtained using fluorescent microscopy were compared and the pixel densities of fluorescence for each CNV were calculated (using Photoshop software). This provided an initial estimate of the effect of CSE treatment. This analysis showed a significant increase in CNV volume. These findings were confirmed by a more in

depth three-dimensional confocal microscopy analysis of the CNV samples. Both methods confirmed a significant increase in CNV volume due to cigarette smoke extract exposure. These findings provide direct *in vivo* evidence supporting higher rates of neovascular AMD observed in cigarette smokers.

The design of our experiments does not directly attribute a cause for the effects seen. One of the weaknesses of using the entire fraction of cigarette smoke is that while the effects are detected, they cannot be attributed to a solitary factor. The acrolein-stained sections indicate that oxidative stress is increased by CSE. It also is possible that effects are mediated through the interaction of multiple chemicals and isolated constituent analysis would not produce the same effects. We consider the isolation of the individual factors within cigarette smoke contributing to the disease to be of limited value in this condition. It is preferable to promote smoking cessation programmes rather than developing a “safer cigarette”.

Patients who continue to smoke during treatment for active CNV may be compromising their responses to anti-VEGF therapies by prolonging CNV growth. At any stage in AMD, active smokers should be counseled and encouraged to quit; however our findings indicate that this is particularly relevant in neovascular AMD. Further investigations are required to examine the effects of smoking on the long-term visual outcomes. On the basis of these *in vivo* findings we hypothesise that active smokers are likely to have a more aggressive CNV manifestation. These patients may require more frequent administration with intravitreal anti-VEGF to stabilize these potentially more aggressive CNV membranes. They may also pursue a more aggressive course, ultimately resulting in worse visual acuity in the long term. A prospective case-controlled study is required to determine these effects. It may be the case that active cigarette smokers might require a different dosing regimen and follow up to non-smokers.

4.6. Figures and tables

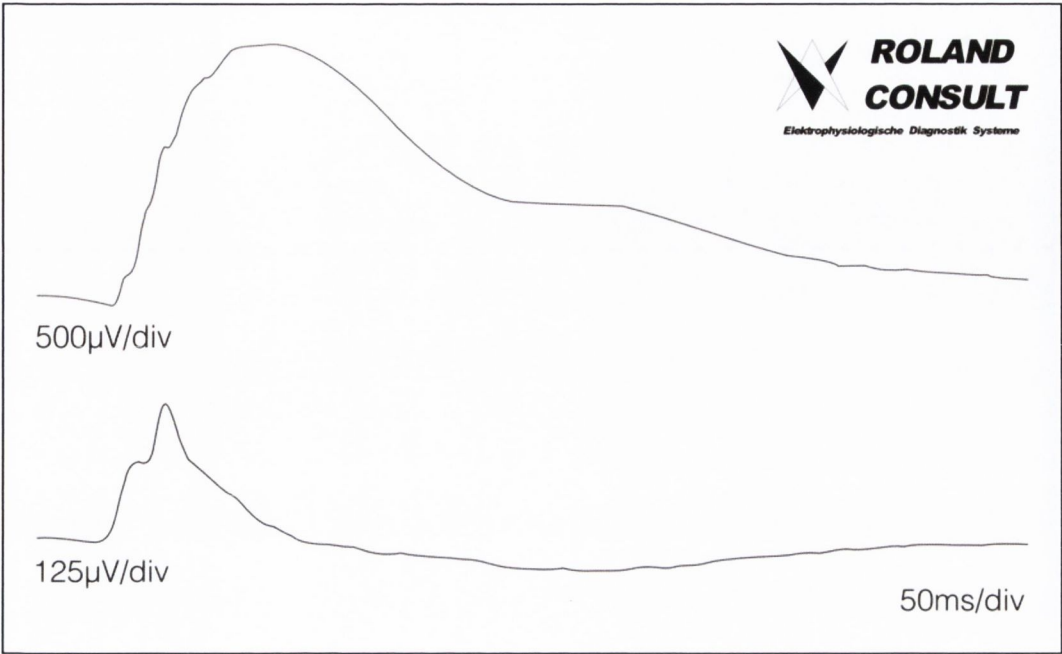


Figure 4.6.1: Normal rod and cone responses recorded in SOD1-/- mouse

Weight in grams	Intravasc. Vol. (0.065ml/g)	20/day constituents per ml	20/day vol.	50/day vol.	100/day vol.
20	1.2	0.00396	2.977443609	7.443609023	14.88721805
21	1.365	0.0045045	3.386842105	8.467105263	16.93421053
22	1.43	0.004719	3.548120301	8.870300752	17.7406015
23	1.495	0.0049335	3.709398496	9.273496241	18.54699248
24	1.56	0.005148	3.870676692	9.676691729	19.35338346
25	1.625	0.0053625	4.031954887	10.07988722	20.15977444
26	1.69	0.005577	4.193233083	10.48308271	20.96616541
27	1.755	0.0057915	4.354511278	10.8862782	21.77255639
28	1.82	0.006006	4.515789474	11.28947368	22.57894737
29	1.885	0.0062205	4.677067669	11.69266917	23.38533835
30	1.95	0.006435	4.838345865	12.09586466	24.19172932
31	2.015	0.0066495	4.99962406	12.49906015	24.9981203
32	2.08	0.006864	5.160902256	12.90225564	25.80451128
33	2.145	0.0070785	5.322180451	13.30545113	26.61090226

Weight in grams	Intravasc. Vol. (0.065ml/g)	20/day constituents per ml	20/day vol.	50/day vol.	100/day vol.
34	2.21	0.007293	5.483458647	13.70864662	27.41729323
35	2.275	0.0075075	5.644736842	14.11184211	28.22368421
36	2.34	0.007722	5.806015038	14.51503759	29.03007519
37	2.405	0.0079365	5.967293233	14.91823308	29.83646617
38	2.47	0.008151	6.128571429	15.32142857	30.64285714
39	2.535	0.0083655	6.289849624	15.72462406	31.44924812
40	2.6	0.00858	6.45112782	16.12781955	32.2556391

Table 4.6.1: Weight based calculations for CSE administration

Animal	Weight	CSE Dose	Cig Equivalent
1	29.1g	4.67µl	20/day
2	35.8g	29µl	100/day
3	34.1g	13.7µl	50/day
4	34.7g	28µl	100/day
5	35.4g	5.64µl	20/day
6	24g	3.87µl	20/day
7	32.1g	12.9µl	50/day
8	35.1g	28.22µl	100/day
9	35.7g	14.51µl	50/day

Table 4.6.2: Weight-based CSE doses for cotinine assessment

Number	Mark	Cage no	DOB	Treatment
1	2REP	1	27 May 2009	CSE
2	REP	1	27 May 2009	Control
3	LEP	1	27 May 2009	Control
4	BEP	1	27 May 2009	CSE
5	REP	2	12 May 2009	Control
6	LEP	2	12 May 2009	CSE
7	LEP	3	28 Apr 2009	Control
8	REP	4	20 Jul 2008	CSE
9	BEP	5	20 Oct 2008	Control

10	LEP	6	28 Apr 2009	CSE
11	LEP	7	9 Sep 2008	CSE
12	REP	8	9 Aug 2008	CSE
13	LEP	8 (RIP)	9 Aug 2008	Control
14	BEP	9 (scarred eyes)	20 Oct 2008	CSE
15	BEP	10	17 Nov 2008	Control
16	2REP	11	22 May 2009	CSE
17	LEP	11	22 May 2009	Control

Table 4.6.3: SOD1-/- Dates of birth and random allocation to treatment groups

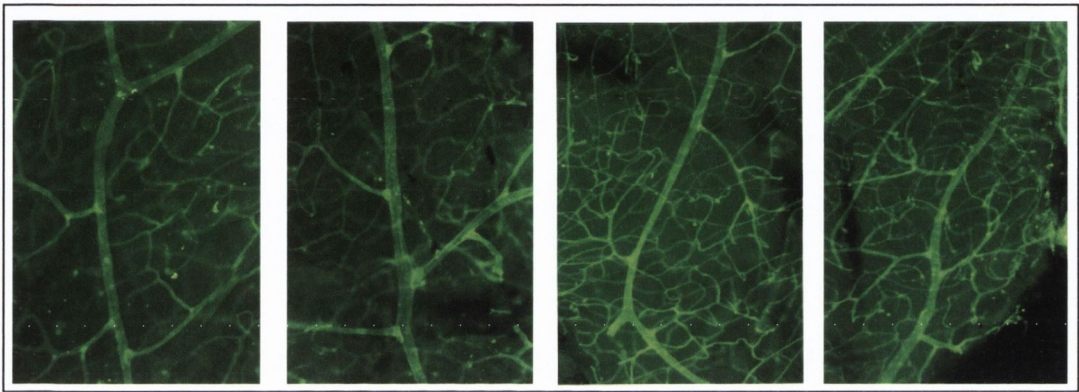


Figure 4.6.2: Retinal vessels stained with FITC Isolectin B4 post CSE exposure 0%, 1.25%, 6.25% and 12.5% concentrations (left to right)

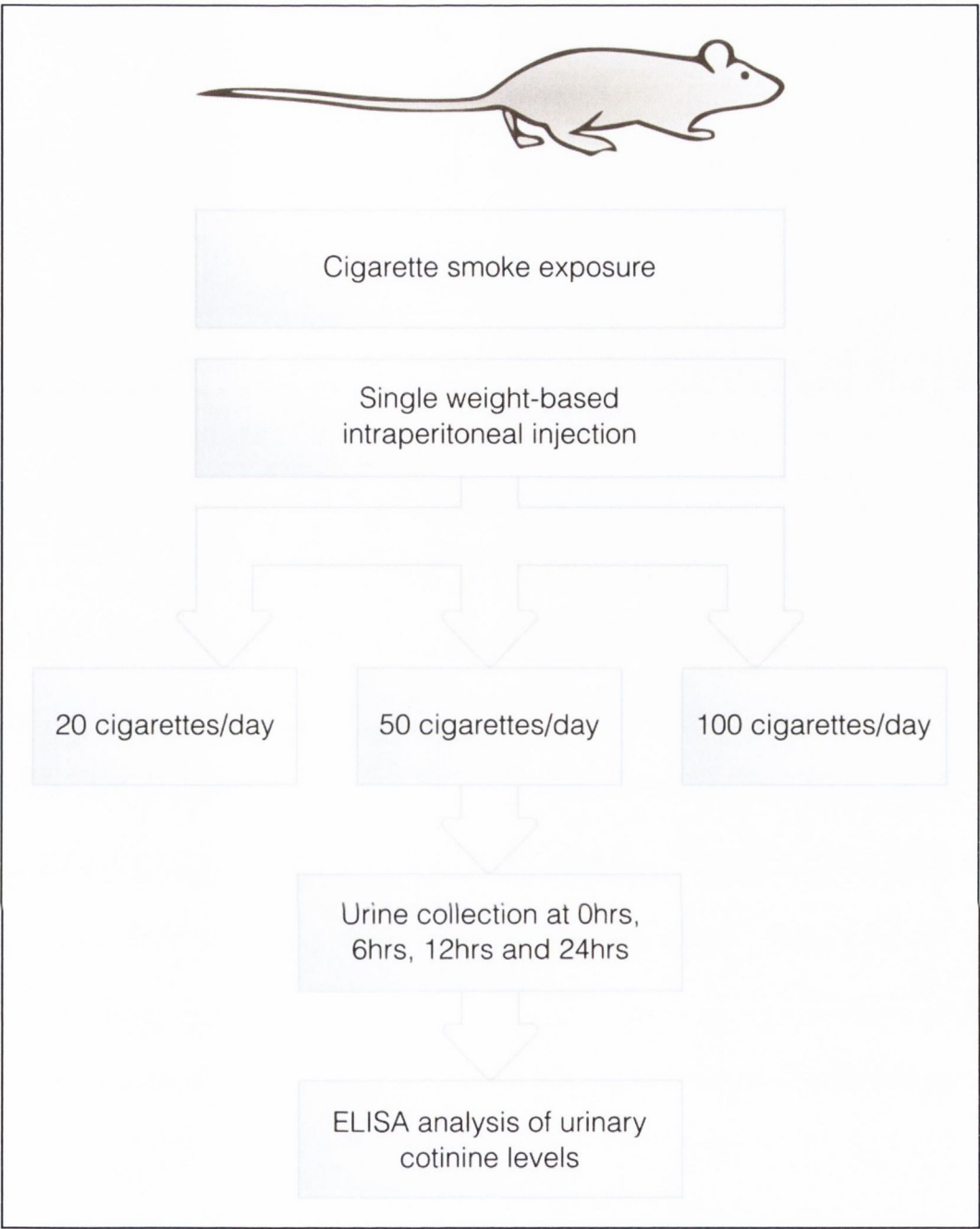


Figure 4.6.3: Study design for validation CSE administration

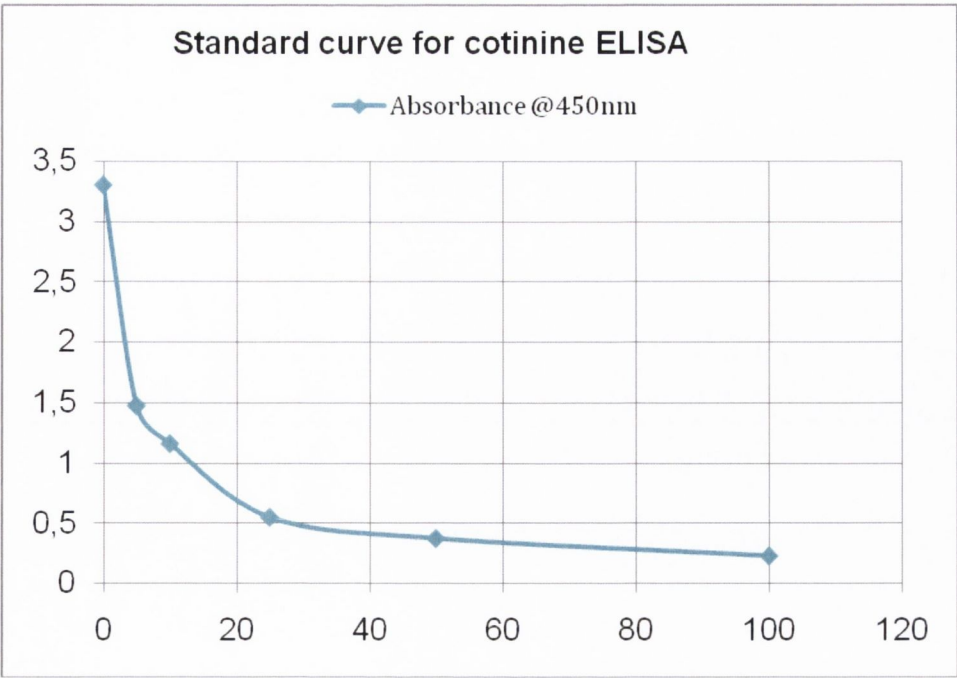


Figure 4.6.4: Standard curve for cotinine ELISA

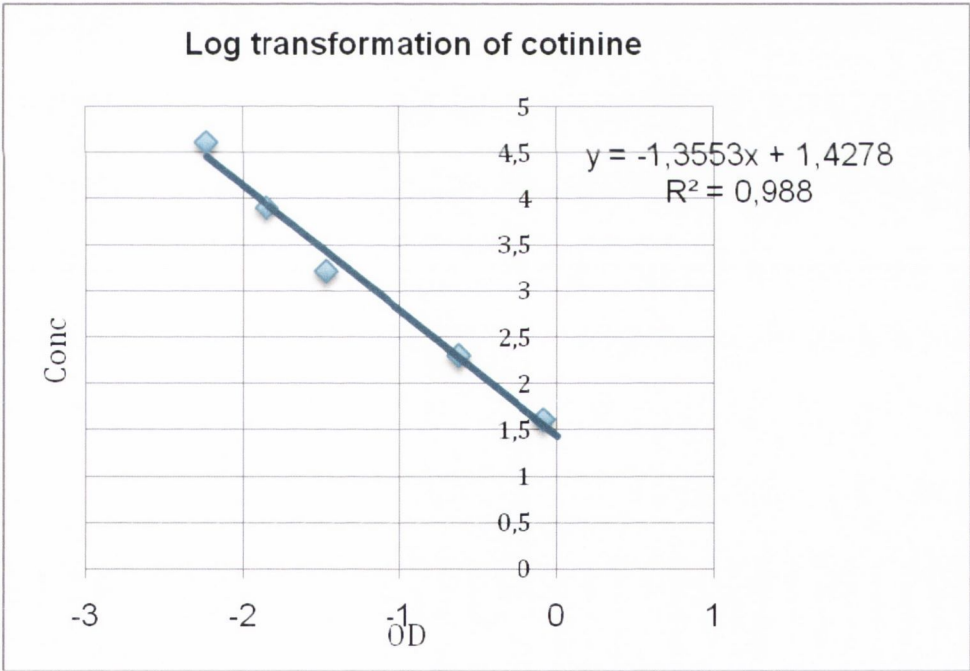


Figure 4.6.5: Logarithmic transformation of cotinine standard curve

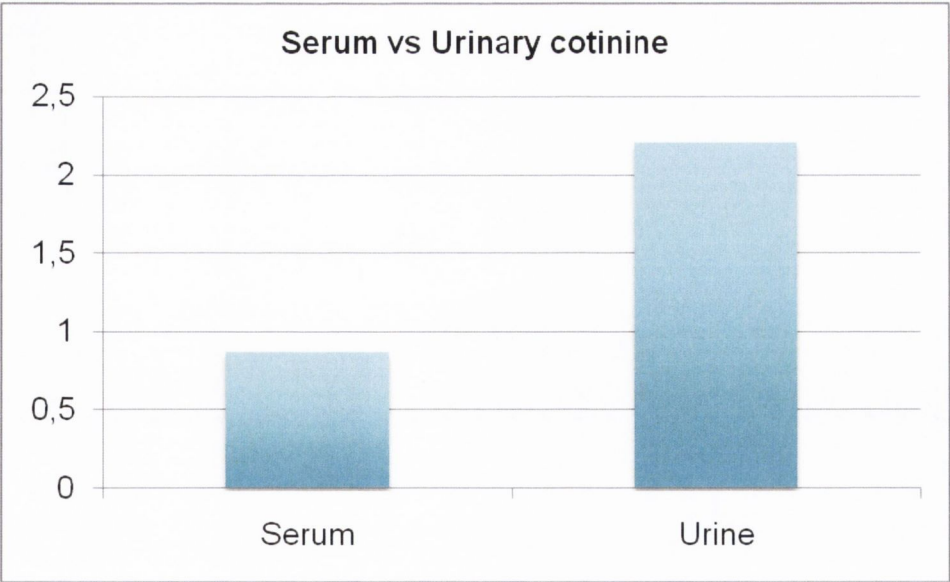


Figure 4.6.6: Serum cotinine concentration and urinary cotinine concentration

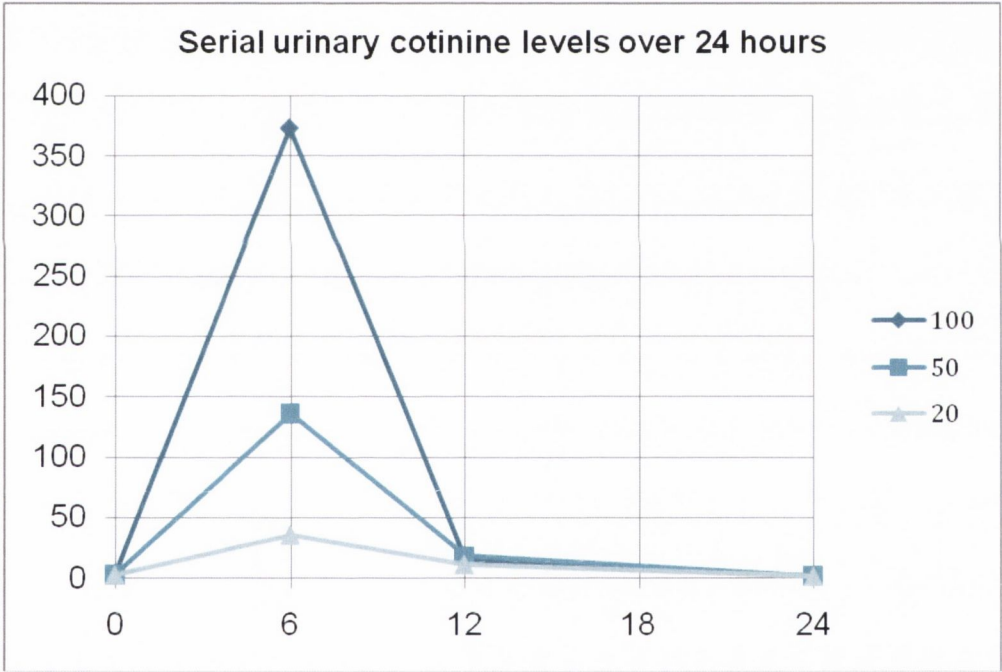


Figure 4.6.7: 24hr Urinary cotinine concentration post CSE exposure

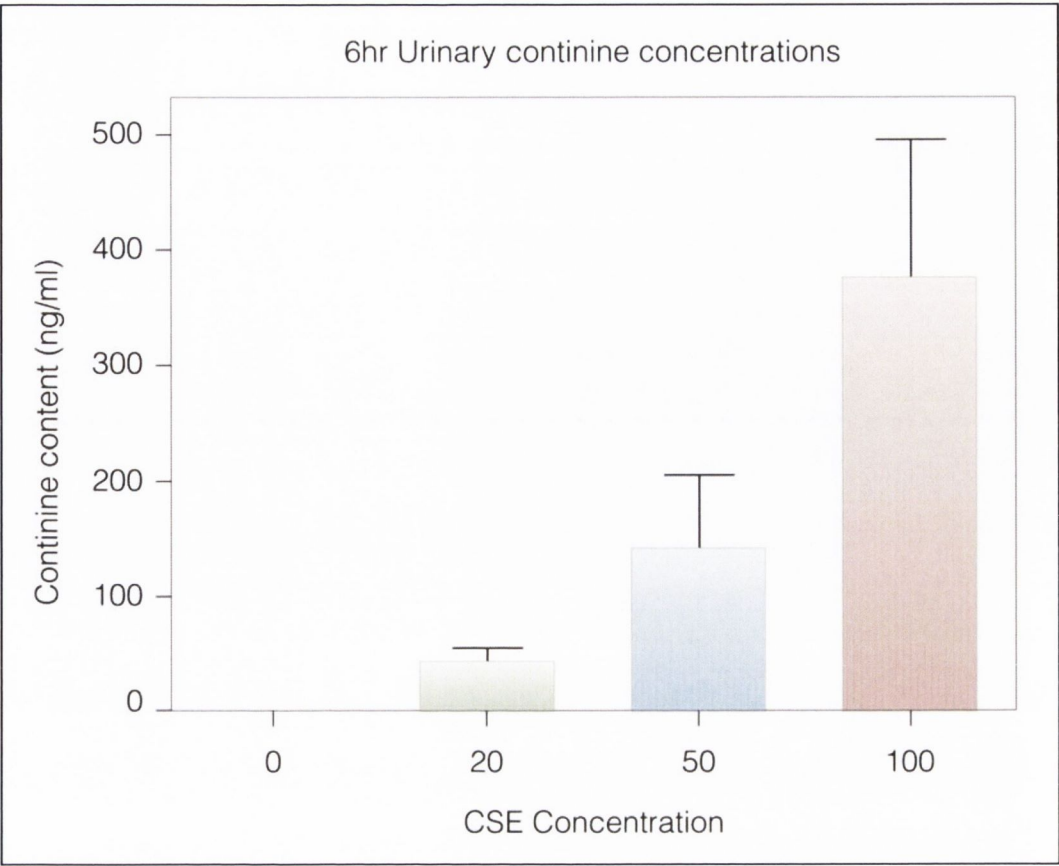


Figure 4.6.8: Comparative urinary CSE concentrations at 6hrs

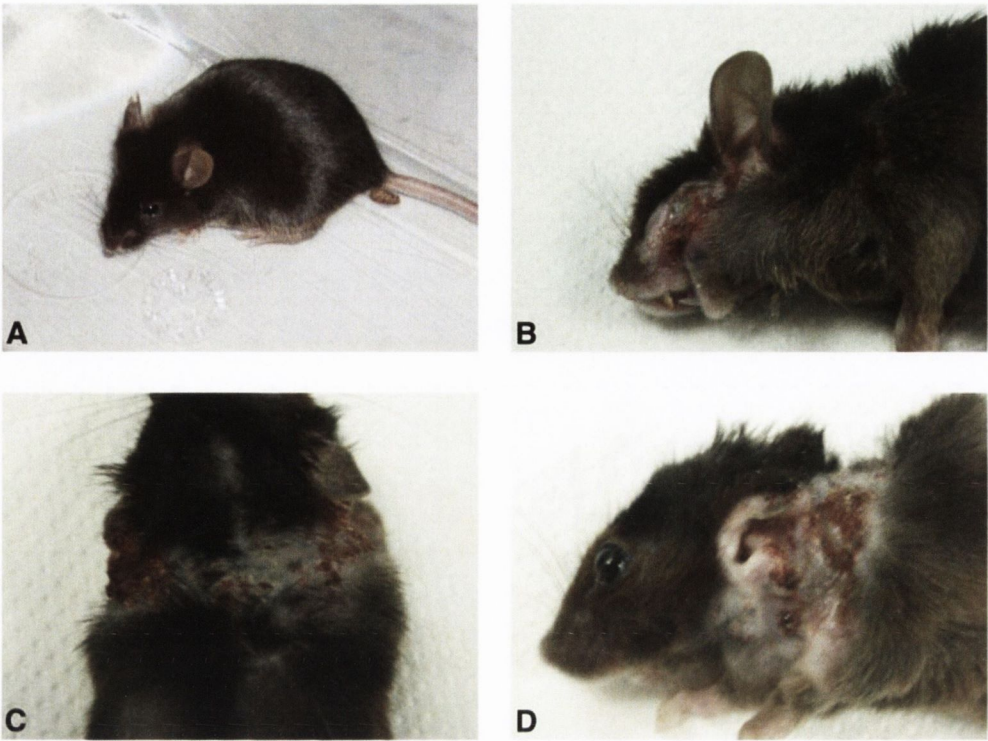


Figure 4.6.9: Mucosal pathology and ulceration in the SOD1^{-/-} mouse.

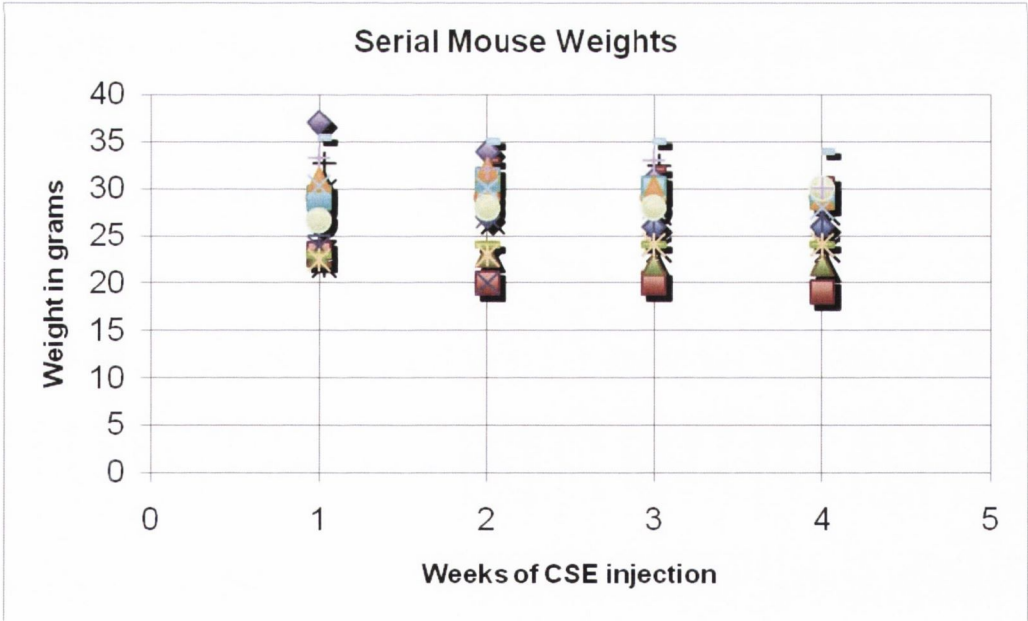


Figure 4.6.10: Serial weight measurements in the SOD1^{-/-} mouse

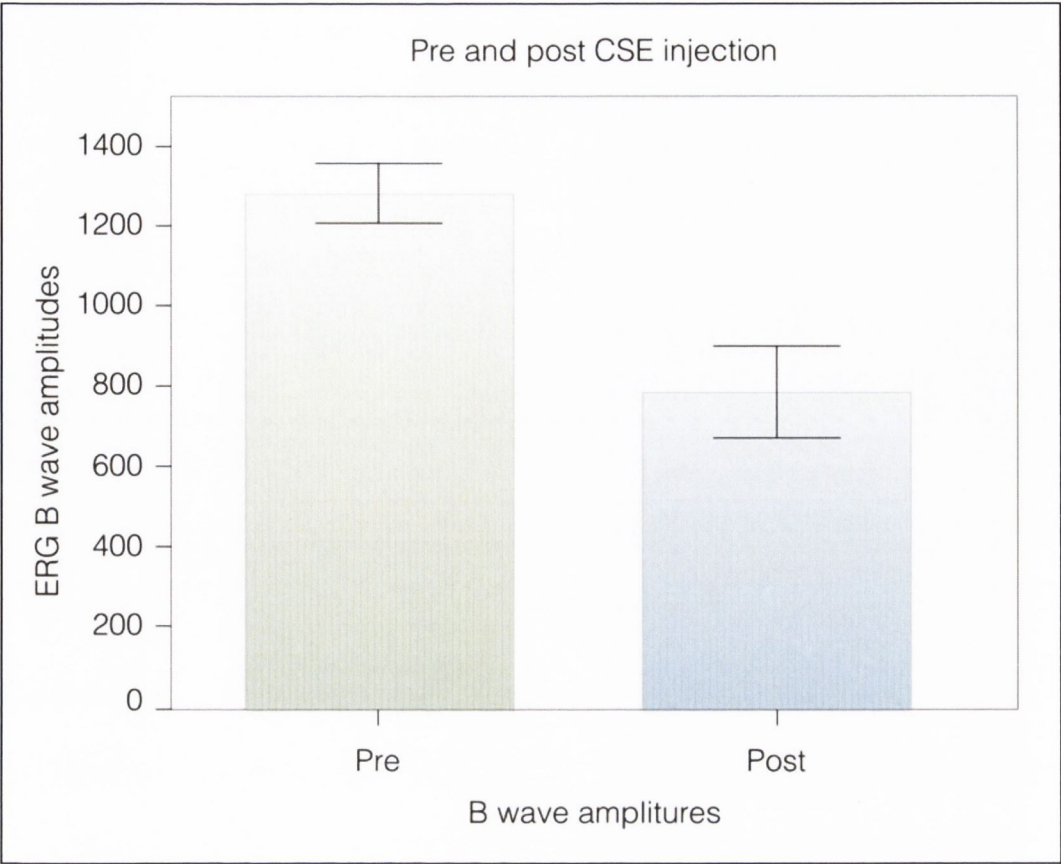


Figure 4.6.11: Dark-adapted rod ERG response pre and post CSE treatment

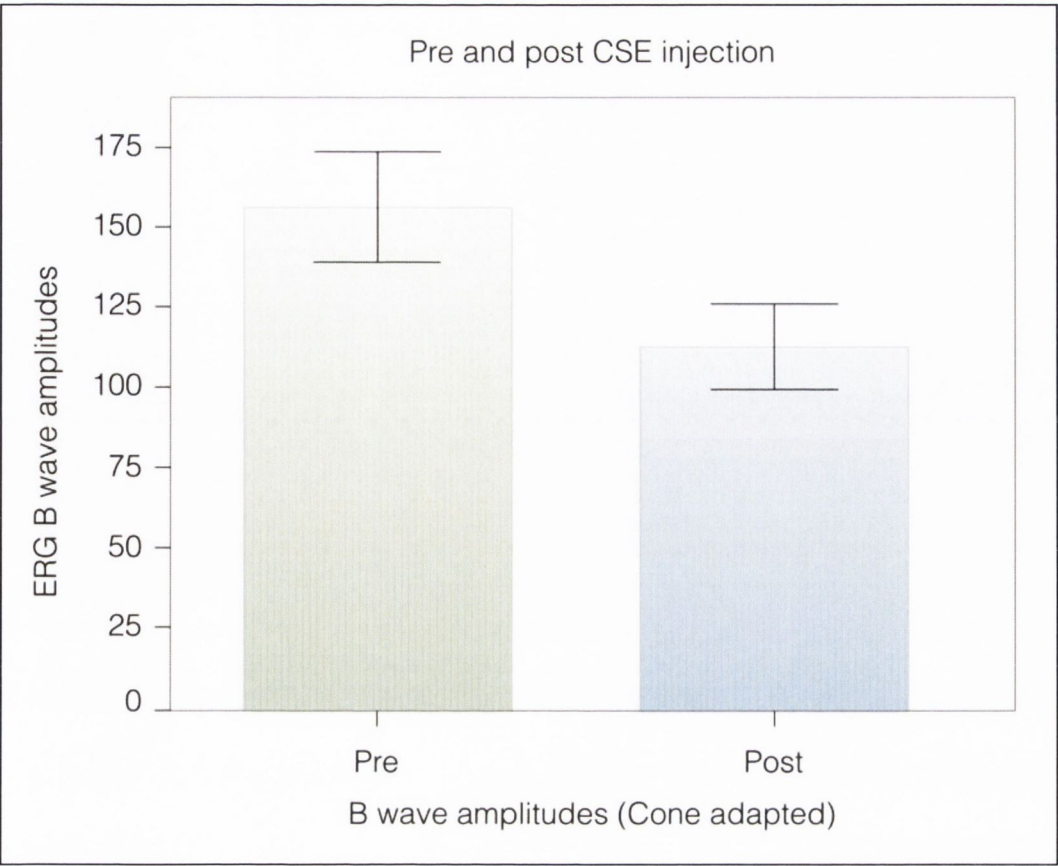


Figure 4.6.12: Light adapted cone ERG responses pre and post CSE treatment.

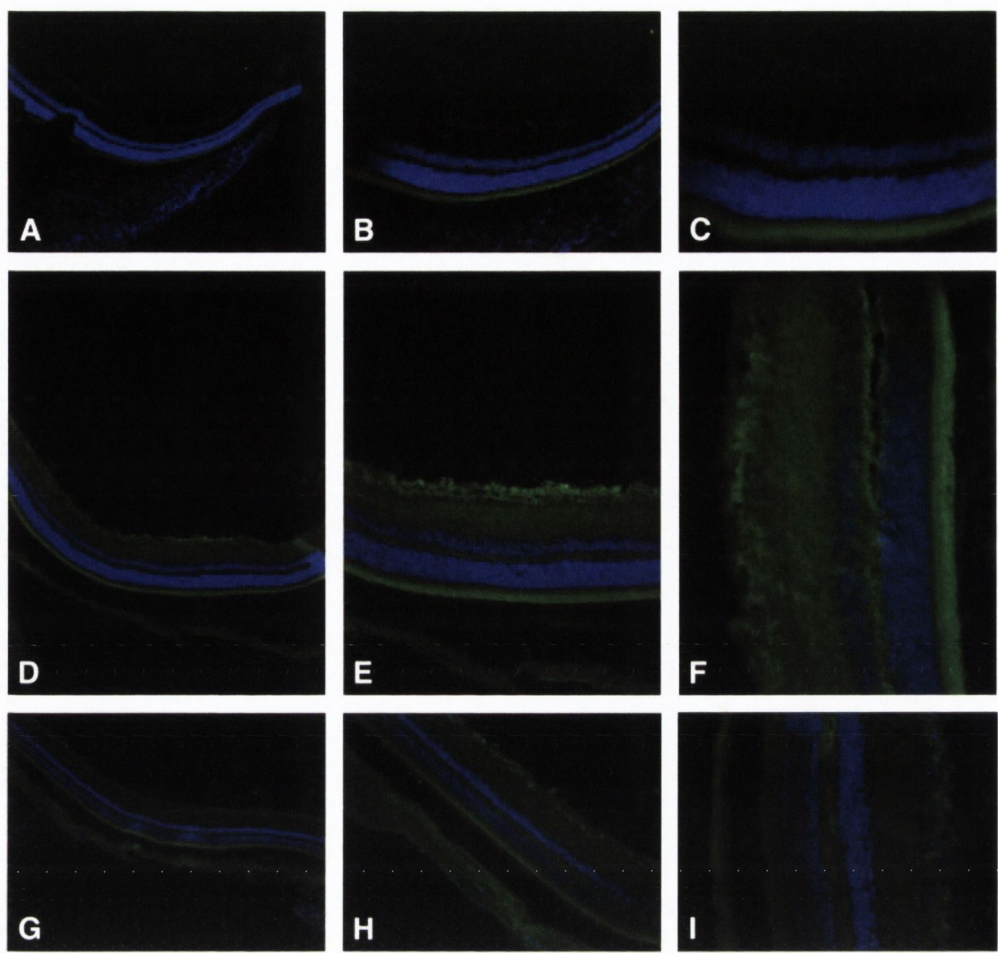


Figure 4.6.13: Serial magnifications of Acrolein stained sections of the *SOD1*^{-/-} retinae **A-C** negative controls **D-F** Positive staining in smoke exposed mice **G-H** Negative staining in control mouse

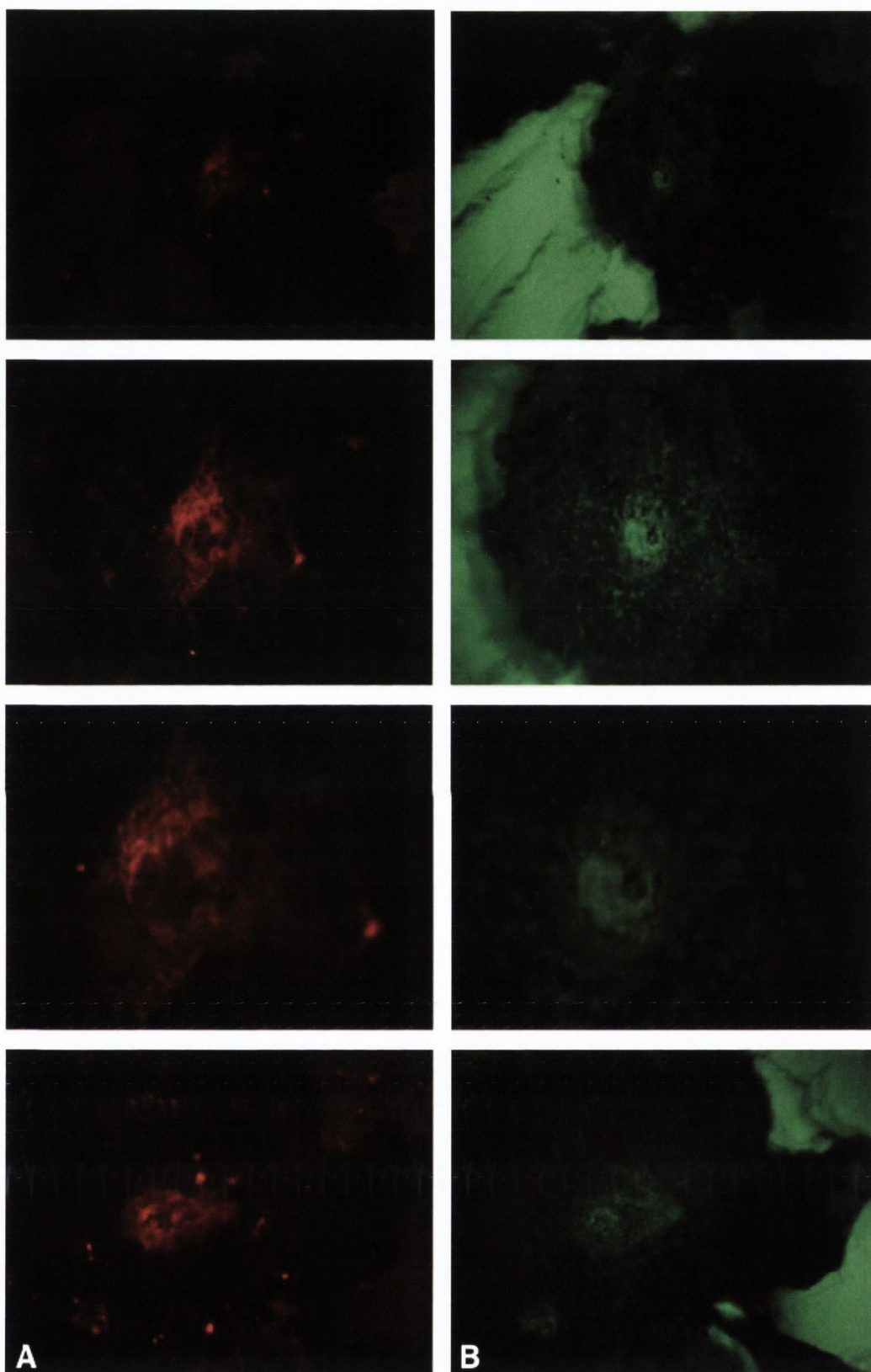


Figure 4.6.14: Comparative analysis of A. CY3 and B. Isolectin B4 Images after laser induced CNV

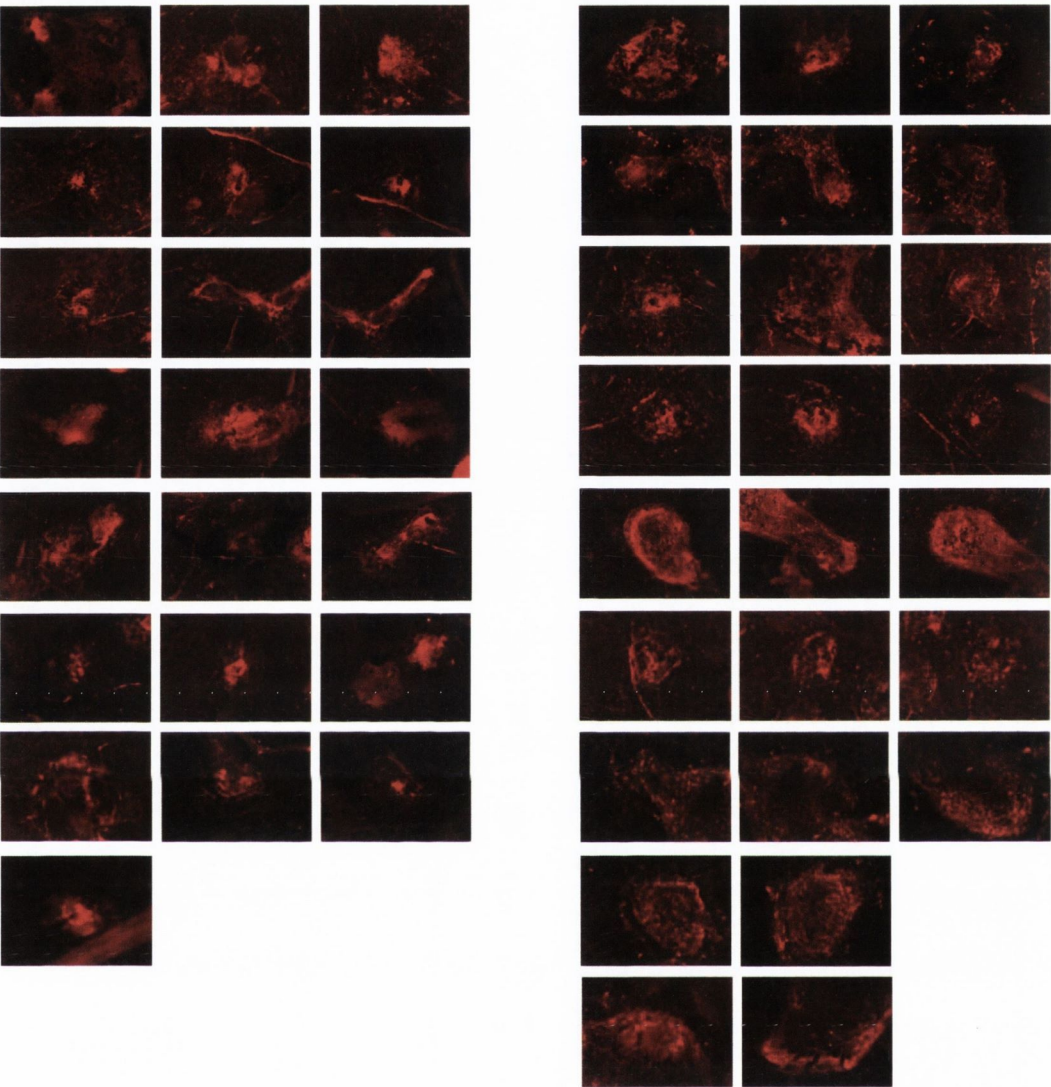


Figure 4.6.15: Stained laser burns post CY3 labeling

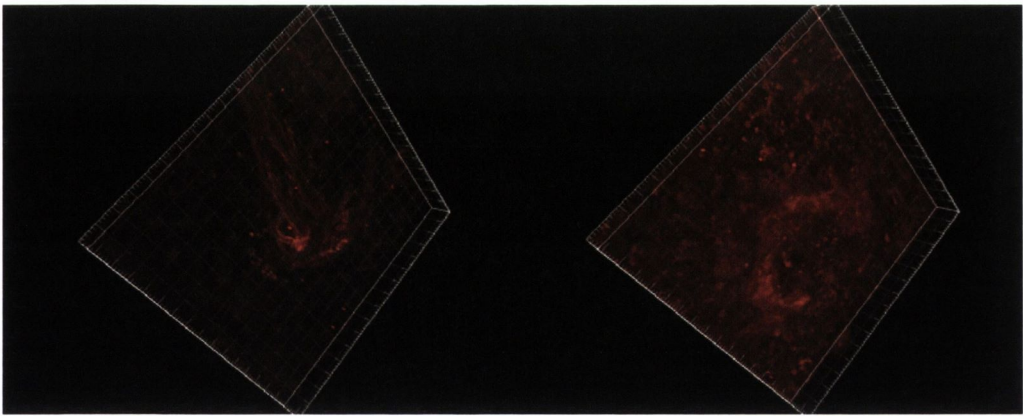


Figure 4.6.16: Representative confocal laser microscopy images of CNV: A. Control. B. CSE injected

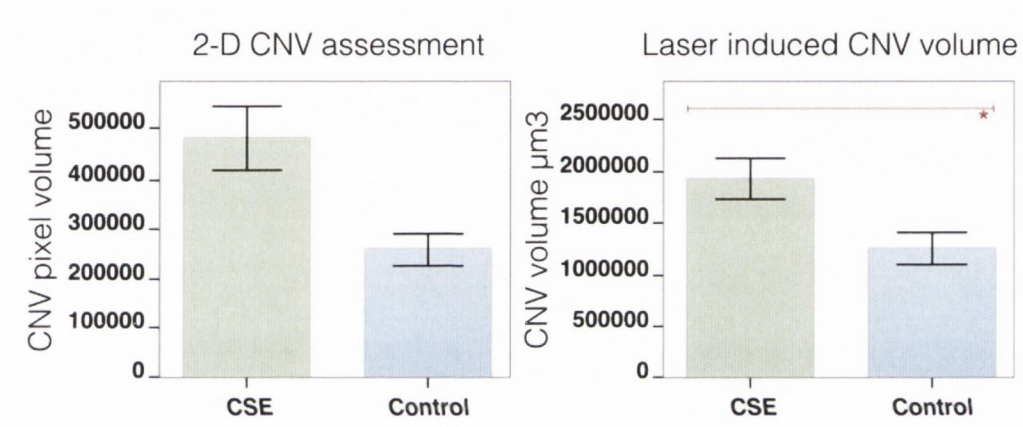


Figure 4.6.17: 2-D and 3-D Volumetric analysis of laser-induced CNV membranes

5.1. Introduction

AMD is a disease determined by both environmental and genetic risk factors. There are approximately 7,000 (of a population of 4.67 million) eligible patients in Ireland for blind registration due to AMD. The risks associated with AMD have been described in the American, Australian, central European, Chinese, Indian and Icelandic populations. The contribution of risk factors observed in these populations to the development of AMD in the Irish population is currently unknown. In this project we aim to characterize the risk factors, both environmental and genetic, in a cohort of Irish volunteers.

5.2. Aims

- 5.1.1 To assess the contribution of non-modifiable risk factors: gender, family history in AMD in the Irish population.
- 5.1.2 To assess the contribution of current cigarette smoking and a past history of cigarette smoking to AMD in the Irish population.
- 5.1.3 To determine the association of additional factors with AMD in the Irish population.
- 5.1.4 To characterise the contribution of four single nuclear polymorphism (SNP) to the risk of AMD in the Irish population.

5.2.1. The association of non-modifiable risk factors to AMD in the Irish population

Family history has long been a recognized association in AMD even prior to the discovery of specific genetic risk factors (Chapter 2.3). There have been no reports of the contribution of family history of AMD in the Irish population. We aimed to report the contribution of positive parental and sibling history in a cohort of Irish patients and control volunteers of who were appropriate age and sex matched.

5.2.2. The association of cigarette smoking with AMD in the Irish population

Cigarette smoking is the most consistent risk factor associated with AMD (Chapter 2.4). In this prospective study, we aimed to identify the contribution of both an ongoing smoking habit and a past history of smoking with the development of AMD.

5.2.3. The association of additional factors with AMD in the Irish population

A number of other modifiable risk factors have been associated with AMD (Chapter 1.7). We aimed to characterise the potential associations of BMI, hypertension, hyperlipidaemia, diabetes, medication use, history of myocardial infarction (MI) or cerebrovascular accident (CVA, stroke) with AMD in the Irish population.

5.2.4. The association of genetic risk factors with AMD in the Irish population

AMD is a complex genetic condition associated with protective and risk conferring alterations of the DNA sequence (Chapter 2.2). These genetic risk factors have been found to be especially relevant to the development of AMD in the Caucasian population. For the purposes of genetic assessment, the Irish population has been considered a homogenous population. There have been no reports on the prevalence of these risk associated SNPs in the general Irish population or in a cohort of AMD patients. We aimed to determine the association of four SNPs associated with AMD and compare associated odds ratios with reports of similar Caucasian populations.

5.3. Material and methods

5.3.1. Clinical participant recruitment

Subjects were recruited from retinal clinics of the Royal Victoria Eye and Ear Hospital in Dublin, Ireland as well as from the surrounding community. Patients were informed of the purpose of the study and informed consent was obtained from each participant (Appendix 2). Controls were recruited from the community by the use of newspaper advertisements and radio announcements and requests. Further participants were recruited by liaising with active ageing groups, church groups and by word of mouth from other participants. Control subjects were provided with additional information about AMD so as to increase awareness of the condition. Participants were informed of the effects of dilation drops on visual acuity and were requested to avoid driving for a period of 4-6 hours. All participants were compliant with this. There were no monetary

inducements for the participation of the controls in this study however coffee and pastries were provided. The project was approved by the Ethics committee of both the Royal Victoria Eye and Ear Hospital as well as by the Ethics committee of Trinity College Dublin and all protocols adhered with the Declaration of Helsinki (update 2008).

Inclusion criteria were as follows:

- Affected patients must be over 45 years old and have the diagnosis of AMD in one or both eyes established by a consultant ophthalmologist based on clinical assessment, OCT and angiography.
- Control patients were individuals who were over 50 years old and had no prior history of ocular pathology.

Exclusion criteria were as follows:

- Retinal pathology that would prevent accurate viewing of the macula. This included dense cataract, pre-existing macular scarring, previous retinal detachment and vascular occlusions.
- Participants were also excluded in cases where informed consent could not be obtained e.g. dementia or severe hearing and visual impairment. There were no restrictions on the basis of best-corrected visual acuity.

Patient questionnaire and sample collection

Venous blood samples were obtained from the peripheral venous circulation either in the antecubital fossa or the dorsal surface of the hand by phlebotomy. Participants completed a questionnaire detailing age, gender, parental and sibling family histories, smoking history, medical history and drug-use history (Appendix 3).

5.3.2. Clinical assessment

Clinical assessment consisted of slit lamp biomicroscopy, intraocular pressure measurement using Goldmann tonometry and an estimation of anterior chamber depth prior to dilation. Participants were then dilated using guttate 1% tropicamide and 2.5% phenylephrine to both eyes and 15-20 minutes elapsed prior to imaging. Patient in the control group underwent photographic assessment only. Control patients who displayed any pathology underwent further assessment with Optical Coherence Tomography (OCT) with fundus fluorescein Angiography (FA). All patients

recruited into the AMD affected group also underwent fundus photography, OCT and FA. On the basis of clinical exam, OCT and FA, patients with diagnosed AMD were subdivided into dry or wet categories. There were no distinctions made on the basis of severity in atrophic ARM/AMD. All patients that met the criteria described in the IntARM guidelines that did not have evidence of late AMD i.e. neovascularisation or geographic atrophy, were placed in the “dry” subcategory.

5.3.3. Fundus photography

Retinal stereoscopic 50 degree and 35 degree colour photographs were obtained digitally using a retinal camera (Topcon 50x) and captured on IMAGEnet 2000 software. Red free and autofluorescent images were obtained in patients that had evidence of retinal changes. Images were stored as high-resolution .tiff files. OCT images were obtained using the direct cross-sectional Stratus OCT (Zeiss). Fundus fluorescein angiography (FFA) was performed by placing an intravenous cannula in the antecubital fossa or dorsal surface of the palm. Patients were positioned in front of the camera and 3mls of 20% (1g/5ml) fluorescein (Martindale pharmaceuticals) was injected. Retinal photographs were taken over the course of 5 minutes. The clinical and FA features of CNV are summarized in Table 5.6.1. For the purposes of risk factor associations, all patients were categorized as either normal or with AMD. There were no distinctions imposed on the basis of AMD type.

5.3.4. Extraction of DNA from patient blood samples

Blood samples

DNA assessments were performed with the support and supervision of Dr. Marian Humphries in the genetics department of Trinity College Dublin. Genomic DNA for each target SNP was amplified using PCR techniques. Samples were stored with anonymous labels in the -80°C freezer located in the genetics department in Trinity College Dublin. PCRs were performed in a manner similar to the *in vitro* assessments. The primers selected to amplify the CFH SNP, HTRA1, LOC and PEDF SNPs are listed (Table 5.6.2). The patient amplifications required 1.5ul of source DNA in the mix described (Table 5.6.2).

5.3.5. Amplification and digestion of the SNPs

SNPs in the patient population were assessed in a manner similar to the *in vitro* experiments. The CFH primers were designed to amplify a gene segment that is 272 base pairs in length. Once PCR was complete, successful amplification was confirmed by loading 5ul of product with 1ul bromophenol blue and electrophoresed on a 2% agarose gel containing 1ul of ethidium bromide. Samples that did not show successful amplification were discarded and PCR was performed again. Samples were digested overnight using the NlaIII restriction endonuclease (New England Biolabs). The results of the digests were imaged by electrophoresis on 2% agarose. Non-variant fragments (T/T) were cleaved by the endonuclease into 187bp and 85bp fragments. Samples containing the C/C variant were not cleaved by the enzyme resulting in the complete 272bp fragment. Samples heterozygous (T/C) for the SNP resolved all three fragments (Fig.5.6.2).

The HTRA1 SNP was amplified and produced a fragment of 385 base pairs in length pairs that was confirmed on a 2% agarose gel as previously described. The successful amplifications were digested overnight using the EagI restriction endonuclease (New England Biolabs). The results of the digests were imaged by electrophoresis on 2% agarose. Non-variant fragments (G/G) were cleaved by the endonuclease into 246bp and 139bp fragments. Samples containing variant (A/A) were not cleaved by the enzyme resulting in the complete 385bp fragment. Samples heterozygous (A/G) for the SNP resolved all three fragments (Fig 5.6.3).

The LOC 387715 SNP was amplified and produced a fragment of 728 base pairs which was confirmed on a 2% agarose gel as previously described. The successful amplifications were digested overnight by the PvuII restriction endonuclease (New England Biolabs). The results of the digests were imaged by electrophoresis on 2% agarose. Samples that did not include the variant SNP were digested into fragments that were 487bp and 241bp in length. The enzyme did not cleave samples containing the T to G variant SNP. Again, samples that were heterozygous for the SNP resolved all three fragments (Fig. 5.6.4).

The PEDF SNP was detected in a similar manner. Samples were amplified and confirmed on 2% agarose gel to yield a 250bp product. Successful products were 250bp in length. Samples were

digested using the BssSI enzyme (New England Biolabs). Amplified DNA segments that did not contain the C to T substitution were cleaved to fragments that were 170bp and 80bp in length. The variant was not cleaved. Heterozygous samples resolved all three fragments (Fig. 5.6.5). All genotype results were analysed using the Prism Stats statistical software. The analysis is discussed in chapter 6.

5.4. Results

5.4.1. Subject recruitment

All participants recruited into the control and AMD groups were of Caucasian/ Irish extraction. 206 participants were recruited into the AMD group and 127 participants in the control group in total. Demographic details for each group are presented below (Table 5.6.4). The ages were matched with an average age of 80.4 years in the AMD group and 75.64 years in the control group. The breakdown of these ages is depicted (Fig. 5.6.6). There was no difference in the breakdown of sex between the AMD and control groups (60.2% vs 62.9%), however a significantly higher number of female participants were recruited in both groups (Fig. 5.6.7).

Weight and height measurements were measured from all participants. All weights were converted to kg and heights converted to cm. Body Mass Index (BMI) is calculated from the following standard formula: $BMI = \text{weight (kg)} / \text{height (m}^2\text{)}$. Participants were classified as underweight (BMI <20), normal (BMI 20-25), overweight (BMI 25-30), or obese (BMI >30). There was no difference detected in average BMI between the AMD (24.87) and control (25.12) groups ($p > 0.05$) (Fig. 5.6.7).

5.4.2. Family history in the Irish AMD population

Familial contribution to the risk of AMD was analysed based on the family histories reported by patients. There were no distinctions made based on the type of AMD reported in the family members. The contribution of a positive parental history (one or both) was recorded in 8.7% of patients with AMD (Table 5.6.5). This was compared with 7.1% in the control group. There was no significant difference detected between these two groups ($p = 0.6822$) (Table 5.6.6)

To establish the sibling risks associated with AMD, participants were questioned on their total number of siblings, living or dead and whether any of them had had AMD. Again no distinction was made based on the type of AMD reported by the patient. The proportion of patients with AMD with at least one brother with a positive history was 8.25% in the AMD group compared with 2.36% in the control group (Table 5.6.5). This was statistically significant ($p=0.032$) conferring an OR of 3.71 and a RR of 1.408 (Table 5.6.6). The proportion of participants with at least one sister with a positive history was 13.1% in the AMD group compared with 3.14% in the control group (Table 5.6.5). This was statistically significant ($p=0.032$) conferring an OR of 4.789 and a RR of 1.489 (Table 5.6.6). The contribution of any sibling, sister or brother, to the development of AMD was subsequently calculated.

Within the AMD group, 17.96% of patients had at least one sibling, sister or brother also diagnosed with AMD. This was compared with 4.72% of control participants. These were significantly different ($p = 0.0003$) and the odds ratio and relative risks associated with a positive family history of any sibling with AMD were 4.415 and 1.477 respectively (Table 5.6.6).

5.4.3. Cigarette smoking in the Irish AMD population

All participants were asked about their smoking history. Current smokers were asked how much they smoke and an estimate of their pack year history was calculated. The pack year history was calculated in pack years where 1 pack year = 20 cigs/day for 1 year. Ex smokers were asked when they quit and for how long they smoked prior to quitting.

Current smokers

Overall, the rate of current cigarette smoking in the entire cohort recruited was 10.81% (Table 5.6.7). The control group had a current smoking rate of 5.51%. The AMD affected group had a significantly higher rate of 14.07% ($p = 0.0008$). This was associated with an OR of 4.19 and a RR of 1.621 (Table 5.6.8). The average pack year history for the total cohort of current smokers was 34.06 pack years. The average pack years in the control group was 27.75 whereas the average pack year history in the AMD group was 35.06 (Table 5.6.7).

Past smokers

When asked about previous smoking history, 39.63% of the entire cohort had a background history of cigarette smoking but had quit prior to assessment (Table 5.6.8). A history of smoking was found in 46.11% of the AMD population but only 29.13% of the control category. This resulted in a significant association with a past history of cigarette smoking ($p < 0.0001$) and the development of AMD with an odds ratio and relative risk of 2.229 and 1.448 respectively (Table 5.6.8).

5.4.4. Co-morbidities in the Irish AMD population

Blood pressure

The rate of diagnosed hypertension in the entire cohort was 51.95% (Table 5.6.9). The prevalence of hypertension in the AMD group was 60.19% which was significantly higher than the 38.58% rate in the control group ($p = 0.0002$). The odds ratio and relative risks associated with hypertension were 2.407 and 1.399 respectively (Table 5.6.10).

Cholesterol

The AMD and the control cohorts reported a similar prevalence when questioned about a prior diagnosis of hypercholesterolaemia (43.69% AMD vs. 40.15% control) (Table 5.6.11). This included a previous diagnosis of hypercholesterolaemia that was either diet or medication controlled. When the strength of association was assessed there was no significant difference between the two groups ($p = 0.5688$). This was reflected in the OR and RR which both contained the null set (Table 5.6.12).

Diabetes

The rates of diabetes in the AMD and control groups were similar at 12.62% and 9.44% respectively (Table 5.6.13). The difference between the groups was non-significant ($p = 0.4784$). The OR and RR were non-significant, both contained the null set (Table 5.6.14).

Medication use

In this study participants were questioned on their current use of the following medications; steroids (oral or inhaled), warfarin, aspirin or any statin drugs (HMG CoA reductase inhibitors).

Similar rates of steroid use were reported in the AMD and the control groups, 7.28% and 4.27% respectively (Table 5.6.15). The percentages of patients on warfarin in the AMD and the control groups were 7.66% and 5.51% respectively (Table 5.6.15). The use of aspirin was reported in 45.63% of AMD and 39.37% of control participants and the use of a statin medication was seen in 45.63% and 39.39% in the AMD and control groups respectively. There was no statistical significance associated with the use of any of the investigated medications and the risk of AMD (Table 5.6.16).

History of myocardial infarction (MI) or cerebrovascular accident (Stroke)

The proportion of participants with a background history of MI was exactly the same in the AMD and control groups, 7.28% and 9.4% (Table 5.6.17). There was no significant difference between the two groups ($p=0.5373$). A past history of stroke was less common than a past history of MI. The proportion of participants who had suffered strokes was still similar, 7.76% and 3.93% in the AMD and control groups (Table 5.6.18). There was no statistical difference between the two groups ($p=0.2451$) (Table 5.6.19).

5.4.5. Prevalence of the SNPs in the Irish AMD population

Prevalence of the CFH (Y402H) SNP in the Irish AMD population

The prevalence of the homozygous non-variant genotype (T/T) CFH allele in the AMD population was 21.12% (Table 5.6.20). The prevalence of the heterozygous genotype (T/C) was 48.08% and the prevalence of the homozygous variant genotype (C/C) was 30.8%. This was compared with the control population. In the control group the prevalence of the homozygous non-variant genotype was 44.09% (Table 5.6.20). The prevalence of the heterozygous genotype was 37% and the prevalence of the homozygous variant genotype was 18.89%. The OR and RR associated with the heterozygous genotype was 2.708 and 1.546 respectively ($p=0.0002$) (Table 5.6.21). The OR and RR associated with the homozygous genotype was 3.394 and 1.653 respectively ($p=0.0001$) (Table 5.6.21)

Prevalence of the LOC387715 SNP in the Irish AMD population

The prevalence of the homozygous non-variant genotype LOC387715 allele (G/G) in the AMD population was 32.36% (Table 5.6.20). The prevalence of the heterozygous genotype (T/G) was 42.99% and the prevalence of the homozygous variant genotype (T/T) was 24.63%. This was compared with the control population. In the control group the prevalence of the homozygous non-variant genotype was 61.41%. The prevalence of the heterozygous genotype was 31.49% and the prevalence of the homozygous variant genotype was only 7%. The OR and RR associated with the heterozygous genotype was 2.527 and 1.482 respectively ($p=0.0002$) (Table 5.6.21). The OR and RR associated with the homozygous genotype was 6.597 and 1.849 respectively ($p<0.0001$) (Table 5.6.21)

Prevalence of the HTRA1 SNP in the Irish AMD population

The prevalence of the homozygous non-variant genotype HTRA1 allele (G/G) in the AMD population was 27.05% (Table 5.6.20). The prevalence of the heterozygous genotype (G/A) was 45.89% and the prevalence of the homozygous variant genotype (A/A) was 27.05%. This was compared with the control population. In the control group the prevalence of the homozygous non-variant genotype was 44.88%. The prevalence of the heterozygous genotype was 47.24% and the prevalence of the homozygous variant genotype was 7.87%. The OR and RR associated with the heterozygous genotype were not significant ($p=0.806$) (Table 5.6.21). The OR and RR associated with the homozygous genotype was 5.802 and 1.717 respectively ($p<0.0001$) (Table 5.6.21)

Prevalence of the PEDF SNP in the Irish AMD population

The prevalence of the homozygous non-variant genotype (C/C) in the AMD population was 9.6% (Table 5.6.20). The prevalence of the heterozygous genotype (C/T) was 48.9% and the prevalence of the homozygous variant genotype (T/T) was 41.4%. This was compared with the control population. In the control group the prevalence of the homozygous non-variant genotype was 12.5%. The prevalence of the heterozygous genotype was 58.92% and the prevalence of the homozygous variant genotype was 28.57%. There was no statistical significance noted between the assessment groups (Table 5.6.21).

5.5. Discussion

In this research we present risk factors associated with AMD in the Irish population. A significant strength of this presented work is the thorough characterization of the control cohort. Patients recruited to the control group underwent full clinical assessment to rule out ARM/AMD. Participants in the control group were also recruited from the general population and not from clinics in the Royal Victoria Eye and Ear Hospital therefore they did not display coexistent ocular disease.

The difficulty in collecting patient candidates – in a tertiary specialist retinal referral centre- is that there is a bias to recruiting AMD cases that require treatment. Though dry AMD is considerably more common in the population, it presents as a more indolent disease. Early dry AMD is often not referred any further than the optician, or community ophthalmic physician. Those that were referred for assessment were sent to rule out neovascular changes. This is a recognized flaw in the patient collection. There is also a bias towards patients requiring active treatment. Anti-VEGF treatment is currently the standard in neovascular AMD. Anti-VEGF treatment has surpassed the previous standard of photodynamic therapy (PDT). Unlike PDT, which was restricted on the basis of morphology to approximately 18% of CNV, the majority of the patients recruited were receiving anti-VEGF treatment.

5.5.1. Family history and AMD

In this study, we did not find any association between parental history and the risk of developing AMD. We believe this to be an inaccurate representation. The average age of all participants in this study was 78.6 years. It may be estimated that the parents of these participants were themselves approximately 20 years old at the time of the participant's birth, placing their own birth ages approximately 98 years ago. The significant problem encountered in characterizing parental contribution to the development of AMD was the shorter life expectancies of the patients' parents. The average life expectancy in Ireland in was less than 65 years up until 1960 (Fig. 5.6.14). It is therefore possible that the parents of these participants died before the disease could manifest. This was compounded by the lack of knowledge as to the specific nature of any eye conditions suffered by their parents and lack of ophthalmic services in Ireland at the time. Patients may not

have been provided the details surrounding any diagnosis in by-gone eras when the disease was less understood and therapeutic intervention was not possible. It was noted, albeit in an anecdotal manner, where patients knew that their parents had a visual impairment they did not know the cause. We believe this to be a significant limitation of all familial risk assessments in AMD. Assessment of sibling history of AMD was not affected by these limitations. There was a significant association noted if a brother in the family had a history of AMD. The association was stronger if a sister in the family had a history of AMD. This may be accounted for by the increased life spans, and higher uptake of health services by females. The odds ratio of a positive sibling history was 4.415 which is consistent with other reported family studies.²²⁰

5.5.2. Cigarette smoking and AMD in the Irish population

The prevalence of current smoking was significantly higher in the affected group when compared to the control cohort, which is consistent with the findings reported by other groups supporting the correlation between smoking and AMD. The OR associated with AMD in this cohort (OR = 4.549) is also consistent with previous reports.¹²¹ Overall, the risk associations seen in this cohort of Irish patients is consistent with previous reports characterizing the Caucasian population. The pooled odds ratio from the Beaver Dam, Blue Mountains and Rotterdam studies of active cigarette smoking and AMD has been calculated as 3.12 (2.10-4.64).¹²¹ Our calculated results of this Irish cohort corresponded to this at 4.196, though it is at the higher end of this confidence interval. The reported odds ratio for a prior history of smoking was 1.36 (0.97-1.90). We report an OR of 2.229 associated with a history of smoking in the Irish population. It is possible that this is an overestimation as patients who had quit fewer than six months prior to the assessment were still stratified as ex-smokers. Though by definition, these patients were analyzed as part of the ex-smoker cohort, they are not likely to be in the same risk band as the ex smokers of 10 year or greater. There is an argument for analyzing them as part of the current smoker cohort as the beneficial effects of quitting are not likely to be seen that early. This study was insufficiently powered to correlate any reduction in risk after smoking cessation over time.

5.5.3. Hypertension and AMD in the Irish population

The reported pooled odds ratio of the association between hypertension and AMD is 1.22 (1.48–1.78).¹⁴⁶ The odds ratio calculated from this Irish cohort is higher (2.407). The contribution of

hypertension to the pathological process of AMD is unclear. In the vascular hypothesis of AMD, increased scleral stiffness leads to increased vascular resistance and reduced choroidal flow. Increased systemic blood pressure would also raise total vascular resistance and further compromise flow. The effect of an increased systemic blood pressure is not accounted for in either the oxidative stress or inflammatory models of AMD.

5.5.4. Cholesterol, diabetes and AMD in the Irish population

Neither hypercholesterolaemia nor diabetes demonstrated a significant association with the development of AMD in this cohort. The reported odds ratio for hypercholesterolaemia in AMD is 0.94 (0.84-1.04). The pooled associated odds ratio for diabetes is 1.09 (0.61-1.92).¹⁴⁶ These pooled non-significant findings are similar to the non-significant ratios we report in this Irish cohort. These non-significant associations are important to consider in the context of disease aetiology. Both hypercholesterolaemia and diabetes are significant risk factors in atherosclerosis and microvascular disease. The vascular hypothesis of AMD links the aetiology of AMD with that of atherosclerosis, however the lack of association of either condition with the development of AMD is a significant weakness of this hypothesis.

5.5.5. Medication and AMD

All participants were asked to detail their medication history in relation to four medications; steroids (which included inhaled steroid preparations), warfarin, aspirin and statins. There was no significant association found between any of the medications and the development of AMD. Previous associations reported between these medications and AMD have been conflicting. A meta analysis, however, failed to find a significant correlation between any of these medications and AMD,¹⁴⁶ however deJong et al recently associated aspirin and an increased risk of AMD in the EUREYE study.³¹⁶ The construction of this study does not discount the possible benefits of these medications in the context of the disease, however, and there appears to be no correlation in terms of baseline risk. Similar to the non-significant results found in the diabetes and hypercholesterolaemia assessments, the lack of benefit from statin medications is inconsistent with an atherosclerotic pathology. Warfarin and aspirin reduce the risk of thrombotic and embolic disease. If these pathological mechanisms were responsible for the development of AMD a reduction in relative risk might have been observed. This was not the case, and although

thromboembolism has not been implicated in the pathogenesis of AMD, the use of anticoagulants may result in increased haemorrhage and as a result worsen visual outcomes in CNV. This study was not constructed in a manner to address the effects of medications on the progression of AMD but it is plausible that anticoagulation could result in poorer visual acuity.

5.5.6. Genetic risk factors in AMD

In this study we reported a statistical association between three SNPs and AMD in the Irish population. The findings confirm that CFH, LOC387715 and HTRA1 have a significant influence on AMD. The odds ratios associated with the risk factors characterized in the Irish population were comparable to the pooled observations reported in the literature.³¹⁷ There does not appear to be any deviations in the homogenous Caucasian Irish population from the American, Australian and European Caucasian populations in respect to the major AMD SNPs. The minor PEDF SNP assessed did not show a significant association with AMD in the Irish population.

The identification and characterization of the genetic contributions to disease risk and progression is important for a number of reasons. It is possible that macular degeneration is a common tissue response to an array of factors or a multitude of disease entities grouped together by the lack of more subtle endophenotypic criteria. Genetic based classifications of the disease may alter the treatments by providing multiple genotypic descriptions of similar disease phenotypes. This provides a possible platform for screening and risk stratification in the presence of early interventions. Accurate screening and risk stratification is a key component in effective primary care. Hageman et al recently reported a series of the 13 most common SNPs associated with AMD where analysis of all genes might provide an optimized risk assessment for all patients with AMD. The isolation of the genes contributing to disease risk also provides a starting point for investigation into the molecular basis of disease. Once a candidate gene is confirmed, the downstream effect on the cellular expression of proteins can be assessed. This assists in forming the disease's molecular pathways. With the success of gene therapies in the treatment of Leber's congenital amaurosis, the possibility of tailored gene therapies for other genetic conditions may also be possible. This could potentially provide a cure, rather than a treatment for, AMD.

5.6. Figures and tables

Clinical Features of Neovascularisation
Subretinal fluid
Subretinal or Sub-RPE blood
Subretinal pigment ring
Irregular elevation of the RPE
Subretinal grey-white lesion
Cystoid macular oedema
Subretinal vessels - sea fan pattern
Fundus Fluorescein Angiogram (FFA) features of Neovascularisation
Classic CNV
Bright, uniform hyperfluorescence
Intensifies in the transit phase
Leakages obscure borders in the late phase
Occult CNV: Fibrovascular PED
Stippled or granular appearance
Progressive leakage in stippled pattern
Not as diffuse as Classic CNV
Occult CNV: Late leakage undetermined source
Fluorescence at the level of the RPE
Best appreciated in late frames
Does not correspond to early leakage features of classic CNV

Table 5.6.1: Clinical features used to identify CNV

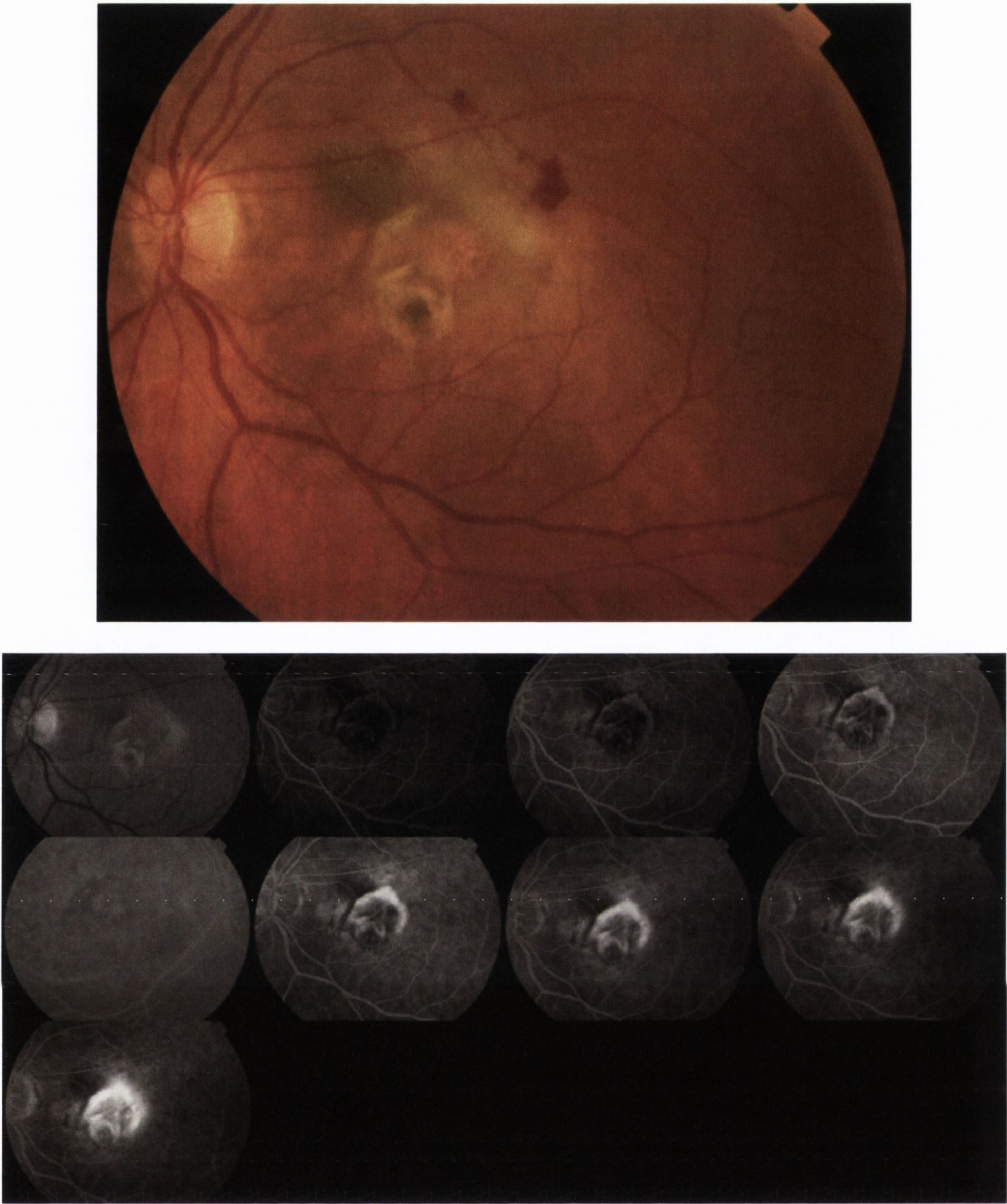


Figure 5.6.1: Choroidal neovascularisation photography and FFA

Gene	Forward Primer 5'-3'	Reverse Primer 3'-5'	Endo-nuclease
CFH	GTTCGTCTTCAGTATACAT	AGACCAGCCATTCTCCAT	NlaIII
HTRA1	ATGCCACCCACAACAACCTTT	CGCGTCCTTCAAACATAATGG	EagI
LOC	CAGCACCTTGTCACCACA	CTAACAACCCGGACGAGAAG	PvuII
PEDF	CAGCACCTTGTCACCACA	CCTCGCTGAACTGAAATGT	BssSI

Table 5.6.2: Primers used for population PCR assessments

PCR mix per sample
DNA 1.5ul
Forward primer 0.25ul
Reverse primer 0.25ul
dNTP 0.5ul
Buffer 2.5ul
GoTag 0.125ul
dH ₂ O

Table 5.6.3: PCR amplification constituents

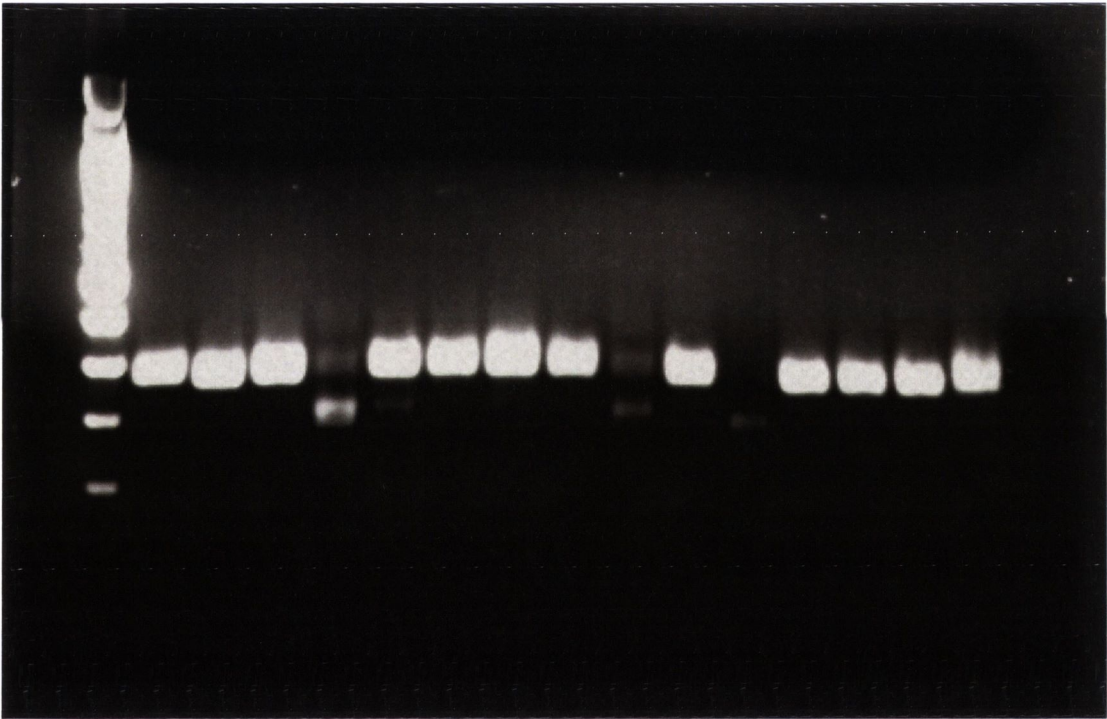


Figure 5.6.2: Example of CFH restriction digest result electrophoresis

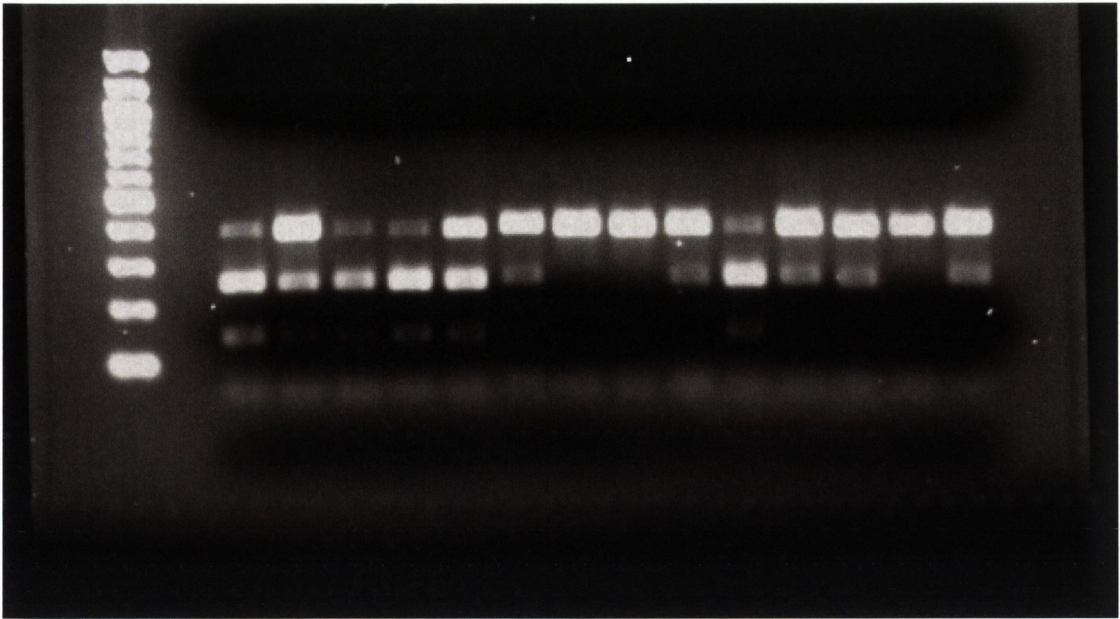


Figure 5.6.3: Example of the HTRA1 digest fragment electrophoresis

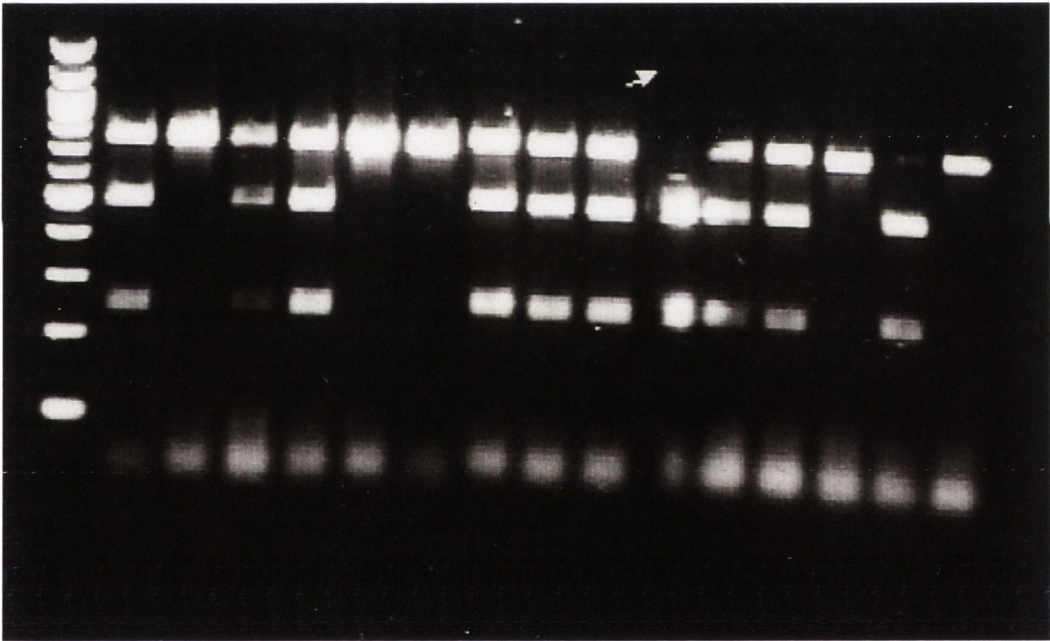


Figure 5.6.4: Example of LOC 387715 digest fragment electrophoresis

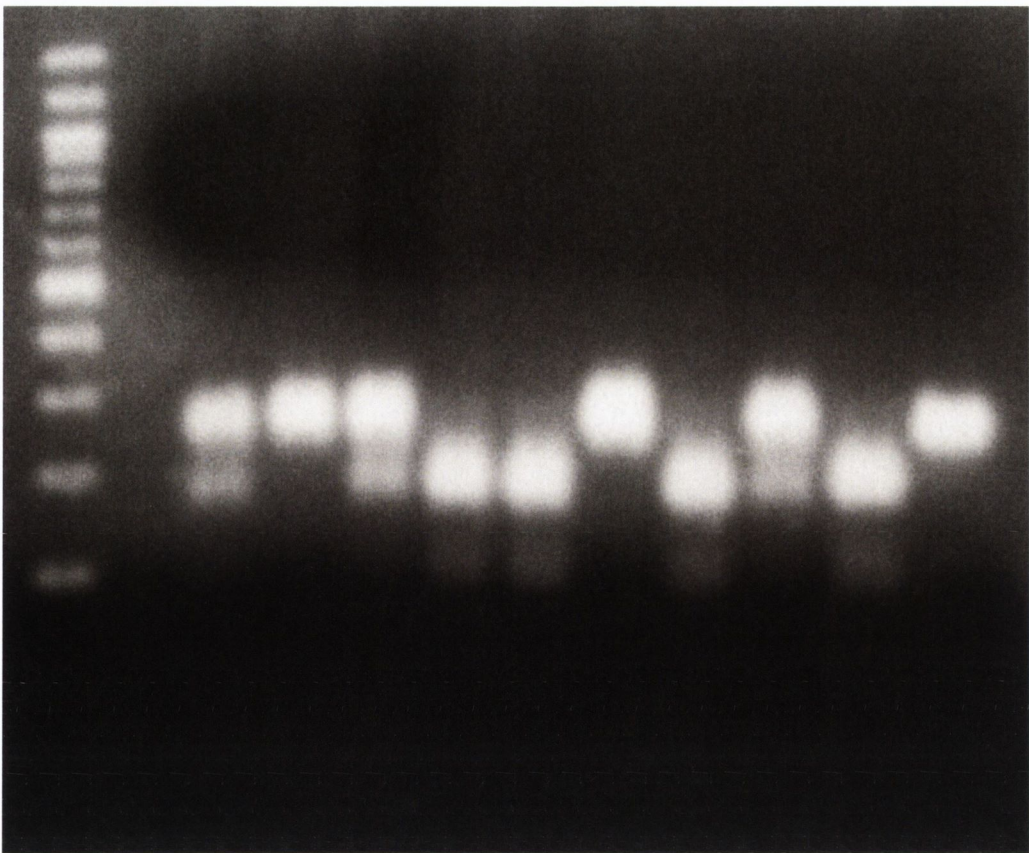


Figure 5.6.5: Example of PEDF digest fragment electrophoresis

	AMD	Control
Number	206	127
Age	80.4 (SE 0.6135)	75.64 (SE 0.6534)
M:F (%F)	82:124 (60.2%)	47:80 (62.9%)
BMI	24.87 (SE 0.2349)	25.12 (SE 0.3487)
Parental hx	8.73%	7.09%
Sibling hx	17.96%	4.72%

Table 5.6.4: Basic demographic data for the AMD and control groups

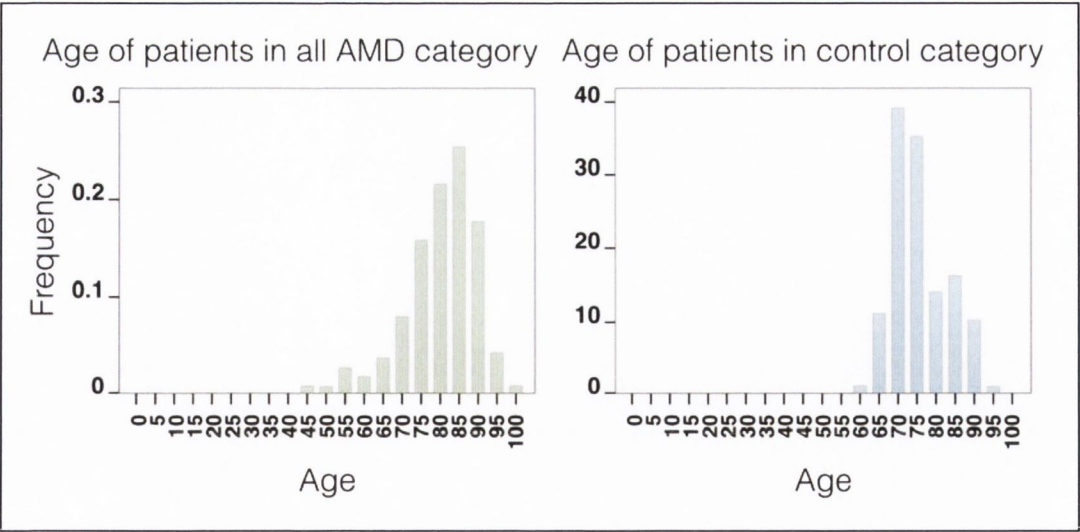


Figure 5.6.6: Histogram of age distribution in the AMD and control groups

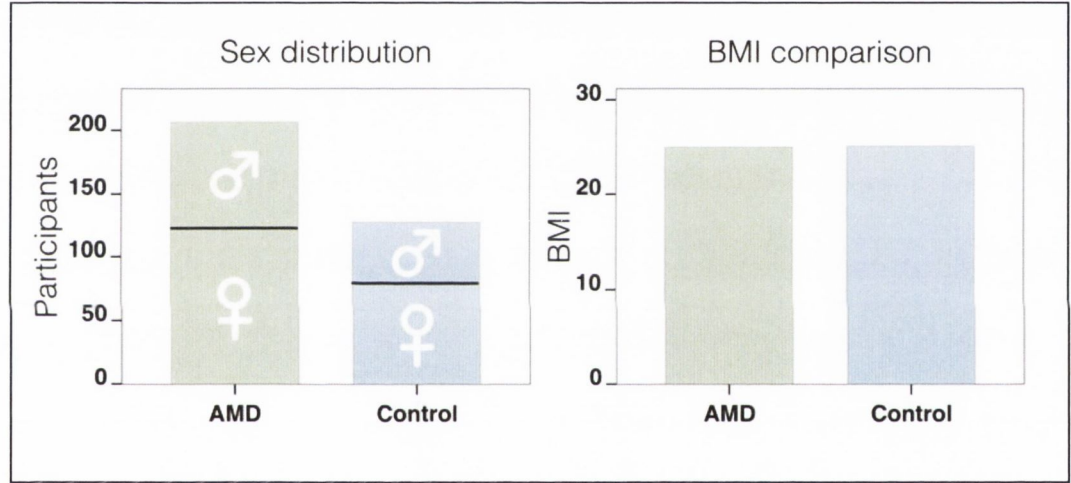


Figure 5.6.7 Sex distribution and BMI comparisons between the AMD and control groups

Family hx	AMD	Control	Total
Parental hx AMD	18	9	27
No parental hx AMD	188	118	306
Total	206	127	333
% Parental hx	8.737864078	7.086614173	8.108108108
% No parental hx	91.26213592	92.91338583	91.89189189
%Total	100	100	100

Family hx	AMD	Control	Total
Brother hx AMD	17	3	20
No brother hx AMD	189	124	313
Total	206	127	333
% Brother hx	8.252427184	2.362204724	6.006006006
% No brother hx	91.74757282	97.63779528	93.99399399
Total	100	100	100
Sister hx AMD	27	4	31
No sister hx AMD	179	123	302
Total	206	127	333
% Sister hx	13.10679612	3.149606299	9.309309309
% No sister hx	86.89320388	96.8503937	90.69069069
Total	100	100	100
Sibling hx AMD	37	6	43
No sibling hx AMD	169	111	290
Total	206	127	333
% Sibling hx	17.96116505	4.724409449	12.91291291
% No sibling hx	82.03883495	95.27559055	87.08708709
Total	100	100	100

Table 5.6.5: Family history on the AMD and control groups

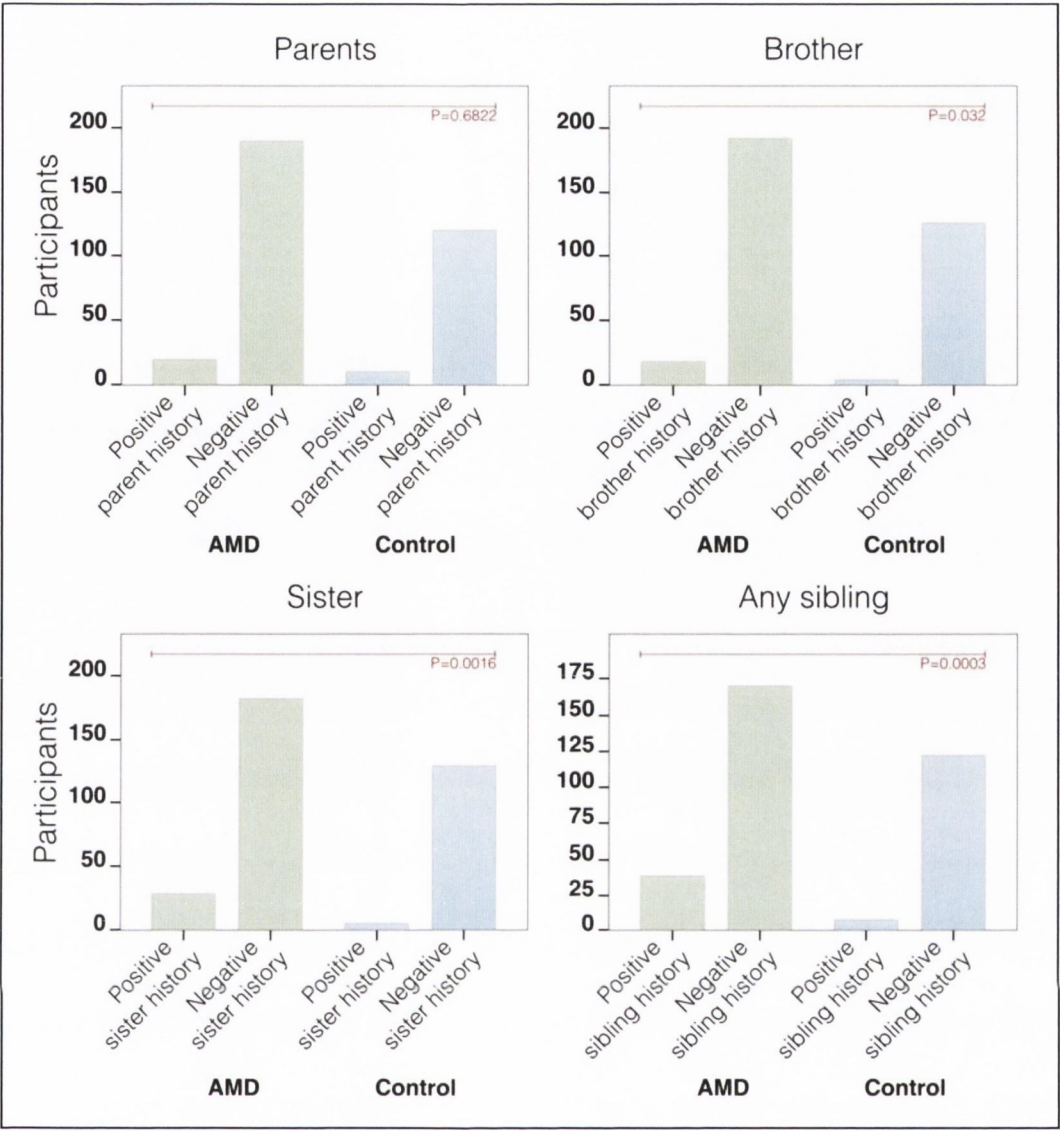


Figure 5.6.8: Contribution of parental and sibling family histories to AMD

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Family History			
Parent	P = 0.6822	1.255(95%CI: 0.5458 to 2.887)	1.085 (95%CI: 0.8191 to 1.437)
Brother	P = 0.032	3.718 (95%CI: 1.067 to 12.96)	1.408 (95%CI: 1.147 to 1.728)
Sister	P = 0.0016	4.789 (95%CI: 1.635 to 14.03)	1.489 (95%CI: 1.262 to 1.756)
Any sibling	P = 0.0003	4.415 (95%CI: 1.806 to 10.79)	1.477 (95%CI: 1.806 to 10.79)

Table 5.6.6: OR and RR of Family history and AMD

	AMD	Control	Total
Current smoker	29	7	36
Ex smoker	95	37	132
Never smoker	82	83	165
Total	206	127	333
% Current smoker	14.0776699	5.511811024	10.81081081
% Ex smoker	46.11650485	29.13385827	39.63963964
% Never smoker	39.80582524	65.35433071	49.54954955
Total	100	100	100
Current smokers			
Mean pack years	35.06	27.75	34.08
Standard deviation	21.59	17.07	20.07
Ex smokers			
Mean years since quitting	22.18	20.06	22.52
Standard deviation	14.3	15.34	14.75
Mean pack years	30.41	20.53	27.6
Standard deviation	28.13	29.72	28.88

Table 5.6.7: Data of cigarette smoking histories in the AMD and control groups

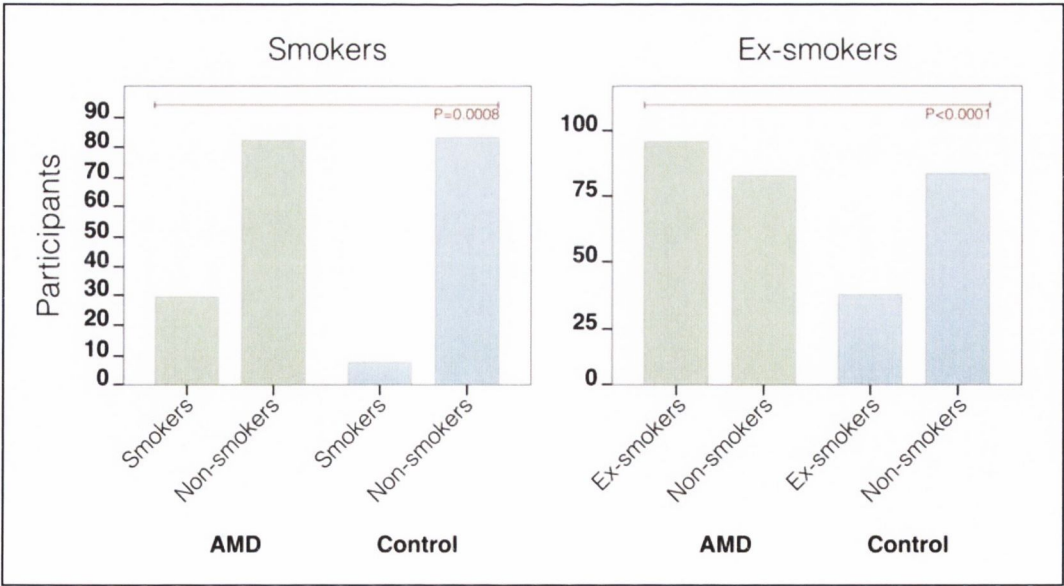


Figure 5.6.9: Smokers and ex-smokers in AMD and control groups

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Smoking			
Current smoker	P = 0.0008	4.196 (95%CI: 1.739 to 10.11)	1.621 (95% CI: 1.298 to 2.024)
Ex smoker	P < 0.0001	2.229 (95%CI: 1.596 to 4.231)	1.448 (95% CI: 1.201 to 1.746)

Table 5.6.8: OR and RR of smoking and the development of AMD

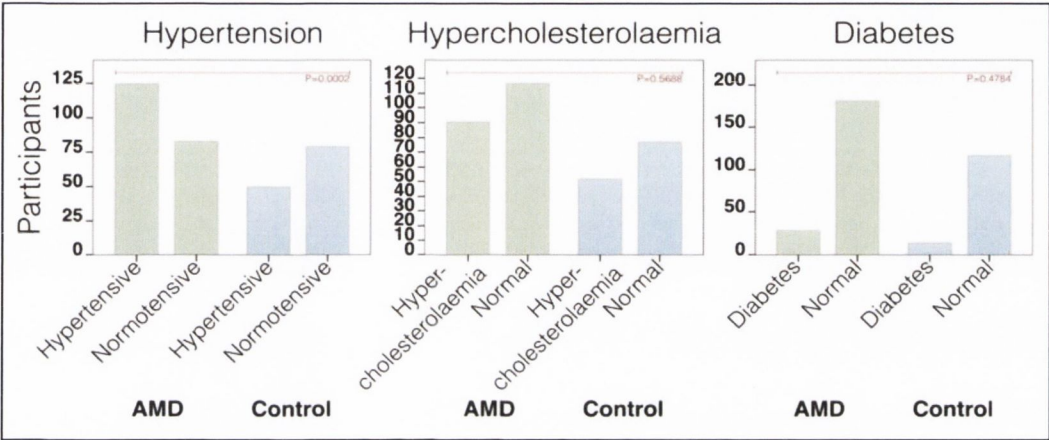


Figure 5.6.10: Hypertension, hypercholesterolaemia and diabetes in AMD and control groups

	AMD	Control	Total
Hypertension	124	49	173
No history hypertension	82	78	160
Total	206	127	333
% Hypertension	60.19417476	38.58267717	51.95195195
% No hypertension	39.80582524	61.41732283	48.04804805
Total	100	100	100

Table 5.6.9: Hypertension in AMD and control groups

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Hypertension	p =0.0002	2.407 (95%CI: 1.171 to 3.788)	1.399 (95%CI: 1.171 to 1.671)

Table 5.6.10: OR and RR of hypertension in the development of AMD

	AMD	Control	Total
Hypercholesterolaemia	90	51	141
No history hypercholesterolaemia	116	76	192
Total	206	117	333
% Hypercholesterolaemia	43.6893203883	40.157480315	42.3423423423
% No hypercholesterolaemia	56.3106796117	59.842519685	57.6576576576
Total	100	100	100

Table 5.6.11: Hypercholesterolaemia in AMD and control groups

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Hypercholesterolaemia	p =0.5688	1.156 (95%CI: 0.7378 to 1.812	1.056 (95%CI: 0.8922 to 1.251)

Table 5.6.12: OR and RR of hypercholesterolaemia and AMD

	AMD	Control	Total
Diabetic	26	12	38
Non-diabetic	180	115	295
Total	206	127	333
% Diabetic	12.6213592233	9.44881889764	11.4114114114
% Non-diabetic	87.3786407767	90.5511811024	85.5885885886
Total	100	100	100

Table 5.6.13: Diabetes in AMD and control groups

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Diabetes	P = 0.4784	1.384 (95%CI: 0.6717 to 2.653)	1.121 (95%CI: 0.8869 to 1.418)

Table 5.6.14: OR and RR of diabetes and AMD

	AMD	Control	Total
<i>Use of medications</i>			
Steroids (Incl inhaled)	15	6	21
No steroids	191	121	312
Total	206	127	333
% Steroids	7.2815533980	4.724409449	6.306306306
% No steroids	92.718446601	95.27559055	93.69369369
Total	100	100	100
Warfarin	16	7	23
No warfarin	190	120	310
Total	206	127	333
% Warfarin	7.766990291	5.511811024	6.906906907
% No warfarin	92.23300971	94.48818898	93.09309309
Total	100	100	100
Aspirin	94	50	144
No aspirin	112	77	189
Total	206	127	333
% Aspirin	45.63106796	39.37007874	43.24324324
% No aspirin	54.36893204	60.62992126	56.75675676
Total	100	100	100
Statin	80	44	124
No statin	126	83	209
Total	206	127	333

% Statin	45.63106796	39.37007874	43.24324324
% No statin	54.36893204	60.62992126	56.75675676
Total	100	100	100

Table 5.6.15: Medication use in the AMD and control groups

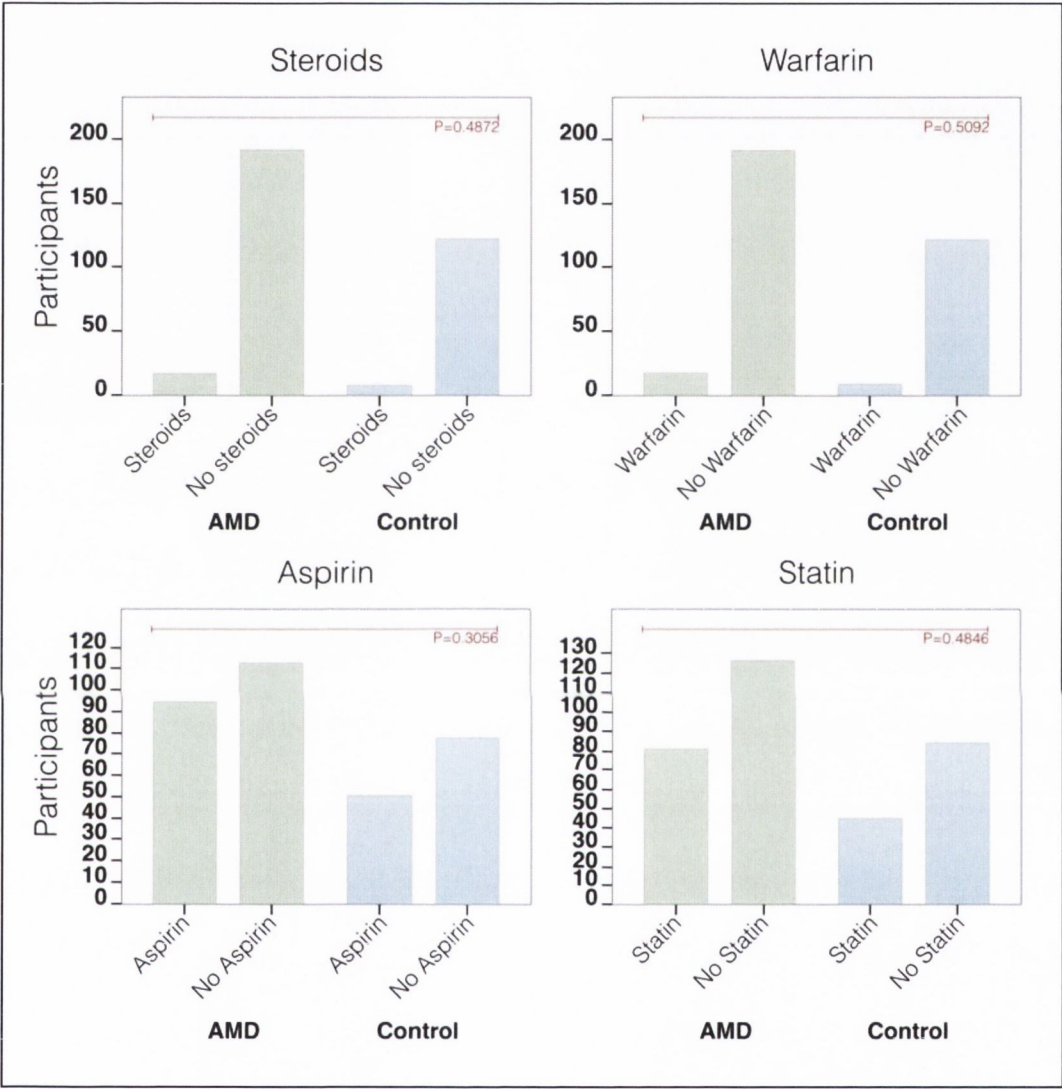


Figure 5.6.11: Medication in the AMD and control groups

Risk factor	Fisher's exact P value	Odds ratio	Relative risk
Medications			
Steroids	P = 0.4872	1.584 (95%CI: 0.5980 to 4.195)	1.167 (95%CI: 0.8778 to 1.551)
Warfarin	P = 0.5092	1.444 (95%CI: 0.5768 to 3.613)	1.135 (95%CI: 0.8540 to 1.509)

Aspirin	P = 0.3056	1.293 (95%CI: 0.246 to 2.026)	1.102 (95%CI: 0.9313 to 1.303)
Statins	P = 0.4846	1.342 (95%CI: 0.8336 to 2.162)	1.070 (95%CI: 0.9022 to 1.269)

Table 5.6.16: OR and RR of medications and AMD

	AMD	Control	Total
Previous MI	15	12	27
No MI	191	115	306
Total	206	127	333
% Previous MI	7.281553398	9.448818898	8.108108108
% No MI	92.7184466	90.5511811	91.89189189
Total	100	100	100

Table 5.6.17: Previous myocardial infarction and AMD and control groups

	AMD	Control	Total
Previous stroke	16	5	21
No stroke	190	122	312
Total	206	127	333
% Previous stroke	7.766990291	3.937007874	6.306306306
% No stroke	92.23300971	96.06299213	93.69369369
Total	100	100	100

Table 5.6.18: Previous stroke in AMD and control groups

Risk factor	Fisher's P value	exact Odds ratio	Relative risk
Vascular events			
Myocardial Infarction	P = 0.5373	0.7526 (95%CI: 0.3403 to 1.665)	0.8901 (95%CI: 0.6282 to 1.261)
Stroke	P = 0.2451	2.055 (95%CI: 0.7337 to 5.755)	1.251 (95%CI: 0.9694 to 1.615)

Table 5.6.19: OR and RR of MI or stroke and AMD

Genotype	AMD	Control	Total
CFH			
T/T	44	56	100
T/C	100	47	147
C/C	64	24	88
	208	127	335
LOC 387715			
T/T	51	9	60
T/G	89	40	129
G/G	67	78	145
	207	127	334
HTRA1			
G/G	56	57	115
G/A	95	60	156
A/A	56	10	66
	207	127	334
PEDF			
T/T	77	32	109
T/C	91	66	157
C/C	18	14	32
	186	112	298

Table 5.6.20: Distribution of SNP genotypes in the Irish AMD and control groups

Genotype	Fisher's P value	exact Odds ratio	Relative risk
CFH			
C/T vs T/T	P = 0.0002	2.708(95%CI: 1.601 to 4.580)	1.546(95%CI: 1.207 to 1.980)
C/C vs T/T	P = 0.0001	3.394 (95%CI: 1.838 to 6.267)	1.653 (95%CI: 1.280 to 2.134)
LOC387715			
T/G vs G/G	P = 0.0002	2.527(95%CI: 1.543 to 4.140)	1.482(95%CI: 1.200 to 1.829)
T/T vs G/G	P < 0.0001	6.597 (95%CI: 3.023 to 14.4)	1.849 (95%CI: 1.498 to 2.259)
HTRA1			
A/G vs G/G	P = 0.0812	1.585 (95%CI: 0.971 to 2.586)	1.229 (95%CI: 0.9816 to 1.538)
A/A vs G/G	P < 0.0001	5.802 (95%CI: 2.695 to 12.49)	1.717(95%CI: 1.390 to 2.121)
PEDF			
T/C vs C/C	P = 0.0806	2.212 (95%CI: 0.987 to 4.954)	1.315 (95%CI: 0.952 to 1.817)
T/T vs C/C	P = 0.1383	1.872 (95%CI: 0.831 to 4.212)	1.256 (95%CI: 0.904 to 1.745)

Table 5.6.21: OR and RR associated with AMD linked SNPs

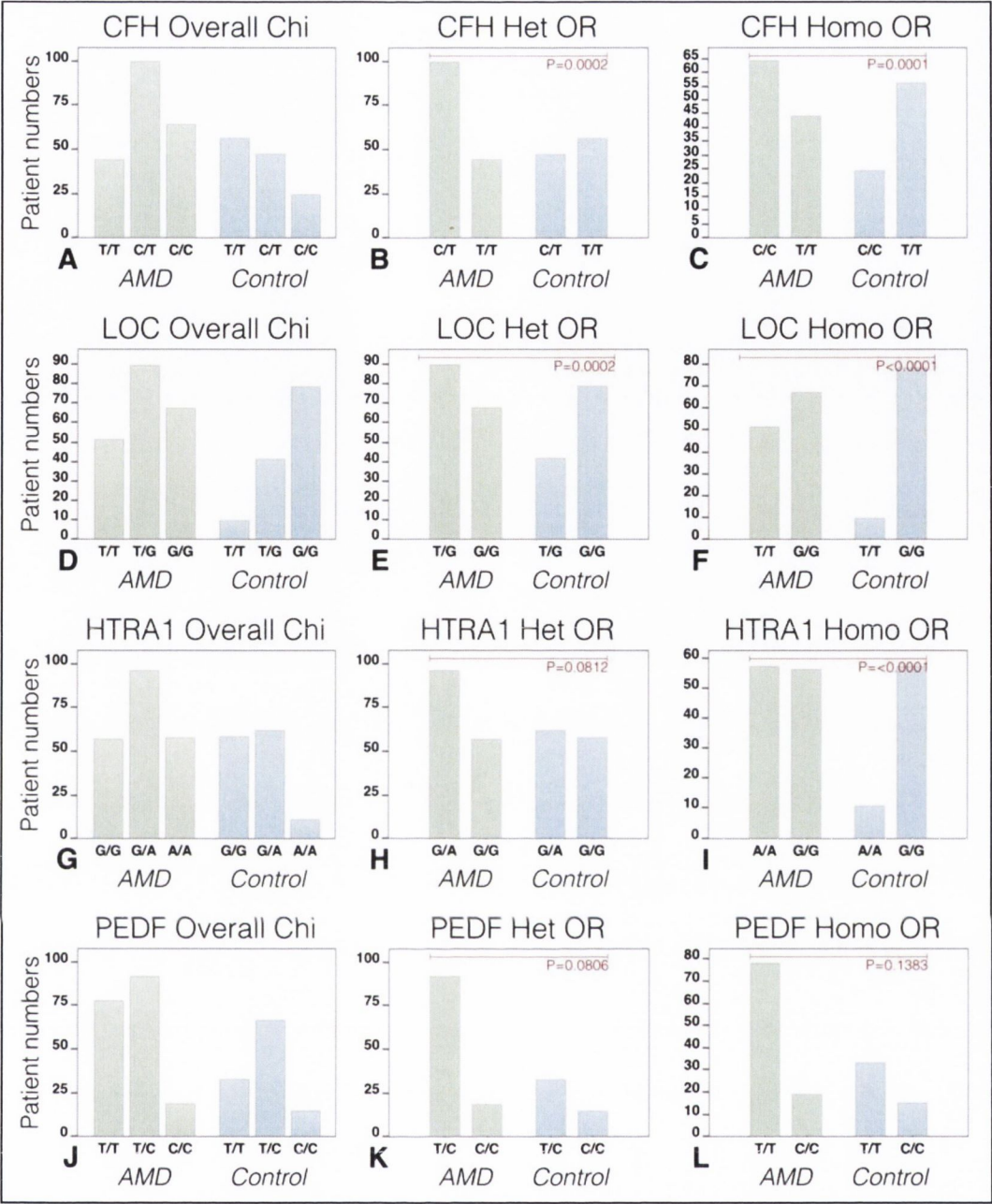


Figure 5.6.12: Genotype distribution in the Irish Age-related Macular Degeneration Cohort

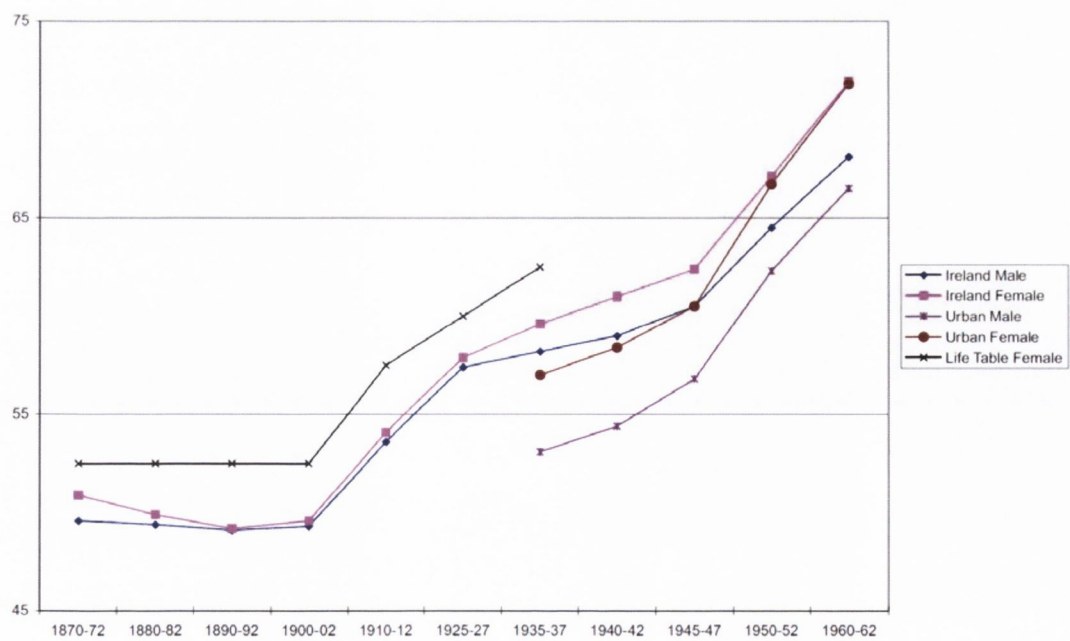


Figure 5.6.13: Life expectancy in Ireland 1870-1960

Chapter 6: 6. Statistical analysis

In vitro studies

The statistical analysis of the *in vitro* findings was performed using GraphPad Prism™ (V4.0b). This analysis included statistical comparison of the BrdU, MTS, bFGF, VEGF and PEDF ELISA assessments. The mean of each result was compared using a one-way analysis of variance (ANOVA) with a Tukey post-test. Data were displayed as mean changes relative to control $\pm$ the standard error of the mean (SEM). Statistical significance was assumed where $p < 0.05$ (*). P values of fewer than 0.01 and 0.001 are denoted by two asterisks (**) and three asterisks (***) respectively.

In vivo studies

Cotinine ELISA studies were analysed in a manner similar to the *in vitro* ELISA assessments as described above. Analysis of the SOD1-/- ERGs were performed in one of two ways. Control and CSE ERGs were compared using an unpaired Student's T test with 9 degrees of freedom. In these ERG assessments the pre treatment ERGs were also used as controls. In this manner a paired pre and post CSE analysis was performed. Despite the fewer degrees of freedom (5dof) the pairing of pre and post treatment ERGs is a more appropriate method of blocking, chosen because it allows each animal to be its own control. Laser-induced CNV is a well-established experimental technique that produces consistent regions of blood vessel growth in the eye. The influence of cigarette smoke was assessed by comparing the volume of CNV growth in smoke-exposed eyes to non-exposed eyes. A parametric one-way ANOVA statistical analysis will be performed on the three sample groups. The mean volume of choroidal neovascularisation induced in the C57LB/6J is 20,400 μ m per laser burn. The approximate standard error of the mean is 1000 μ m. An increase or decrease in CNV volume of over 10% was considered biologically significant. Predicted animal numbers ($\alpha = 0.05$, $\beta = 0.1$, Power = 0.9) Mean = 20400 SD = 1000 D = Difference of 10%, $n = 6$ per group. The daily dosing protocol will need to be changed if intraperitoneal injection is not successful. An increased dose at a longer interval is not as accurate but would be required if the intravascular approach was used.

Population studies

Demographic information was assessed by comparing an affected AMD cohort with an age-matched control group. Where individual characteristics were numerical rather than proportional's (e.g. Age, BMI, cigarette pack year history, number of brothers or sisters), normal distribution was assumed and values were compared using an unpaired two-tailed Student's T test with a 95% confidence interval. Where risk factors were represented as proportions, the difference in the prevalence of the risk factor was assessed using Fisher's exact test, which is more accurate in smaller sample sizes than the Chi square assessment. If the risk factor attained statistical significance (95%CI), odds ratio (OR) and relative risk (RR) statistics were subsequently calculated.

Genotype frequency differences between patient and control groups were compared using the Fisher's exact test for the comparison of proportions and Chi square analysis of proportions.

Chapter 7: Conclusions

7.1. Summary of results

The *in vitro* studies presented describe three baseline genetic risk factors associated with AMD in a frequently used cell line. Here we present the first report of the effects of acute cigarette smoke exposure on an aged post confluent, cell line. Unlike previous studies, the cells did not die in response to the toxic insult. At low concentrations, normal cell mechanisms accommodated to the insult with no evidence of any stress. At higher concentrations, cells displayed a transient stress response. This corresponded to an increase in the release of bFGF and suppression in VEGF. This effect was transient as the cells returned to a static level within 48 hours. The cigarette smoke exposure experiments describe the earliest observed changes in the protein expression of the ARPE-19 cell line.

The *in vivo* studies focused on a later stage of disease manifestation. The development of CNV is a hallmark of late disease. We have established a model of cigarette smoke exposure in the mouse that more cost effective and with less operator exposure than the standard established methods. Absorption of the intraperitoneal cigarette smoke extract was confirmed by detection of urinary cotinine. We propose that the biomolecular pathways responsible for the toxic effects of CSE in AMD are dependent on the stage of disease pathology. Early damage may not be due to oxidative stress however as damage accumulates, cells undergo apoptosis and the stimulus for the release of VEGF is increased. The development of CNV requires VEGF but it is not VEGF alone that is responsible for the neovascularisation.

The population-based studies describe the risk associations that have been associated with AMD in other Caucasian populations. The Irish population was found to be similar to other Caucasian populations. Patients who were active cigarette smokers were significantly associated with AMD. It was also noted that patients who had significant smoking histories were at an increased risk. A history of hypertension was the only other highly significant risk factor isolated. There was no association found with diabetes, steroid use or hypercholesterolaemia. The genotype analysis

found an association between the CFH, the LOC387715 and the HTRA1 SNPs in the Irish population. There was no contribution detected from the PEDF SNP assessed.

7.2. Future directions

The work we have described provides significant scope for further investigation. Further assessment of the effects of cigarette smoke toxicity on the retinal pigment epithelium is required. There is a point at which the defense mechanisms of the RPE will no longer maintain viability in response to CSE. When the cells capacity of remove the toxic insult it overwhelmed, intracellular damage accumulates. When intracellular damage becomes significant and irreversible, the cell becomes committed to the apoptotic pathways. The signals governing this threshold, determine the point at which sub lethal injury becomes irreversible and would contribute greatly not only to the knowledge of cigarette smoke induced changes in AMD but may be potentially applicable to all AMD.

7.2.1. Optimized treatment for smokers

We suspect that cigarette smokers, in the absence of the ability to quit smoking may need to be assessed differently to non-smokers. Smokers with CNV are more likely to have a worse clinical visual outcome. Active smokers with CNV should be followed up in a long-term study to determine an optimal treatment protocol of anti-VEGF medications. These patients probably need more frequent injections than non-smokers.

7.2.2. Correlation of genotype with outcome

Currently there is no role for routine genotyping of all patients with AMD. Genetic risk factors have not been correlated to a different visual outcome in neovascular AMD therefore knowledge of genetic risk factors is not of clinical significant. There is no proven preventative measure or early intervention for AMD therefore there is no current role for routine genotyping in screening. It has been suggested that knowledge of genotype may provide additional motivation to stop smoking.

7.2.3. Potential gene therapies in AMD

Targeted gene therapy represents a new treatment modality in medicine. The first successes in the area were in the field of Leber's Amaurosis indicating that genetic based treatments in the 21st

century may revolutionize medicine like antibiotics in the 20th century. A genetic treatment of the underlying condition holds the promise of a cure rather than treatment of AMD.

7.2.4. Multi target antibody treatment

There is also significant potential in targeting multiple vasoactive mediators of angiogenesis. It has been hypothesized that increased VEGF expression is a late event in AMD. This is consistent with the late AMD presentation of CNV. By the time that CNV develops in an eye there has already been significant structural damage to the RPE and Bruch's membrane. Even complete regression of the pathological membranes will not likely result in full restoration of vision. In this work we provide evidence for protein expression prior to frank neovascular AMD. It is possible that earlier intervention to intercept the biological pathway at this stage would be of greater benefit in AMD.

The specific targeting of a single mediator of CNV will only treat some aspects of the pathology. Further benefit may be derived from the use of multiple agents that target different components of the inflammatory cascade. Combination therapy with both anti-VEGF and photodynamic therapy is beneficial in the treatment of AMD.³¹⁸ Photodynamic therapy is a non-specific vascular coagulative therapy that causes localized tissue destruction. Molecular treatments administered intravitreally are not locally destructive. The optimal treatment regimen would be one that allowed a single dosing regimen of a non-destructive modality. Modern biological interventions represent a more targeted approach to treatment. An earlier target in the molecular pathway that occurs in AMD could provide a more effective point of intervention. In our reported *in vitro* work we have discovered an early rise in bFGF that occurs prior to significant cellular morphological damage. The combination of bFGF and VEGF has shown to be of additional benefit *in vivo*.

These findings provide *in vitro* support for the direct influence of cigarette smoke in the initiation of a stress response, which may contribute to AMD. In this *in vivo* study we examine the effects of cigarette smoke on a later event in AMD. Our results indicate that once the endothelial cells begin proliferation in the formation of CNV, cigarette smoke exposure facilitates the development of larger volume CNV responses. This would correlate to more advanced disease in the human eye.

A full understanding of these pathways provides greater targets for interventional therapeutics at more specific, earlier target sites in the disease's process. The future of clinical genetics may include personalized treatment regimens in response to patient genotypes. Research is ongoing into whether the known genetic risk factors for the development of AMD also determines the progression of the disease and influences the response to anti-VEGF treatment. The effect of genotype on treatment response may be significant. It may transpire that pre-treatment work up for intravitreal injections will require genotyping that may require different injection regimens. A greater understanding of the subtle differences and contribution of genetic and environmental risk factors will improve understanding of this common disease and ultimately preserve vision and improve quality of life.

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Appendix 1: SOD1-/- pathology report

Appendix 2: Consent form for AMD study

Appendix 3: Patient questionnaire

Pathologist:
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UNIVERSITY VETERINARY HOSPITAL

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Report to:

Mr. Peter Nowlan Mvb, Msc, Certlas, Mrcvs
Bioresource Unit
Trinity College Dublin
Dublin 2

Owner Name: Peter Nowlan MVB, MSc
Animal Name: sodi -1-
Case No: 63006
Species: mouse
Breed:
Sex:
Colour:
Date of Birth:

Admitting V.S.:

Date Received 14/4/2009

Pathology Report 14902

Visit Number 89438

Referring V.S.:

BioResource Unit

Trinity College Dublin
Dublin 2

Block Number: N145/09

N145/09

This mouse was submitted fixed whole in 10% formalin. On external examination multifocal large areas of ulceration with granulation tissue evident on the head, around the periorbital areas and on the dorsal aspect of the neck.

Histopathologically, the epidermis exhibited multifocal areas of acanthosis and spongiosis with variable orthokeratotic hyperkeratosis. There were multifocal to coalescing, areas of ulceration covered by cellular debris, dead and dying neutrophils and occasionally bacterial cocci. Subjacent to ulcerated areas the superficial dermis was thickened by densely cellular neutrophil rich infiltrate within areas of fibroplasia and angiogenesis (granulation tissue). Throughout all sections there were multifocal dilated hair follicles with no hair shafts and often containing keratin. There were also multifocal microabscesses within the dermis in sebaceous glands and hair follicles. Diffusely there was thickening of the superficial dermis subjacent to the intact epidermis by fibrous tissue (dermal fibroplasia). Superficial dermis also had mast cells infiltrates, occasional multinucleated giant cells and diffuse melanin incontinence.

The main lesions identified in this animal are

MULTIFOCAL EPIDERMAL ULCERATION with GRANULATION TISSUE
DIFFUSE DERMAL FIBROPLASIA
MULTIFOCAL DERMAL MICROABSCESSES

The lesions outlined above involve all areas and elements of the skin, they are chronic persistent changes of an inflammatory nature. Given the overall picture it is unlikely that the changes are primarily due to the occasional colonies of cocci seen,

Appendix 2. Consent form for AMD study

ROYAL VICTORIA EYE AND EAR HOSPITAL

Adelaide Road

Dublin 2

CONSENT BY SUBJECT OF RESEARCH

INVESTIGATION/EXPERIMENT/CLINICAL TRIAL

The Frequency of Gene Polymorphisms (variations) in Age-related Macular Degeneration in an Irish Population

I, _____ of _____
hereby consent to participate in the research investigation entitled above to be carried out

1. I confirm that the consent which I am giving is voluntary and I have not been subjected to any force, constraint, coercion, duress or any inducement whatsoever which has led me to give this consent.
2. The nature, purpose, duration, method, means, inconveniences and hazards reasonably to be expected, as well as the possible effects upon my health or person arising out of my participation have been fully explained to me by _____ and accordingly I understand that I am making a fully informed decision to consent.
3. I confirm that I have full legal capacity to give consent in that I am not a minor under 18 years of age, my mental capacity is not impaired in any way and I am not suffering from any learning disability, mental illness or other illness whatsoever.
4. I confirm that I have been given a reasonable time to consider the

information provided to me in connection with this consent and I am giving this consent having given careful consideration to the information with which I have been provided.

5. I confirm that my language is English and I fully understand the contents of this consent form.

or

_____ Translator (here relevant)

I confirm that I attended when the medical practitioner explained to the Subject what is involved in the research investigation/experiment/clinical trial and that the Subject indicated that he/she fully understood the information provided to him/her by the medical practitioner.
by my signature I am witnessing the valid, informed and voluntary consent of the Subject.

Date:

Signed: _____

Independent Witness

Appendix 3. Patient AMD questionnaire

Macular Degeneration Study
Participant Questionnaire

Date of Questionnaire

Personal Information

Surname		First Name	
Address			
County		Telephone	
Gender		Height	
		Weight	

Family History

Father has/had Macular Degeneration? Yes/No		Mother has/had Macular Degeneration? Yes/No	
Number of brothers (living or deceased)		Number of sisters (living or deceased)	
Number of brothers with Macular Degeneration?		Number of sisters with Macular Degeneration?	

General Health History

Do you smoke (cigarettes or pipe) now? Yes/No If you smoke now, how many cigarettes or ounces of tobacco per day?

If you smoked in the past but have stopped, please answer the following questions:

How many years since you smoked? How many cigarettes or ounces of tobacco did you smoke per day?

Are you being treated for any of the following conditions?

High Blood Pressure? Yes/No		High Cholesterol? Yes/No		Diabetes? Yes/No	
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Are you taking any of the following medications?

Steroids? Yes/No		Warfarin? Yes/No		Low dose Aspirin? Yes/No		Statins? Yes/No	
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Have you suffered from any of the following?

Heart Attack? Yes/No		Stroke? Yes/No	
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Thank you for completing this questionnaire.
The information you give is completely confidential.

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