UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical Neurosciences

Age-related Macular Degeneration, Complement Factor H and Liver Transplantation

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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Samir Khandhadia

The purpose of this study was to investigate whether modification of liver complement factor H (CFH) production, by alteration of liver *CFH* Y402H genotype through liver transplantation (LT), influences the development of Age-Related Macular Degeneration (AMD).

223 Western European patients at least 55 years old, who had undergone LT at least 5 years previously, were recruited. AMD status was determined using a standard grading system. Recipient *CFH* Y402H genotype was obtained from DNA extracted from recipient blood samples. Donor *CFH* Y402H genotype was inferred from recipient plasma CFH Y402 and H402 protein levels, measured using enzyme-linked immunosorbent assays (ELISA). This approach was verified by genotyping donor tissue from a subgroup of patients. Systemic complement activity was ascertained by measuring levels of plasma complement proteins using ELISA, including substrates (C3, C4), activation products (C3a, C4a and terminal complement complex) and regulators (total CFH, C1 inhibitor).

AMD in LT patients was associated with recipient *CFH* Y402H genotype ($p=0.036$, odds ratio (OR) 1.6, 95% CI: 1.0-2.4) but not with donor *CFH* Y402H genotype ($p=0.626$), after controlling for confounders including age, gender, smoking status, and body mass index. Recipient plasma CFH Y402 and H402 protein levels predicted donor *CFH* Y402H genotype with 100% accuracy ($n=49$). Plasma complement protein levels were similar in LT patients with and without AMD. On controlling for confounders, decreased plasma C4a levels were associated with AMD. Compared to previously reported prevalence figures (Rotterdam Study), LT patients demonstrated a high prevalence of both AMD (64.6% vs 37.1%, OR 3.09, $p<0.001$) and the *CFH* Y402H sequence variation (41.9% vs 36.2%, OR 1.27, $p=0.014$).

Presence of AMD was not associated with modification of hepatic CFH production, and was not associated with increased systemic complement activity in LT patients. These findings suggest local intraocular complement activity is of greater importance in AMD pathogenesis. The high prevalence of AMD in LT patients may be related to the high prevalence of the *CFH* Y402H sequence variation. LT patients should undergo regular optometrist monitoring for AMD.

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Declaration of authorship

I, Samir Khandhadia declare that the thesis entitled "Age-related macular degeneration, complement factor H and liver transplantation" and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work (see appendix) have been published as:

1. Khandhadia S, Hakobyan S, Heng LZ, Gibson J, Adams DH, Alexander GJ et al. Age-related Macular Degeneration and Modification of Systemic Complement Factor H Production Through Liver Transplantation. *Ophthalmology* 2013.
2. Khandhadia S, Cipriani V, Yates JR, Lotery AJ. Age-related macular degeneration and the complement system. *Immunobiology* 2012;**217**:127-46.
3. Khandhadia S, Lotery AJ. Prevalence Of AMD In A Liver Transplant Cohort. *ARVO Meeting Abstracts* 2011;**52**:1233.

Signed: ……..

Date:……

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Commonly used abbreviations

AMD	Age-related macular degeneration
AREDS	Age-Related Eye Disease Study
BM	Bruch's Membrane
CFB	Complement Factor B
CFD	Complement Factor D
CFH	Complement Factor H
CFI	Complement Factor I
CNV	Choroidal Neovascularisation
CNVM	Choroidal Neovascular Membrane
EDTA	EthyleneDiamineTetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assays
FFA	Fundus Fluorescein Angiogram
FFPE	Formalin-Fixed And Paraffin-Embedded
GA	Geographic Atrophy
HH402	"High risk" homozygous CFH Y402H genotype (alleles CC) - associated with increased AMD risk
KASP	KBioscience competitive Allele-Specific PCR
LT	Liver transplant
MAC	Membrane-attack complex
MAF	Minor allele frequency
OCT	Optical Coherence Tomogram
OD	Optical Density
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Principal Investigator
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
rpm	Revolutions per minute
rs	Reference SNP
SNP	Single Nucleotide Polymorphism
Taq	Thermus Aquaticus
TCC	Terminal Complement Complex

U/L	Units per litre
VEGF	Vascular Endothelial Growth Factor
YH402	Heterozygous CFH Y402H genotype (alleles CT or TC)
YY402	“Low risk” homozygous CFH Y402H (alleles TT) – associated with low AMD risk

CHAPTER 1 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world.[1] Although new treatments can treat some forms of advanced AMD, there is still no satisfactory way of preventing AMD.

AMD is a complex multifactorial disease, associated with a range of environmental and genetic risk factors.[2] The complement system in particular appears to play a key role in the pathogenesis of AMD. Sequence variation in several genes coding for complement proteins have been associated with AMD,[3] including the complement factor H (*CFH)* gene.[4-6]

The liver is the primary producer of circulating complement proteins, including CFH.[7;8] However CFH protein is also produced in the eye at considerable levels.[9] What is not known is whether intraocular or systemic CFH, or indeed the complement system in general is more important in AMD pathogenesis. Furthermore this is important since complement inhibitors are being developed commercially, and as yet there is no supporting evidence on whether intraocular or systemic complement inhibition would be more effective.

The research question to be answered in this thesis is whether systemic or local intraocular CFH production is more important in AMD pathogenesis.

The introduction includes background information and review of the current literature relevant to this thesis. First a brief overview of genetics is presented, followed by a description of AMD, including a literature review of the current theories of AMD pathogenesis.

The complement system, in particular, the structure and function of CFH are then described. This is followed by a literature review of the associations of the complement system, in particular the *CFH* gene Y402H sequence variation, with AMD. The evidence of intraocular and systemic CFH production and the importance of each to AMD are discussed, followed by the possible role of liver transplantation in modifying systemic CFH production. This leads onto the aims, hypothesis and objectives of this thesis. The introduction ends with a brief outline of the structure of the rest of the thesis.

1.1 Genetics

DNA structure and genetic epidemiology are briefly discussed in this section as background information to supplement the rest of the thesis.

Reference used for this section: Strachan T. Human Molecular Genetics. 4th ed., 2011[10]

1.1.1 Structure of DNA

All nuclei of cells contain DeoxyriboNucleic Acid, or DNA. This forms a template for protein production for the entire body. DNA is composed of two polymer chains (i.e. is double-stranded). Each strand contains a sequence of nucleotides.

A nucleotide contains a five carbon sugar (deoxyribose), with a phosphate group attached to carbon atom 5', and a nitrogenous base attached to carbon atom 1' (Figure 1.1). Successive nucleotides on a single DNA strand are linked together by the phosphate group, through "phosphodiester" bonds to carbon atoms number 3'and 5' on successive carbon sugars. This creates a sugar-phosphate backbone (Figure 1.2). The nitrogenous base contains carbon and nitrogen atoms, and includes the purines (Adenine – A, and Guanine – G), and the pyramidines (Cytosine – C, and Thymine – T). The term "nucleoside" refers to the sugar with its attached base. The term "nucleotide" refers to the sugar plus base plus the phosphate group. A nucleotide is the basic repeat unit of DNA. The various names of the bases, nucleosides and nucleotides are given in Table 1.1.

Figure 1.1: Structure of a single nucleotide

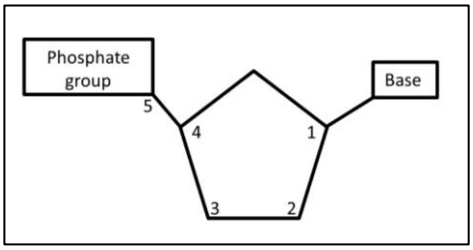


Diagram showing the structure of a single nucleotide within a DNA strand. A nucleotide contains a five carbon sugar (deoxyribose). A base is attached to carbon atom 1', and a phosphate group to carbon atom 5'. Drawn based on information obtained from Strachan T. Human Molecular Genetics, 2011.[10]

Figure 1.2: Linking of nucleotides on DNA strand

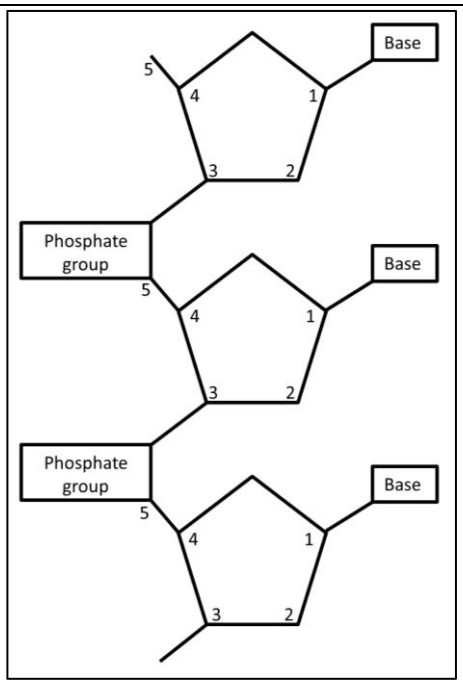


Diagram showing linking of successive nucleotides on a single DNA strand by a phosphate group between the 3' and 5' carbon atoms on successive carbon sugars (the "phosphodiester bond"). Drawn based on information obtained from Strachan T. Human Molecular Genetics, 2011.[10]

Table 1.1: Nucleosides versus nucleotides

BASE CATEGORY	BASE	DNA UNIT	
		Nucleoside = Base + sugar (deoxyribose)	Nucleotide = Base + sugar (deoxyribose) + phosphate
PURINES	Adenine	Deoxyadenosine	dAMP (Deoxyadenosine monophosphate)
	Guanine	Deoxyguanosine	dGMP
PYRIMIDINES	Cytosine	Deoxycytidine	dCMP
	Thymine	Deoxythymidine	dTMP

Table showing nucleoside and nucleotide nomenclature

Double DNA strands form as individual base pairs from each strand bind to complementary bases on the opposing strand. The base pairs bind via weak hydrogen bonds, according to the Watson-Crick rules: A – T (U in RNA), and C – G (Figure 1.3). The phosphodiester bond which forms between carbon atoms number 5' and 3' of successive sugar residues also denote the two ends of a DNA strand. At the 5'end of a DNA strand, carbon atom number 5' on the terminal sugar residue is free. At the 3' end, carbon atom number 3' is free. This creates two antiparallel strands – the strands bond together in opposite directions. One strand is in the direction of 5' – 3', the other 3'– 5'.The individual strands are said to be "complementary" to each other. The association of two complimentary DNA strands with one another is known as "annealing". The 5' – 3' direction is also the direction of DNA replication and RNA transcription. The two polymer chains are wrapped around each other to form a double helix.

Figure 1.3: Double stranded DNA formed by hydrogen bonds between complementary base pairs

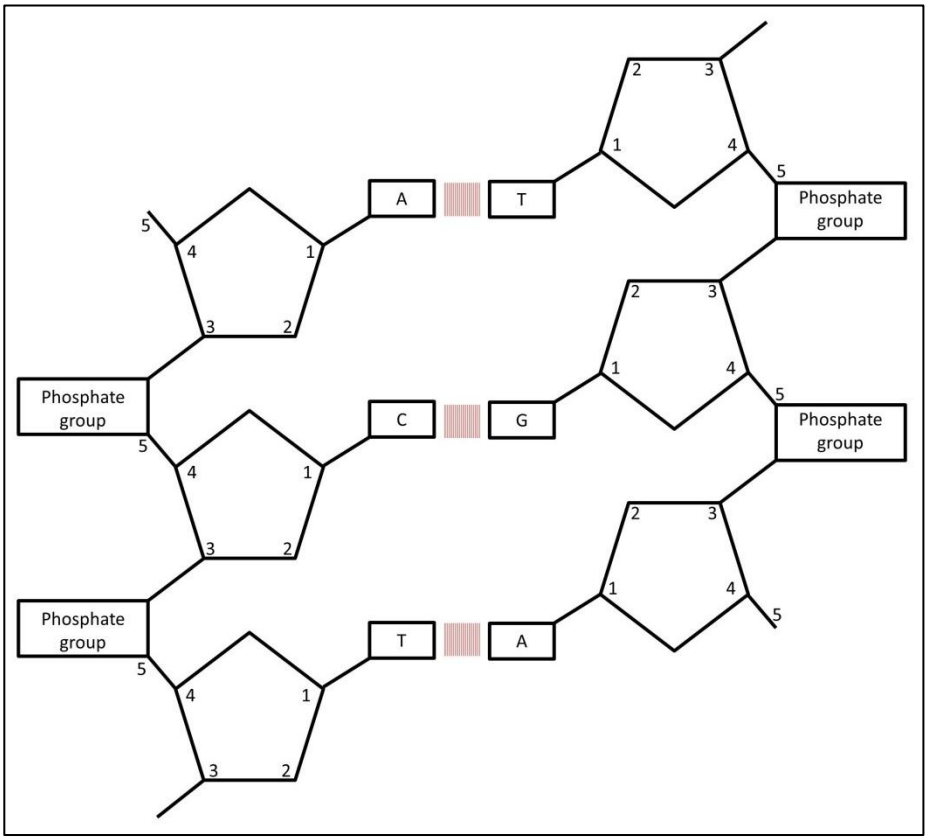


Diagram showing bonding between 2 complementary DNA strands. Hydrogen bonds (in pink) form between complementary bases, according to the Watson-Crick rules. Drawn based on information obtained from Strachan T. Human Molecular Genetics, 2011.[10]

1.1.2 Genetic epidemiology

Some definitions of terms used in this thesis are given below, followed by a discussion of genetic variation.

Locus / allele / genotype:
A locus is a unique chromosomal location defining the position of an individual gene or DNA sequence. Alleles are alternative versions of a gene. Genotype is a list of alleles present at one or a number of loci. Humans have two copies of each chromosome and correspondingly two copies of each gene side by side. A person

is homozygous if both alleles at a particular locus are identical, and heterozygous if alleles are different.

Phenotype / Mendelian / multifactorial inheritance:
The phenotype (or character / trait) are the observable properties of an individual organism. A trait is “dominant” if it is apparent in a heterozygous individual, and is “recessive” if not. A trait is Mendelian if its pattern of inheritance suggests it is caused by a variation at a single locus. Such monogenetic traits include ABO blood grouping, governed by A, B or O alleles at a single locus. Most human traits are not Mendelian, but are multifactorial. Although DNA sequence variations are inherited in a simple Mendelian pattern, manifestation of a particular phenotype usually involves a complex balance of factors. These include contribution of multiple gene loci (polygenic), post-translational protein modification, unique protein interaction within a particular cellular environment, and environmental factors. Complex multifactorial traits include birth defects (eg. cleft palate, spina bifida), IQ levels, and development of AMD.

Haplotype:
During meiotic cell division, pairs of homologous chromosomes line up to allow exchange of genetic material. Equivalent chromosomal segments are exchanged, therefore there is no net increase or decrease in genetic material. Several alleles on a particular chromosomal segment which are transmitted together are known as a haplotype. A particular haplotype can be used to track a particular chromosomal segment through populations.

Linkage disequilibrium:
2 alleles at separate loci which are statistically associated with each other are said to be in linkage disequilibrium; such alleles are usually within an ancestral haplotype.

Minor allele frequency:
The prevalence of a particular genotype in a population is usually expressed as the “minor allele frequency” (MAF). This is the frequency of the less common (minor) allele.

1.1.3 Genetic variations

A spectrum of genetic variations can occur, from the level of the chromosome to individual nucleotides. Chromosomal abnormalities are traditionally defined as those which are visible (involving up to 4 million bases of DNA), although newer imaging techniques enable changes at a molecular level to be detected. Numerical and structural chromosomal abnormalities can exist.

The DNA sequence can also vary at the nucleotide level from one individual to another in several ways. More than six billion nucleotides are present in a single human cell. This genetic sequence is remarkably similar from one person to another (99.9%). However 1 in 300 nucleotides are different, and form the most common type of genetic variation (a "point mutation", or single nucleotide polymorphism - SNP). SNPs are defined as occurring in more than 1% of the population[11]. There are about ten million SNPs present in the human genome, representing most of the inter-individual genetic variation. Most of these have been identified by the HapMap project.[11;12] These variations account for the individual differences in physical characteristics (phenotype). Some variations lead to disease, and are referred to as genetic mutations. SNPs can be present within exons or introns. Those within exons may lead to a change in amino acid, and are referred to as "non-synonymous" coding SNPs, which in turn may cause a functional variation in the translated protein. Non-synonymous coding SNPs can be either "missense" (change in amino acid to another one) or "nonsense "(change in amino acid to a stop codon, leading to a shortened protein). An exonic SNP, however, may not cause any change in amino acid, and this is known as a "synonymous" coding SNP. SNPs are referred to by "rs" (reference SNP) numbers, which are allocated by the National Centre of Biotechnology Information SNP database ("dbSNP", www.ncbi.nlm.nih.gov/snp).

A simple association between a non-synonymous SNP in a particular gene and a specific disease does not necessarily implicate the gene in the pathogenesis of the disease. However such associations do warrant further exploration with functional cell/animal/human based studies, to investigate first of all how the affected protein's function may change with a non-synonymous SNP, and secondly how this alteration in function may be implicated in the pathogenesis of the disease in question. Many associations between SNPs and diseases have been

discovered, with ever more efficient genotyping (such as next generation sequencing), and the use of large collaborative multicentre case-control populations. However there is often a lack of full understanding of the actual role of an implicated gene in the pathogenesis of a particular disease. For example, a SNP in the *ARMS2* gene is often reported as the most strongly associated SNP with AMD. Although the ARMS2 protein has been localised to mitochondria, the exact function of this gene and its role in AMD pathogenesis is still not clear.[13]

SNPs associated with a particular disease can also act as disease markers in future studies. Furthermore, certain SNPs may always be inherited together with other particular sequence variations in more distal genes (in linkage disequilibrium), and exploration of these distal genes may also provide clues to the pathogenesis to a particular disease.[14]

Other DNA variations can also occur. “Indels” are small insertion/deletions. Variable repeats are sequences of nucleotides which are repeated several times, also called “microsatellites”. If repeated many thousands of times, these are called “variable number tandem repeats”.

1.1.4 DNA replication

The purpose of DNA replication is to form two daughter DNA helices, in order to enable duplication of the parent cell during mitosis (cell division). DNA replication begins by the unwinding of the two strands of DNA of each chromosome. Each DNA strand acts as template for a complementary DNA strand, formed by the enzyme DNA polymerase. Two daughter DNA duplexes are formed, each identical to the parent; one strand is from the parent, the other is newly formed. This results in a Y-shaped “replication fork”. However since DNA replication only occurs in the 5’ – 3’ direction of the original DNA strand, only one strand (the “leading” stand) is synthesised continuously. Replication of the other strand (the “lagging” strand) will occur in sections, only when enough DNA has unwound, resulting in “Okazaki” fragments. These short fragments are eventually joined up through the action of the enzyme DNA ligase.

DNA polymerase adds a nucleotide (deoxyNucleotide MonoPhosphate - dNMP) complementary to the DNA template, according to the Watson-Crick rules, and

then moves on to the next nucleotide. Nucleotides are obtained from a pool of dNTP (triphosphates - two extra phosphate groups are cleaved off before use). DNA polymerase can only begin replication if a primer is present. This is a short sequence of a base-paired RNA strand with a 3-OH group (i.e. a hydroxyl group on carbon atom number 3). One is required for each strand (i.e. the forward and reverse primers). Primers are denoted conventionally in the 5'-3' direction.

1.2 Age-related macular degeneration

Age-related macular degeneration (AMD), as its name indicates, is an age-related condition causing degeneration of the macula, the centre of the retina. AMD has a considerable impact on patient quality of life, despite recent advances in treatment. AMD is the leading cause of blindness in the developed world, accounting for 50% of all blindness in Europe, USA and Australia.[1] Visual impairment from AMD can also cause considerable morbidity.[15] This significant impact on quality of life makes AMD an important disease to study.

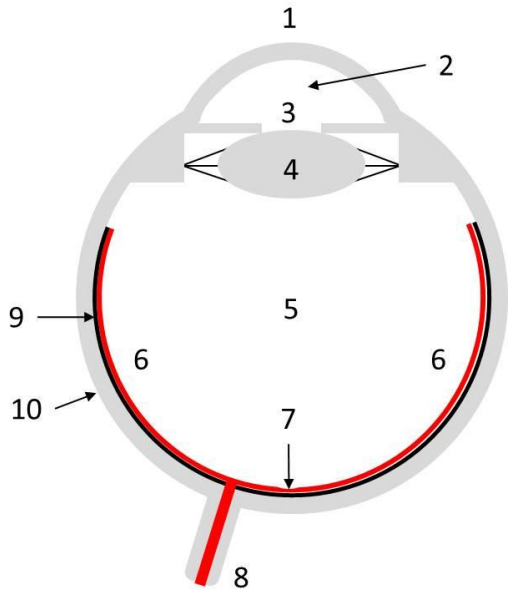
1.2.1 Epidemiology

The prevalence in adults of all grades of AMD is around 3%[16], and increases with age - almost two thirds of the population over 80 years old have some signs of AMD.[17;18] AMD is more common in white populations compared to other ethnic groups.[19] Some studies suggest women may be more susceptible to AMD.[20]

1.2.2 Normal retinal anatomy

The macula is an area at the centre of the retina which receives the main focus of incoming light, and is responsible for central vision and fine detail such as that required for reading. The retina forms the innermost of the three layers constituting the wall of the eyeball (Figure 1.4).

Figure 1.4: Cross-section of the eyeball



1: The cornea – the clear window at the front of the eye. It's main role is to focus light onto the macula

2: Anterior chamber – a small chamber at the front of the eye just behind the cornea, containing clear aqueous fluid

3: Iris – a concentric muscle with a hole in its middle (pupil) – regulates the amount of light entering eye

4. Lens – clear crystalline structure required for fine-tuning focusing

5. Vitreous – central cavity in the eyeball containing clear jelly-like substance

6. Retina (in red) – a thin layer of nerve tissue at the back of the eye, containing photoreceptors.

7. Macula – the centre of the retina upon which incoming light is focused by the optics of the eye (see Figure 1.5)

8. The optic nerve – light signals pass from the retina posteriorly to the brain via the optic nerve, which is surrounded by the optic sheath

9. The choroid – external to the retina is the vascular choroid (in black) which supplies the retina with nutrients and oxygen, whilst removing toxic waste products. A modified basement membrane (Bruch's membrane) lies between the choroid and retina.

10. The outermost wall of the eye consists of the tough white fibrous sclera, which acts to protect the eye and maintain its shape.

Diagram drawn based on information from Clinical Anatomy Of the Eye, Snell 1998.[21]

1.2.3 Clinical anatomy

Figure 1.5 illustrates the structures seen on clinical examination of the back of the eye (fundus). The optic disc is a pink-yellow circular structure, which acts as a landmark during retinal examination. The optic disc represents the beginning of the optic nerve. The retinal arteries emanate from the centre of the optic disc, then branch out in four diagonal directions to end in capillaries. These then drain into retinal veins that also form four branches running alongside the retinal arteries. These leave the eye through the centre of the optic disc. The centre of the retina is the macula, identifiable as an area about 3 mm in diameter, temporal to the optic disc. The temporal retinal artery branches arc around the macula (the 'arcades').[22] The centre of the macula is the fovea. This is the point on which light rays from the external sources are focussed by the eye's optical system. The fovea appears darker clinically than the surrounding retina, since pigment levels are higher here.

Figure 1.5: A normal fundus

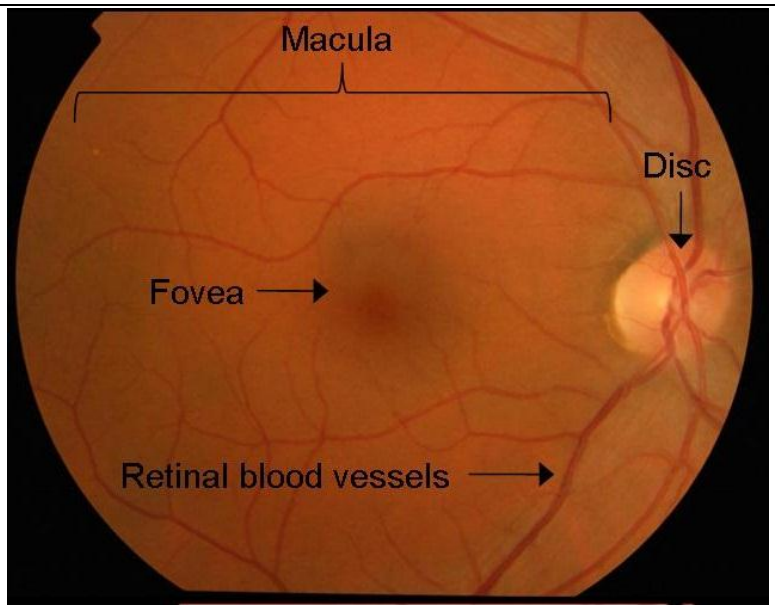
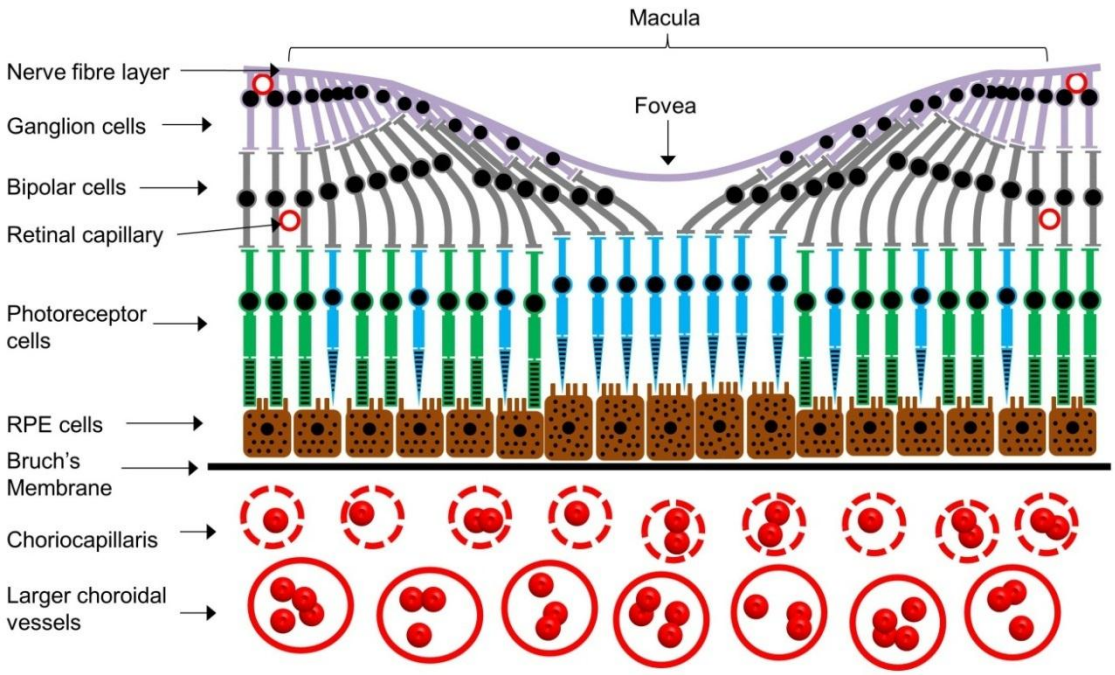


Image obtained from Southampton Eye Unit database.

1.2.4 Microstructure and ultrastructure of the retina

The retina consists of two layers, the inner neurosensory retina, and the outer retinal pigment epithelium (RPE) (Figure 1.6). The neurosensory retina contains photoreceptor cells (cones and rods), which detect incoming light and convert this to neuronal signals. These signals are then relayed for processing, through bipolar and ganglion cells in the neurosensory retina, to the optic nerve. Signals then pass to the visual cortex, located in the posteriorly situated occipital cortex. The RPE layer, despite being only one cell thick, performs important supporting functions, including supporting the photoreceptor layer, recycling of outer segment discs, and maintenance of the blood-retinal barrier.[22;23]

Figure 1.6: Structure of macula



Sandwiched between the RPE and choroid, lies Bruch's membrane (BM). This consists of collagen, with a central layer of elastin fibres. The choroid itself sits just external to BM. It is a highly vascular tissue and consists of an inner capillary layer, the choriocapillaris and an outer large vessel layer. The capillaries within the choriocapillaris are fenestrated, allowing free passage of substances up to a certain size between the intra-capillary and the extravascular space. The retina has a dual blood supply. The inner retina is supplied by its own vascular system, situated within the neurosensory retina. The choroid supplies the outer retina, via passive diffusion and active transport through BM and the RPE layer. Tight junctions between the RPE cells restrict movement and constitute the outer blood-retinal barrier. These barriers are important to ensure regulation of transport of fluids, nutrients and waste products. BM also acts as a physical barrier to restrict abnormal neovascularisation from the choroid (see below). The endothelial cells of this vascular system, together with tight junctions, compose the inner blood-retinal barrier, corresponding to the blood brain barrier.[22] Image drawn by author, reprinted with permission from: Khandhadia and Lotery (2010)[24]

1.2.5 AMD – classification

Several attempts have been made over the years to create a standardised classification system for both clinical and research use. Recognising the lack of consistency of AMD classification used in studies and in clinical practice, the International Classification and Grading System study group in 1995 suggested a more standardised, but rather complex, grading system for AMD.[25] This has been simplified in later studies, such as the Rotterdam and Eureye Studies.[26] [27]

The Age-related Eye Disease Study (AREDS) research group modified the classification system to enable reliable detection of change indicating progression to advanced AMD (one of the main study outcomes).[26] Differences of grading definitions do exist between the International Classification and AREDS system. Recently a further report aiming to standardise all AMD classification in a more clinically useful manner has been suggested by the Beckman Initiative for Macular Research Classification Committee.[28]

All classification systems generally agree on several aspects. The macula is defined as an area delineated by a circle 2 disc diameters from the fovea, abutting on the temporal edge of the disc. Early AMD is characterised by drusen and/or pigmentary changes (Figure 1.7a-c). Drusen, containing a multitude of proteins and lipids, result from accumulation of extracellular material between the RPE layer and BM. The exact reasons for the formation of drusen and their role in AMD pathogenesis are as yet not clear.[29] Increasing drusen size (as measured by the greatest linear diameter), indicate increasing severity of early AMD. Large drusen are defined as >125μm. Pigmentary changes (hyper/hypopigmentation) are due to RPE hypertrophy/atrophy, and are thought to occur due to local insult / reactive change. Large drusen and pigmentary changes are risk factors for progression to advanced AMD.[20]

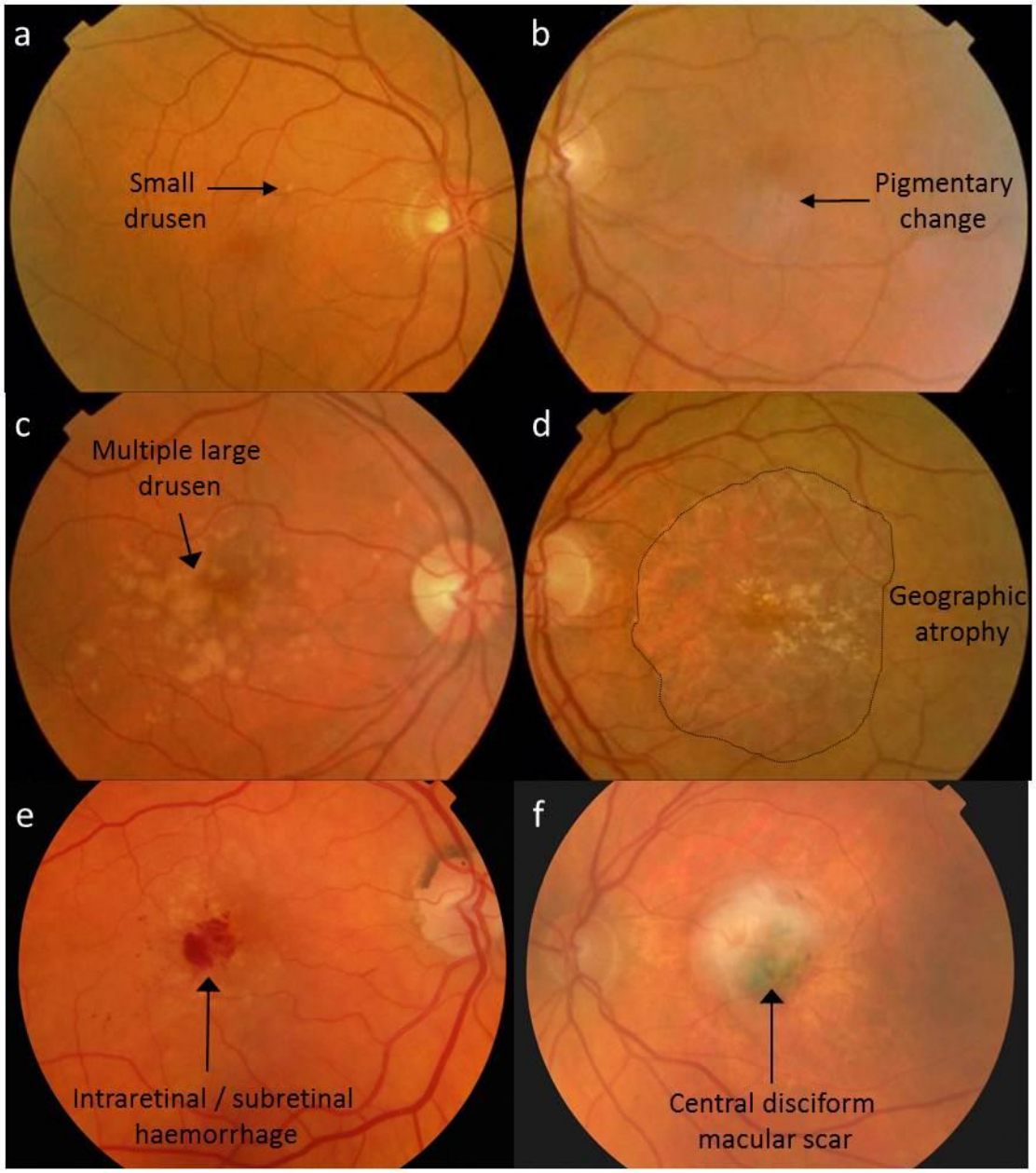
Advanced AMD includes geographic atrophy (GA) or neovascularisation. GA describes a well demarcated region of significant RPE atrophy at the macula, with visible underlying choroidal vessels Figure 1.7d). Neovascularisation can originate from the highly vascular choroidal layer (choroidal neovascularisation, CNV), breaching the normal barrier function of BM to proliferate under the RPE and neurosensory retina. Alternatively, neovascularisation can originate from within

the retina (retinal angiomatous proliferation, or RAP). The endothelia of these neovascular structures are highly permeable and prone to extravasation of fluid and blood, which can lead to significant visual symptoms (Figure 1.7e). Fundus fluorescein angiogram (FFA) confirms the diagnosis, aided by cross-sectional scans of the macula obtained by optical coherence tomography (OCT) (Figure 1.8). Neovascularisation eventually undergoes fibrosis, and if large a "disciform" scar may result (disc-like in shape) (Figure 1.7f). In a prevalence study of Europeans over 65 years old, around 4.5% of all patients with AMD demonstrated nvAMD. Overall, the prevalence of advanced AMD in the UK in patients 50 years and over is around 2.4%.[30] Figure 1.9 and Figure 1.10 summarise the changes seen within the retina in early and neovascular AMD.

1.2.6 Dry / wet AMD

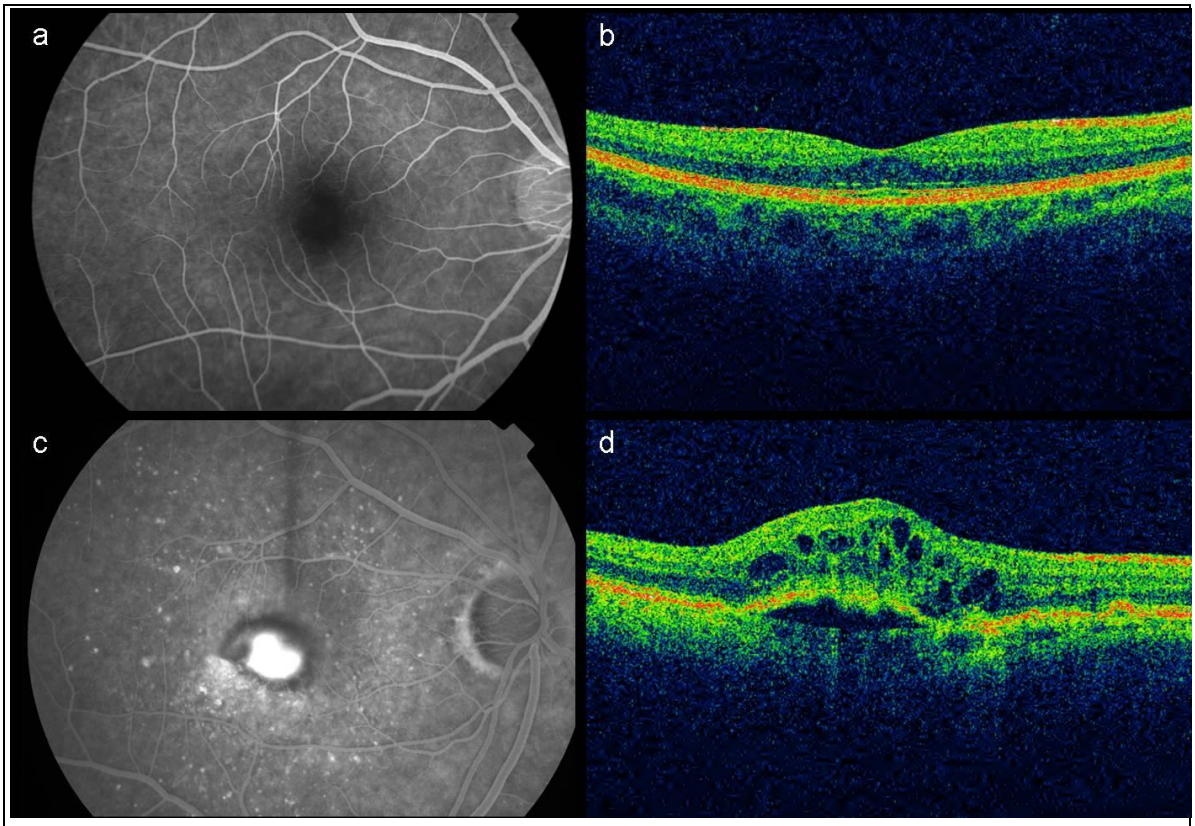
A classification often used by patients for simplicity describes AMD as "dry" or "wet", based on the absence / presence of neovascularisation. However this classification can be confusing since patients are unaware that "wet" AMD is usually always accompanied with underling dry changes, and also patients with dry AMD are sometimes falsely misled to believe that significant visual deterioration cannot occur (which can happen with GA).

Figure 1.7: Clinical features of AMD



Clinical appearance of AMD at different stages. a-c: Early AMD; d-f: Late AMD
a: Small Drusen. A single small druse at the macula is present, superotemporal to the fovea. b: Early pigmentary changes at fovea (hyperpigmentation surrounded by ring of hypopigmentation). c: Multiple large drusen at macula. d. Geographic atrophy. A well demarcated area of RPE atrophy affecting the macula, revealing the underlying choroidal vessels. e. Neovascularisation. Bleeding at the fovea is seen, occurring from a choroidal neovascular membrane.
f: Disciform scar- end stage of macular neovascularisation.
Photographs obtained from those taken from patients recruited in this study.

Figure 1.8: Fundus fluorescein angiograms (FFA) and Optical coherence tomograms (OCT) of CNV compared to a healthy eye



Fundus fluorescein angiograms (FFA) and Optical coherence tomograms (OCT) of CNV compared to a healthy eye. FFA involves injection of fluorescein (a fluorescent dye) intravenously, usually via the antecubital fossa. This dye passes through the systemic circulation and as it passes through the eye, photos are taken with a fundus camera equipped with barrier filters. Thus reflected light is excluded and a clear image of fluorescent light is obtained. Usually the dye passes through the retinal vessels without leaking. However, the presence of CNV is demonstrated by the presence of leakage from choroidal vessels at the macula. Currently an FFA is usually augmented by a non-invasive test called the Optical Coherence Tomogram (OCT). This uses low intensity laser to produce high resolution cross-sectional images of the macula. Evidence of CNV is inferred by observing the presence of intraretinal or subretinal fluid

a) FFA of healthy macula. b) OCT of healthy macula. c) FFA of eye with CNV. Note hyperfluorescent area at fovea, resulting from fluorescein dye leaking from choroidal neovascular membrane. d) OCT of eye with CNV. Note thickened retina, presence of intraretinal cysts full of fluid and sub-retinal pigment epithelial collection of fluid. Images taken from Southampton Eye Unit archives.

Figure 1.9: Schematic diagram of retinal changes in early AMD

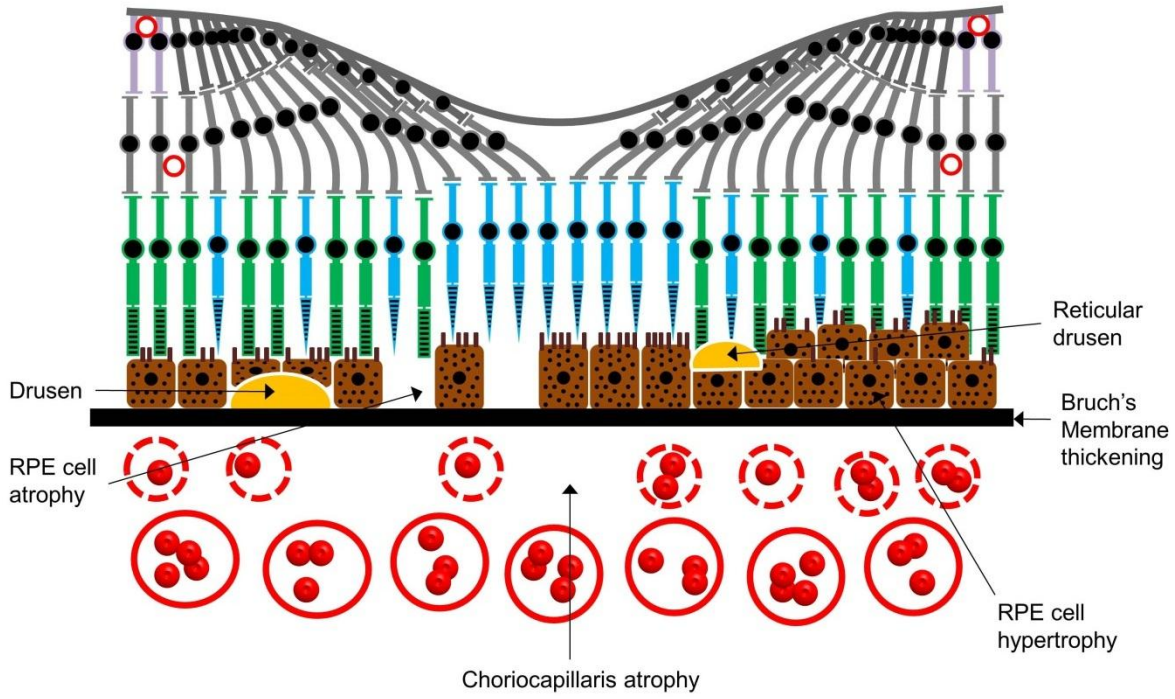


Diagram shows cross-section of macula depicting the typical changes seen in early AMD. Drusen (yellow) usually form between the RPE layer and Bruch's Membrane (BM), although can also form between the neuroretina and RPE layer (reticular drusen). RPE atrophy and hypertrophy/hyperplasia also occur, together with choriocapillaris atrophy. Image drawn by author, reprinted with permission from: Khandhadia and Lotery (2010)[24] © 2010 Cambridge University Press.

Figure 1.10: Schematic diagram of retinal changes in NVAMD

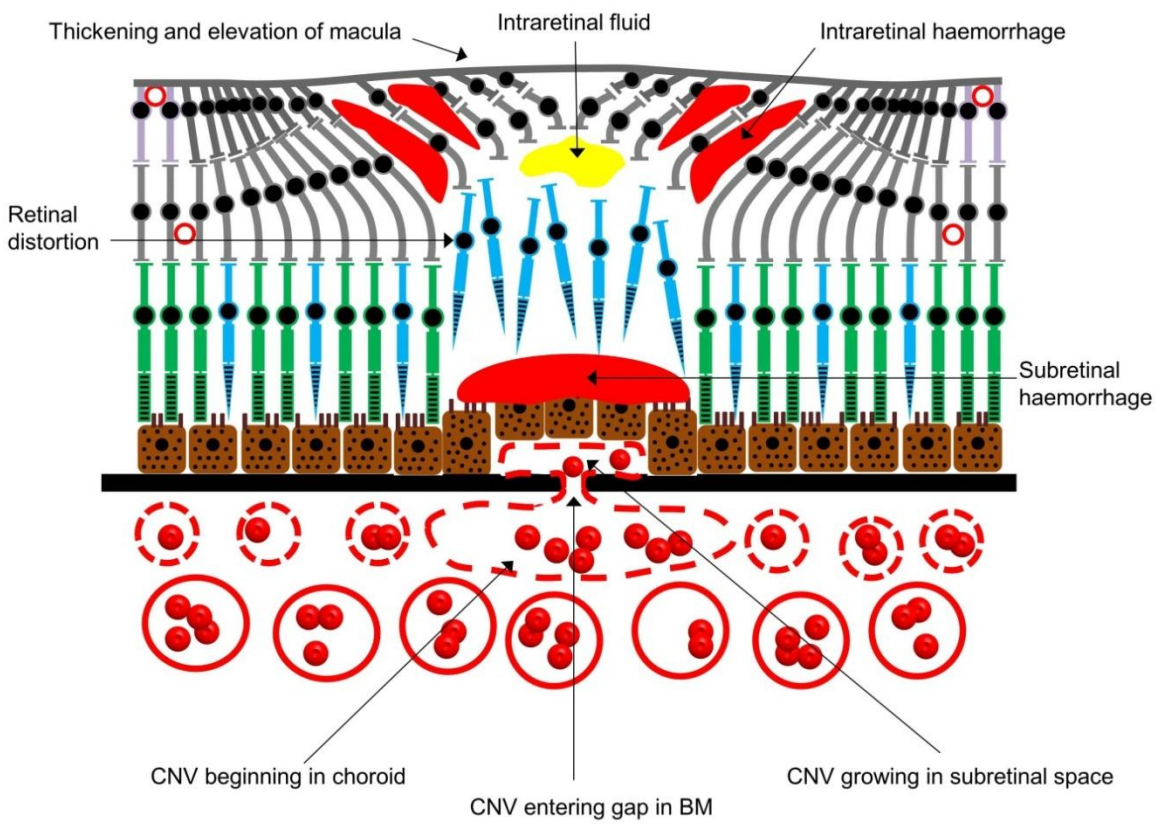


Diagram shows cross-section of macula depicting the typical changes seen in nvAMD. Choroidal neovascularisation (CNV) breaks through a gap in Bruch's Membrane (BM) to proliferate under the RPE and neuroretinal layers. Extravasation of fluid and blood can occur leading to thickening and distortion of the retinal architecture, with resulting impairment of central vision. Image drawn by author, reprinted with permission from: Khandhadia and Lotery (2010)[24] © 2010 Cambridge University Press.

1.2.7 Clinical features

Early AMD is often asymptomatic. Patients with early AMD are warned they may develop gradual deterioration of central vision and increasing distortion, over years. Advanced AMD is associated with significant central visual deterioration. GA is insidious, but neovascularisation can present suddenly with acute increase in distortion / reduction in central vision / a central blind spot.

1.2.8 Management of AMD

Presently early AMD and GA is not treatable, although several clinical trials of investigational products are on-going. Addressing modifiable risk factors, including smoking and reduction in body mass index may help to reduce progression.[2] Oral anti-oxidant supplements may reduce the risk of progression of established early AMD. The AREDS study found patients with high risk early AMD (presence of multiple large drusen with RPE changes) were less likely to exhibit AMD progression if taking high doses of oral vitamins A, C, E and zinc.[31] Increasing consumption of fresh fruit, vegetables and oily fish may also reduce AMD progression.[32-34]

Patients with non-nvAMD are advised to report any new central visual symptoms, especially distortion, which may represent conversion to nvAMD.[35] An Amsler grid, consisting of small squares, can be used regularly by the patient to detect new distortion and metamorphosia.

Until recently no satisfactory treatment existed for nvAMD and patients inevitably irretrievably lost sight. Since 2006, the development of intravitreal injections of antibodies against vascular endothelial growth factor (VEGF) has radically changed the management of CNV.[36;37] These injections can stabilise sight deterioration for many patients and in some cases sight can be partially restored. However often many injections are required once a month, each one not without its complications (for example, endophthalmitis, or infection inside the eye can lead to blindness very quickly and has a 1 in 1000 risk per injection).

About 3% of all patients with AMD will suffer significant visual impairment, associated with a reduced quality of life. These patients may require socio-psychological support.[15] Magnifying aids, telescopes, closed circuit television, talking books and domestic modifications may be beneficial. Some patients report benefit from intra-ocular placement of a magnifying intraocular lens.[38] Extensive retinal rotation surgery has also been attempted, but is associated with a high rate of complications.[39]

1.2.9 Risk factors for AMD

AMD is a complex multi-factorial disease. Risk factors associated with AMD include demographic, environmental/behavioural, ocular, systemic and genetic factors.

1. Demographic risk factors

Ageing is the most significant risk factor for AMD. AMD prevalence increases with advancing age.[40] Increasing age may be accompanied by alteration in gene expression and cellular functions, including deteriorating mitochondrial function, increased protein degradation and apoptosis.[41;42] RPE phagocytosis of photoreceptor outer segments diminishes with age, leading to the build-up of undigested material called lipofuscin within RPE and photoreceptor cells[43–45]. This contains several toxic substances including retinoids, produced as by-products of the visual cycle, along with modified proteins and lipids.[46] Another consequence of ageing is the appearance of drusen. How drusen may precipitate RPE changes and neovascularisation is as yet not clear.

There is some evidence to suggest nvAMD is more common in older females.[47] The reason for this is unknown, but may be related to hormone levels. Espinosa-Heidmann et al found oestrogen supplementation in mice increases size of laser-induced CNV.[48]

AMD prevalence differs according to ethnic group. A retrospective longitudinal study based on hospital billing records in the USA showed that Black, Latino and Asian populations had less risk of developing AMD compared to the White population.[49] Other studies have shown AMD is more common in White compared to Black or Asian populations.[17;50]

2. Environmental / behavioural factors

Smoking in particular is a major risk factor in AMD.[51–53] As mentioned above, dietary intake can influence AMD development[31–34] , and high BMI is also associated with AMD[2].

3. Ocular factors

Although cataract surgery has been associated with AMD[54], only limited information is presently available from prospective randomised controlled trials.[55]

4. Systemic factors

AMD is associated cardio/cerebrovascular disease.[56–59] This may be partially explained by the association of individual cardiovascular risk factors with AMD, such as systemic hypertension, dyslipidaemia, diabetes, raised BMI, high alcohol consumption and smoking.[57;60]

5. Genetic susceptibility

Sequence variations in several genes have been associated with AMD. The proteins coded by these genes tend to fall into well-defined groups, which may provide clues to the underlying mechanisms of AMD pathogenesis. These include genes involved in the complement pathway (including complement factor H[4–6], complement C3[61], complement factor B[61], CFH-related genes 1+3[62], complement factor I[63], C1 inhibitor[64]), lipid metabolism (hepatic lipase C[65], cholesterylester transfer protein[66] and apolipoprotein E[67]), extracellular matrix (tissue inhibitor of metalloproteinase 3[66], collagen genes COL8A and COL10A[68]), and angiogenesis (VEGFA[68]). However the function of the gene most strongly associated with AMD (HTRA1/ARMS2 gene) is as yet unknown.[13;69;70]

1.2.10 Pathogenesis of AMD

As yet the exact pathological mechanisms involved in the development of AMD are not fully understood. However current thinking, as suggested by Ambati and Fowler in 2012, implicates two main pathological processes in AMD development, namely immune-mediated and oxidative stress.[71] The cells of the retina, in particular the RPE layer, are highly susceptible to damage from the effects of both these processes. Since the RPE cell is pivotal in maintaining the health of the retina, RPE dysfunction and damage can have a profound effect.

The function of the immune system is to protect the host from pathogens; however in doing so unintended local tissue destruction can occur. Components of the immune system, which may impair retinal cell viability and function, include immune cells (macrophages, microglia and neutrophils), and protein

mediators (cytokines, complement proteins).[72] The complement system in particular has been associated with AMD. This will be discussed later in the introduction and in more detail in the appendix ("Age-related macular degeneration and the complement system" - published review article, Khandhadia et al).[3]

Oxidative stress refers to cellular damage exerted by reactive oxygen species (ROS), which are highly reactive oxygen-containing atoms/molecules/ions. ROS are produced during physiological conditions mainly by cellular respiration (glycolysis and Kreb's cycle), especially by mitochondria.[73;74] An antioxidant system within the body protects host cells from the deleterious effects of ROS.[73] However when the local antioxidant capacity is exceeded, damage to cells can occur, especially to mitochondria and lipids. Damage to mitochondria can lead to functional mutations in mitochondrial DNA[75] as well as release of apoptosis-inducing enzymes, such as caspase and cytochrome C.[76;77] Peroxidation of lipids, especially within cell membranes leads to further toxic products which damage other molecules, especially proteins.[78] ROS are also produced by macrophages to combat pathogens, and through environmental factors, for example smoking, pollution and radiation. The association of oxidative stress and AMD is discussed in more detail in the appendix ("Oxidation and age-related macular degeneration: insights from molecular biology" - published review article, Khandhadia et al).[24]

Immune system, oxidative stress and development of AMD

Drusen are the first sign of AMD, however it is not known whether drusen form as a result of local immune-mediated or oxidative stresses, or whether drusen actually induce an immune response or ROS production. The observation that that larger drusen are associated with increased risk of progression to advanced AMD suggests that drusen do have an important role in AMD pathogenesis.[20] Drusen contain many constituents associated with the immune system, including immunoglobulins and complement proteins.[79-81] A druse may act as a nidus of on-going inflammation within the retina.[80;81] The high lipid content of drusen may also induce inflammatory changes, similar to that occurring in atherosclerosis.[29]

Sufficient immune-mediated or oxidative stress may induce RPE and secondary photoreceptor cell apoptosis, eventually leading to the GA phenotype. A major component of lipofuscin is *N*-retinylidene-*N*-retinylethanolamine (A2E),

associated with both complement activation and oxidative stress, and has been found to cause DNA damage and apoptosis of RPE cells in vitro.[82;83] Furthermore, cytokines also have a deleterious effect on RPE integrity and function.[84]

Immune and oxidative stress also may induce angiogenic factor production from retinal cells. In particular the predominant stimulus appears to be VEGF-A, a cytokine normally produced by RPE cells. Physiological production of VEGF by the RPE cells maintains the choriocapillaris[85], but up-regulation induces angiogenesis and increased vascular permeability.[86] Indeed as discussed previously, monoclonal antibody fragments, blocking the action of VEGF-A at their receptors, are the present mainstay of treatment for CNV.[36;37]

CNV development itself exhibits many characteristic feature of inflammation, including increased vascularisation, exudation and leukocyte chemotaxis.[87] Immunohistological examination of surgically excised CNV membranes demonstrate presence of immunoglobulins, complement proteins and HLA-expressing cells.[88] Oxidative stress may also play a key role in CNV development. Carboxyethylpyrrole (CEP), produced by oxidation of phospholipids within photoreceptor cell membranes, binds to proteins and modifies protein function.[78] Ebrahim et al found mice given subretinal injections of CEP-adducted albumin demonstrated exacerbation of laser-induced CNV.[89] Furthermore, mice genetically engineered without the essential antioxidant enzyme superoxide dismutase (SOD) *(SOD1* gene double-knockout) demonstrate features of AMD, including development of CNV, suggesting a lack of antioxidant capacity to mop up ROS may be a key driver in neovascular AMD.[90]

Other theories of pathological mechanisms in AMD

Other pathological mechanisms postulated to be associated with AMD include choroidal ischaemia, and changes in BM.

Choroidal ischaemia

The main watershed area of the posterior choroid (i.e. the area in between the two main circulations of the posterior choroid) lies between the fovea and optic disc and therefore the macula is vulnerable to ischaemia.[91] Choroidal blood flow reduces with age, presumably as a result of atherosclerosis or atrophy.[92]

Choroidal ischaemia may be one factor contributing to the risk of AMD. Patients with lower choroidal blood flow are more likely to develop CNV.[93]

BM changes

With age, BM increases in thickness due to collagen remodelling and lipid deposition. This leads to decreased hydraulic conductivity impairing transport of solutes between the choroid and RPE.[94] Reduced RPE function can result, especially since RPE cells may already be compromised due to lipofuscin accumulation.

Breaks in BM are a key factor in CNV development. The evidence for this is compelling. CNV growth is a risk of therapeutic thermal laser applied on the retina, due to BM disruption. Indeed, animal models of CNV are often created experimentally using retinal laser.[95] Breaks in BM are also features of non-AMD conditions associated with CNV growth, including high myopia, angioid streaks (seen, for example, in pseudoxanthoma elasticum) and eye trauma.[96] As mentioned above, the central component of BM is elastin. Interestingly, genetic variations in the fibulin-5 gene, which controls elastogenesis, have been associated with AMD.[97;98] This may lead to weakening of BM, thus increasing susceptibility to CNV.

The exact mechanism for the breakdown of BM in CNV formation is yet unknown. Present studies suggest that with age, BM may become increasingly fragile due to lipid-build-up, calcification and fragmentation.[99;100] Increased degradation of BM may occur from increased matrix metalloproteinase (MMP) activity, reduced activity of Tissue Inhibitors of MMP (TIMP) and from increased cytokine release from macrophage build-up.[101-103] Of note, SNPs in the *TIMP-3* gene have been associated with AMD.[101] Contradicting this, decreased MMP activity occurs with increasing age within the macula and may explain the characteristic accumulation in extracellular matrix and BM thickening.[104] CNV formation may be associated with a more localised increase in MMP activity prior to BM rupture. Curcio et al recently postulated a key role for lipoprotein build-up within BM in AMD pathogenesis.[105]

GA and nvAMD – 2 distinct phenotypes?

Although the two end stages of advanced AMD, GA and nvAMD are very differing phenotypes, they may share a common effector pathway. Both GA and nvAMD

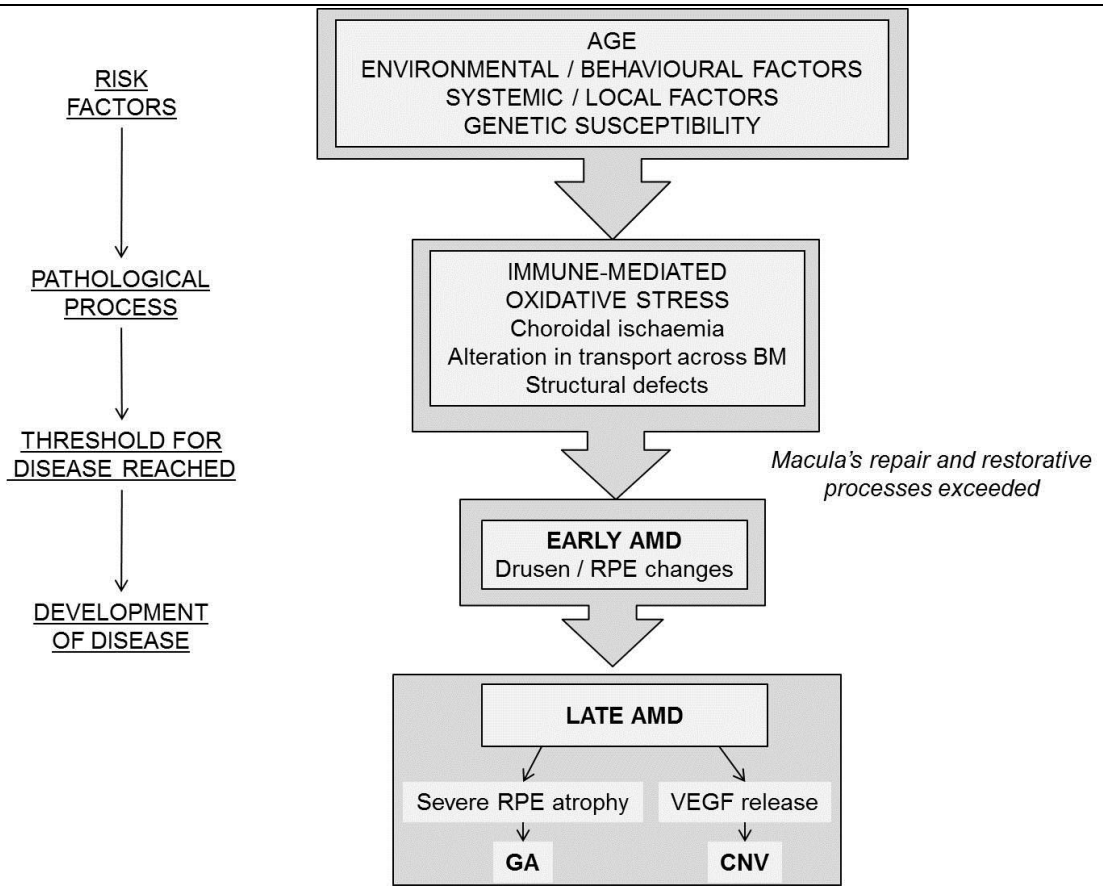
share a common early phase (early AMD) which is indistinguishable clinically and pathologically. Furthermore, the genetic and environmental factors predisposing to each phenotype appear to be very similar. At some point a cellular event must occur to trigger specific mechanisms, which presumably encourages development of a specific advanced AMD phenotype (i.e. GA or nvAMD). It is likely there is some genetic influence on this, since monozygotic twins demonstrate similar AMD phenotype, and patients tend to have symmetrical AMD phenotype between eyes.[106;107] The definitive triggers are as yet undefined.

One possibility may be a difference in VEGF gene expression. Although increased RPE production of VEGF is associated with nvAMD[108], Saint-Geniezet al demonstrated that decreased VEGF expression may precipitate RPE and choriocapillaris atrophy.[109] GA has been associated with toxic accumulation of AluRNA in RPE cells due to reduced levels of DICER1, a micro-RNA processing enzyme[110]; as yet this has not been reported in nvAMD. It is as yet unclear how important and to what extent such interactions are in the pathogenesis of either nvAMD or GA.

Model of AMD pathogenesis

A suggested model of AMD pathogenesis based on the literature review is shown in Figure 1.11.

Figure 1.11: Model of AMD pathogenesis



The phenotype of AMD is triggered once the cumulative effect of multiple risk factors crosses a threshold beyond the capabilities of the body's own repair and regenerative processes. Abbreviations: BM = Bruch's Membrane. GA = Geographic atrophy. CNV = Choroidal neovascular membrane. RPE = Retinal pigment epithelium. VEGF = Vascular endothelial growth factor.

1.3 The complement system

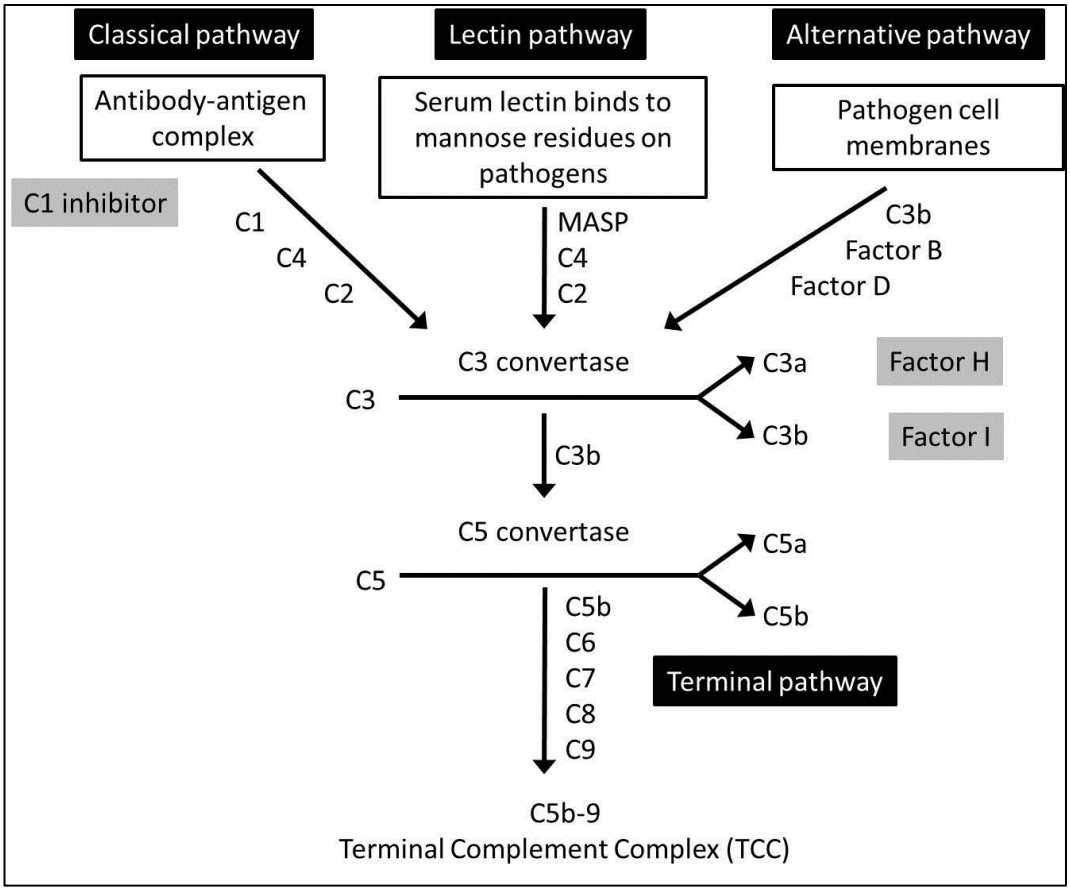
Reference used for this section: Wood P. Understanding immunology. 2nd ed., 2006.[111]

The complement system refers to a number of proteins normally found in circulating blood. This is part of the innate immune system, but can be recruited by components of the adaptive immune system, therefore effectively "complementing" the latter. The complement proteins are referred to as heat-labile, since they are deactivated by heat. Many of the complement proteins are pro-enzymes, which require activation either via conformation changes induced by binding, or cleavage from another protease. Activation of one complement protein leads to activation of several complement proteins further down the cascade, leading to significant amplification following the initial trigger.

The key complement proteins are named in numerical order based on when they were discovered, and (with one exception) this is also the order in which they are activated in the classical pathway (see below). Cleaved complement proteins are denoted by the addition of a small letter (e.g. C2a, C3b). Additional proteins required in the alternative pathway are designated capital letters (e.g. Factor B).

There are three pathways: the classical, alternative and lectin pathways. Each has different triggers, but all end in a common terminal pathway. The classical complement pathway is activated by an antibody-antigen complex. The lectin pathway is activated by mannose residues, which form part of polysaccharides or glycoproteins found on pathogenic surfaces. The alternative pathway is activated by microbial cell membranes. All three pathways produce a C3 convertase, which then in turn produces a C5 convertase. This subsequently activates the terminal pathway, which results in the formation of the terminal complement complex (TCC) (Figure 1.12).

Figure 1.12: The complement pathways



Complement pathway regulators shown in grey boxes. Modified from Zhou et al 2011[112]

1.3.1 The classical pathway

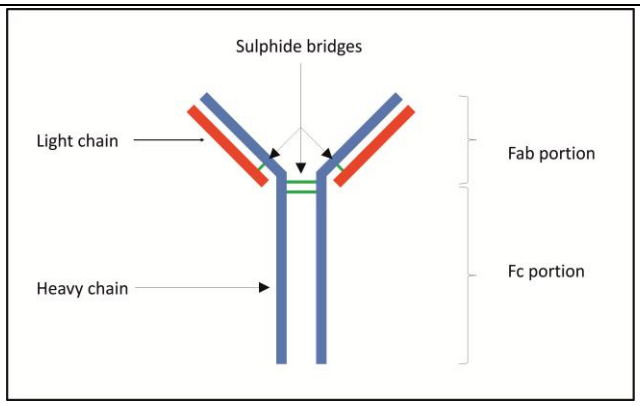
The classical complement pathway is activated by an antibody-antigen complex. An antigen is a molecule, usually a polysaccharide, which is recognised by an antibody (Figure 1.13). An antigen can be in solution, or present on a solid surface. To activate the complement pathway the antigen needs to be present on a solid surface, for example the cell membrane of a bacterium, or the surface of a particle. Circulating antibodies (IgM or IgG) bind to the surface-antigen, which then allows binding to the first complement protein, complement C1. This is called "complement fixation" (Figure 1.14A). (C1 is made up of three separate components, C12q, C1r and C1s). C1 is then activated, which in turn cleaves circulating C4 into two smaller protein fragments, C4a and C4b. C4a has no further role in the cascade, but functions as an anaphylatoxin, inducing mast cell activation and degranulation. C4b binds to the cell / particle surface to which the

antibody is attached (Figure 1.14B). Circulating C2 binds to surface-bound C4b, and is cleaved into C2a and C2b. C2a stays attached to C4b, to form C4b2a, a C3 convertase. C2b is released, with no obvious important function (Figure 1.14C).

C3 is a key protein in all three complement cascades. Normally some C3 spontaneously hydrolyses in serum to form C3a and C3b. However normally C3b is inactivated rapidly either via hydrolysis in serum, or by cell surface regulatory proteins. The classical pathway C3 convertase, C4b2a, substantially increases conversion of C3 to C3a and C3b, and very little is then inactivated. Over 200 molecules of C3b can be generated from one molecule of C4b2a. C3a plays no further part in the cascade. However, similarly to C4a, C3a functions as an anaphylatoxin, inducing mast cell activation and degranulation. Some C3b binds to C4b2a on the cell/particle surface to form C4b2a3b, the classical pathway C5 convertase. The majority of C3b acts as an opsonin, one of the most important functions of complement. Opsonisation enables certain white blood cells to bind to pathogens to enable phagocytosis. Furthermore, C3b reduces deposition of harmful insoluble immune-complexes in blood vessels, which otherwise can cause prolonged localised inflammatory damage (Figure 1.14D).

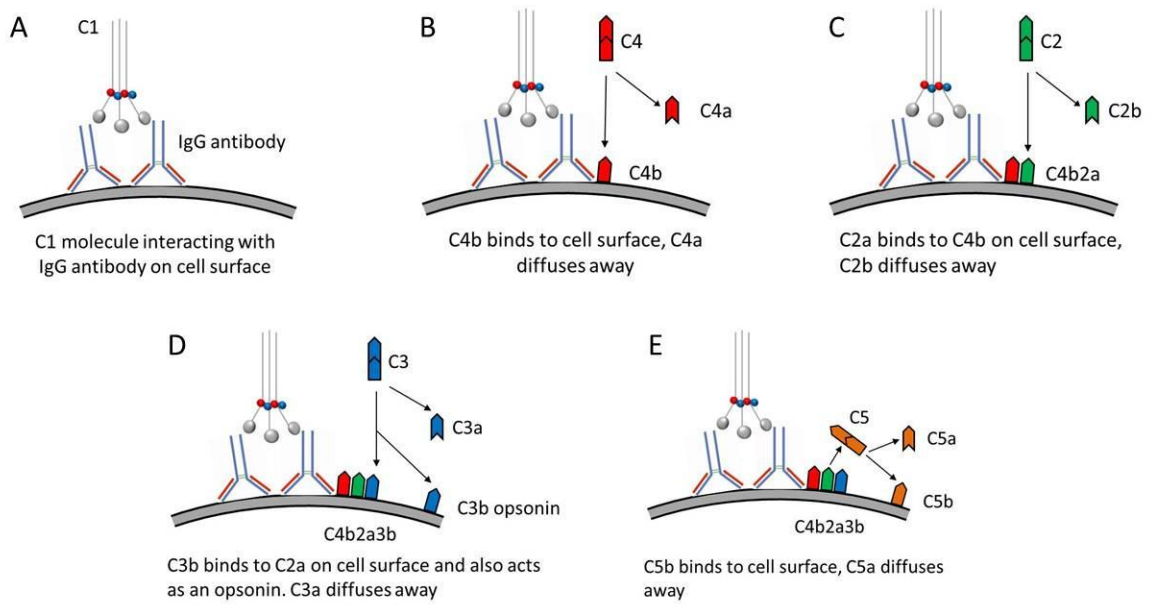
The classical pathway C5 convertase, C4b2a3b, binds and cleaves C5 into C5a and C5b. C5a diffuses away, but has several other important functions. It is a chemotaxin, attracting leucocytes. It functions as an anaphylatoxin, similar to C4a and C3a. It also encourages neutrophil hydrolytic enzyme production. C5b binds to the cell / particle surface (Figure 1.14E). Production of C5b initiates the common terminal pathway (see Figure 1.12).

Figure 1.13: Antibody structure



A single antibody molecule comprises four chains – two identical heavy chains and two identical light chains. Sulphide bridges connect the chains together. An antibody molecule is cleaved into two fragments by the enzyme papain (derived from papaya). The antigen-binding fragment (Fab) carries antigen epitope recognition sites. The Fragment crystallisable (Fc) is so-named so due to its property to crystallise at 4°C. A monoclonal antibody (mAB) carries specific Fab epitope recognition sites to enable binding to only one epitope.
Redrawn based on Wood 2006[111]

Figure 1.14: The classical complement pathway



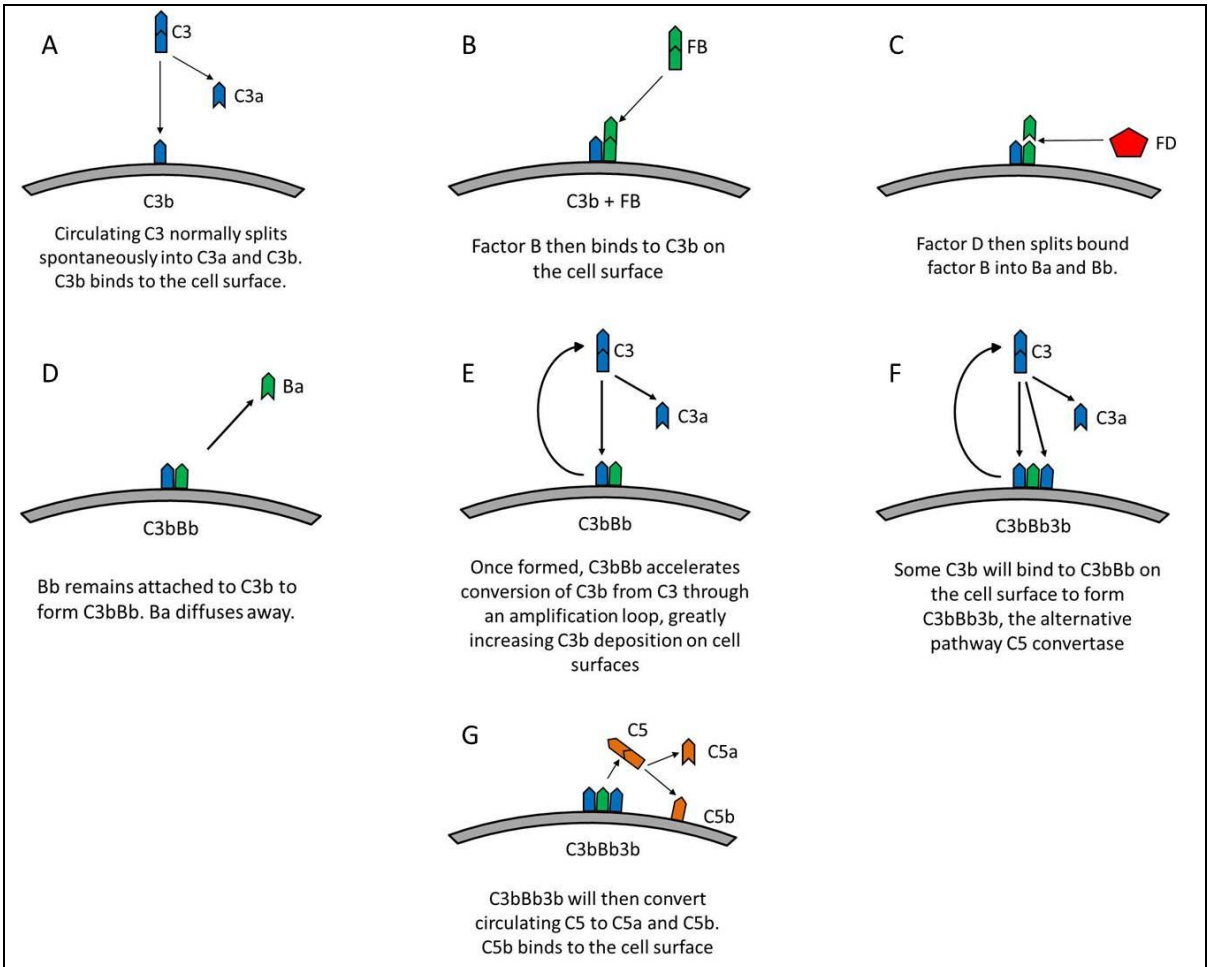
Figures A-E show the sequential steps of the classical pathway.
Redrawn based on Wood 2006[111]

1.3.2 The alternative pathway

Circulating complement C3 normally splits spontaneously into C3a and C3b. C3b binds to the cell surface Figure 1.15A). Normally C3b would be inactivated by cell surface regulatory proteins. However, many microbes do not possess these surface proteins, and therefore C3b remains active. C3a is a chemotaxin and anaphylatoxin. Factor B then binds to C3b on the cell surface (Figure 1.15B), which is then cleaved by Factor D to produce Ba and Bb (Figure 1.15C). Bb remains attached to C3b to form C3bBb, the alternative pathway C3 convertase (Figure 1.15D). Ba diffuses away.

C3bBb cleaves C3 into C3a and C3b through its C3 convertase action. Therefore even more C3b is formed through a self-amplification loop (Figure 1.15E). It is estimated that within five minutes, more than a million C3b molecules can bind to a microbial surface. Some C3b will bind to C3bBb on the cell surface to form C3bBb3b, the alternative pathway C5 convertase (Figure 1.15F). C3bBb3b will then convert circulating C5 to C5a and C5b (Figure 1.15). C5a is a potent chemotaxin and inflammatory mediator. C5b binds to the cell surface, and initiates the terminal pathway.

Figure 1.15: The alternative complement pathway

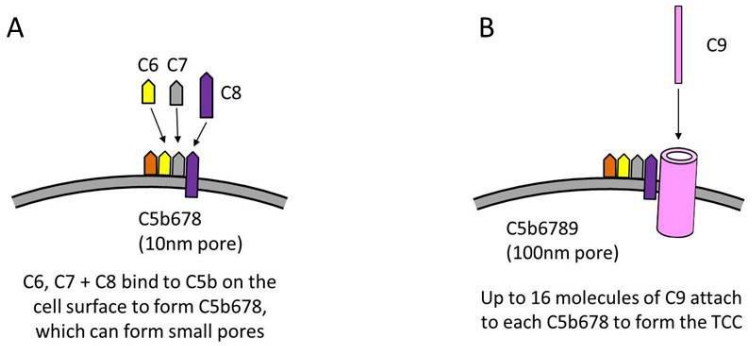


Figures A–G show the sequential steps of the alternative pathway.
Redrawn based on Wood 2006[111]

1.3.3 The terminal pathway

Complement C6, C7 + C8 bind to C5b on the cell surface to form C5b678 which can form small pores in the cell membrane (Figure 1.16A). Up to 16 molecules of C9 attach to each C5b678 to form the terminal complement complex (TCC), also called the membrane attack complex (MAC) (Figure 1.16B). This can form pores in cell membrane, which are large enough to allow transport of water and ions, but not protein. This creates an osmotic gradient, resulting in water entering the cell, which eventually "bursts". This is a key mechanism of pathogen destruction by the complement system.

Figure 1.16: Terminal complement pathway



Figures A-B shows the sequential steps of the terminal pathway. TCC =Terminal complement complex. Redrawn based on Wood 2006[111]

1.3.4 The lectin pathway

This pathway is similar to the classical pathway, with one important difference. The initial trigger is induced by mannose residues, which form part of polysaccharides or glycoproteins found on pathogenic surfaces. Mannose-binding protein (MBP), normally found in serum, binds to these mannose residues. MBP then binds to MBP-associated serine protease (MASP), also normally found in serum, to form an MASP-MBP complex. This then cleaves C4 to C4a and C4b; the rest of the pathway follows that of the classical pathway.

1.3.5 Regulators of the complement pathways

Complement can be harmful to the host cell, especially near the initial site of complement activation. Therefore the body has evolved a regulatory system to keep complement activation in check. Complement regulators are either found in solution in serum or extracellular fluid, or are membrane-bound. Most complement regulators are involved in both classical and alternative complement pathways. The primary sites of regulation occur at the point of C3 convertase and TCC formation.

a. Regulation of C3 convertase.

C3 convertase can be regulated either by reduction of its formation, or increasing its degradation. Reduction of formation C4b2a (classical C3 convertase) occurs by the attachment of various proteins to C4b (including complement receptor 1, membrane co-factor protein (MCP) or C4b-binding protein), preventing the

binding of C4b to C2a. Complement Factor I (CFI) then cleaves C4b, which can no longer form C3 convertase. Reduction of the formation of C3bBb (alternative C3 convertase) occurs via binding of proteins (complement receptor 1, MCP or complement factor H) to C3b. C3b is now unable to bind to factor B. CFI then cleaves C3b into iC3b and C3f, leading to permanent inhibition of C3bBb formation. iC3b is eventually converted to C3c and C3dg.[113]

Increased decay of C3 convertase in both classical and alternative pathways is accelerated by "decay accelerating factor". This splits C3 convertase into its constituents. The classical C3 convertase C4b2a is degraded into C4b and C2a, and the alternative convertase C3bBb degraded into C3b and Bb.

b. Inhibition of TCC formation.
Formation of the TCC can be inhibited either by blocking its attachment to cell membranes, or inhibiting binding of its constituent proteins. Vitronectin, or S-protein, is a soluble protein which binds to C5b67 as it forms, reducing further attachment to the cell membrane. CD59 is a protein found bound to the cell surface, which prevents C8 and C9 binding to C5b67.

c. Other sites of regulation
C1 inhibitor (C1i) is a regulator of the classical pathway, through the inhibition of C1 protease cleavage activity.

CFH, as indicated above, plays an important role in the regulation of the alternative pathway. The production, structure and function of the CFH protein will now be described in more detail.

1.3.6 Complement production

Systemic complement production
The liver is the primary producer of most circulating complement proteins, including CFH.[117] The production of hepatic CFH is demonstrated by the abundant secretion of CFH from primary human hepatocyte cultures, and CFH expression in human liver cDNA libraries.[7;8] Furthermore, Schmeling et al showed that plasma CFH allotype converted entirely to that of the donor in six out of six liver

transplant (LT) recipients[118], demonstrating the vital contribution made by the liver to circulating CFH levels.

Extra-hepatic production

Although most organs rely on hepatic complement production via the systemic circulation, certain organs with limited access to circulating proteins have developed local "extra-hepatic" complement synthesis.[117] One example is the brain, in which the blood-brain barrier restricts systemic complement protein entry. Evidence of separate intra-cerebral complement synthesis is demonstrated by the production and secretion of a range of complement proteins by human astrocyte-derived tumour cell lines and primary human fetal astrocytes in culture.[119] The blood-retinal barrier is considered to exert a similar restrictive effect on access of systemic proteins to the retina.[120]

Intraocular complement production

Complement is normally produced within the eye itself. Local production of a particular protein can be determined by examining the products of gene expression from local cells, through isolation of RNA followed by real-time quantitative PCR or microarray analysis. Complement deposition within the retina can also be detected and measured quantitatively using immunohistochemistry.

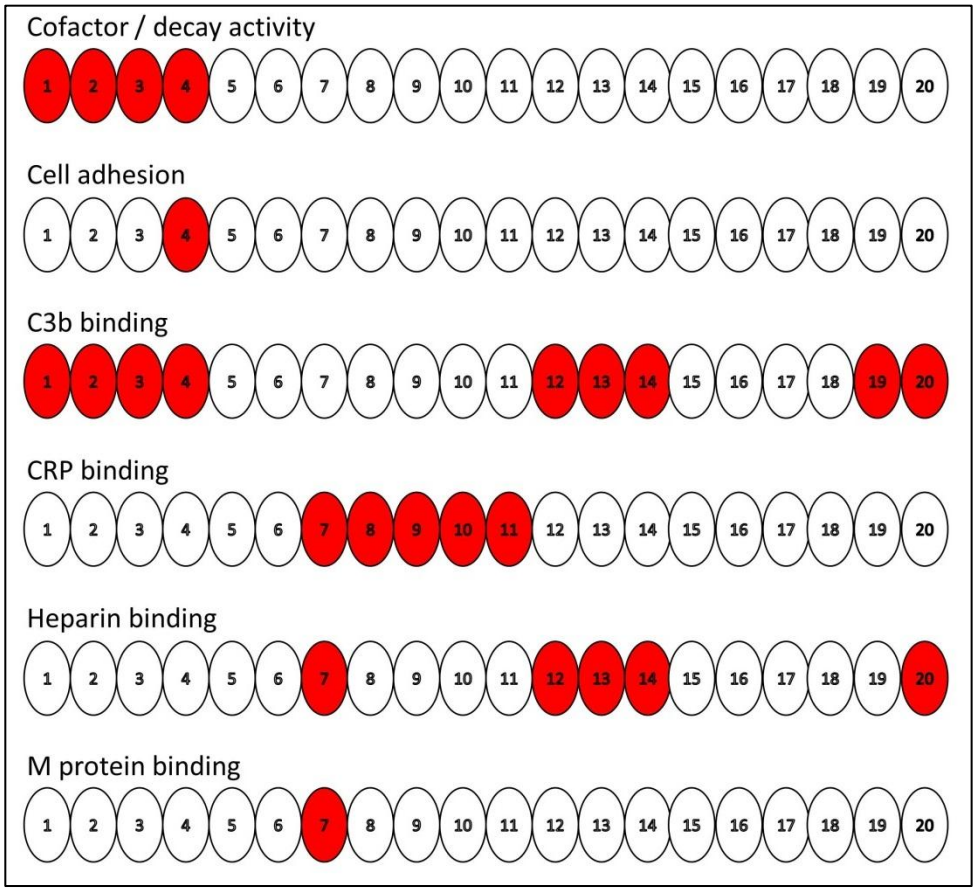
Chen et al found expression of genes encoding for C1q, C1r, C1s, C2, C3, C4b and CFB in retinal tissue in mice eyes.[121] Booij et al reported high levels of complement gene expression in RPE cells from healthy human donor eyes aged 63-78, including genes encoding for C1q, C2, C3, CFB and CFH.[122] Gold et al also found C2 and CFB are expressed in neural retina, RPE and choroid from human donors.[61]

The CFH protein is produced at high levels within the eye. Hageman et el quantified CFH transcripts *in vitro* in the eye using real time PCR, and found RPE cells and the choroid produced CFH protein at levels approaching that found in the liver.[9] CFH accumulates in RPE cells, the sub-RPE space, the inter-photoreceptor matrix and in the choroid.[9;123] Levels are also high in other parts of the eye, including the lens, optic nerve, sclera and ciliary body.[124]

1.3.7 CFH protein structure

CFH is a plasma protein of approximately 150 kiloDaltons in size. The CFH polypeptide chain is arranged into 20 similar units, called short consensual repeats (SCR), each containing approximately 60 amino acids.[114] The CFH molecule has multiple binding sites, and is able to bind to numerous ligands, including C3b, C-reactive protein (CRP), heparin, and streptococcal M protein (Figure 1.17).[115]

Figure 1.17: Binding sites of ligands to CFH protein



Redrawn / modified from Zipfel et al 2002.[116]

Figure showing the short consensual repeats (SCRs) 1-20 of the complement factor H (CFH) protein. The SCRs which are involved in binding to specific ligands are highlighted in red.

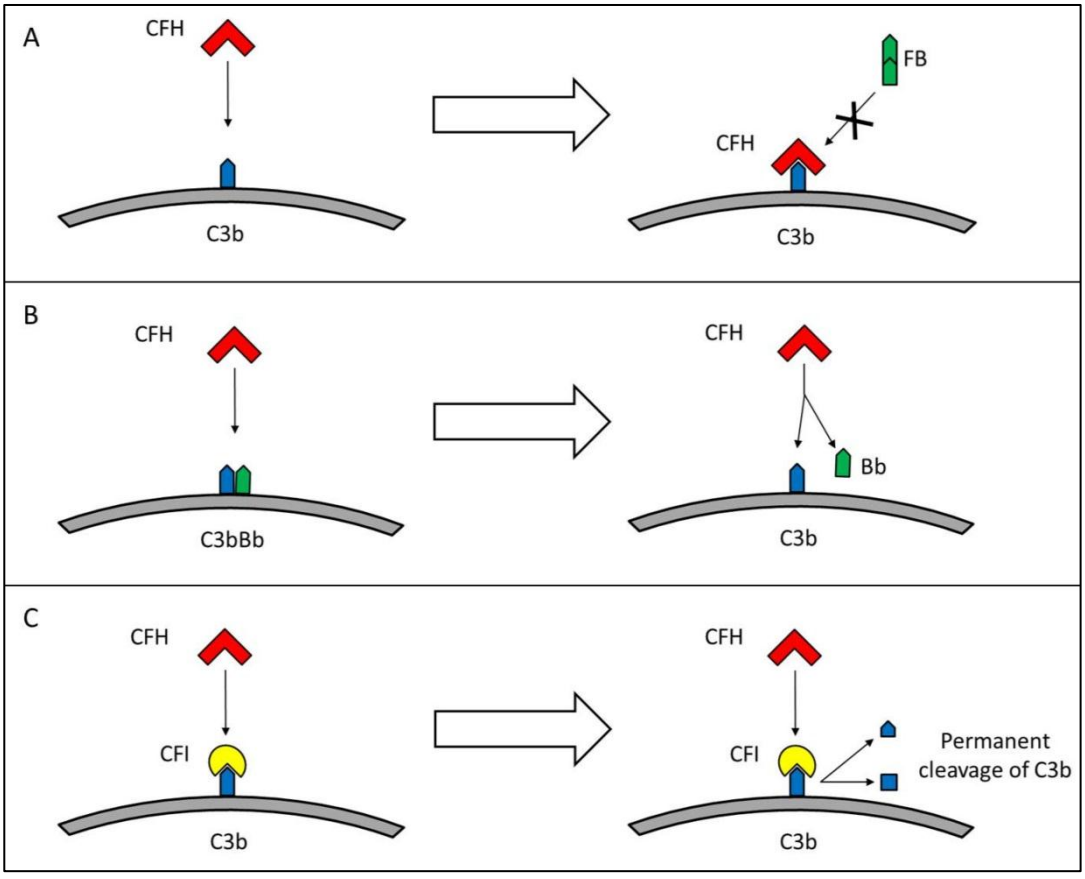
1.3.8 CFH protein function

CFH acts on C3b and on C3bBb (the alternative pathway C3 convertase), in one of three ways (Figure 1.18):

1. It prevents binding between C3b and CFB.
2. It accelerates decay of alternative pathway C3 convertase (C3bBb) into C3b and Bb.
3. It acts as a cofactor for complement factor I (CFI) inactivation of C3b.

These actions are controlled by SCRs 1–4. C3b binding also occurs at SCRs 12–14, and 19–20 (Figure 1.17).[116]

Figure 1.18: Mechanism of action of CFH



A–C shows the three mechanisms of action of complement factor H (CFH).
A: CFH prevents binding between C3b and CFB. B: CFH accelerates the decay of the alternative pathway C3 convertase (C3bBb). C: CFH acts as a cofactor in CFI-mediated cleavage of C3b. Drawn based on information from Wood 2006[111]

1.3.9 Role of CFH in the eye

Studies of *CFH* gene knock-out animal models demonstrate absence of CFH can lead to significant retinal pathology. Coffey et al reported that two year old *CFH* gene double knockout (-/-) mice exhibited several significant anatomical, histological and functional changes, including increased deposition of auto-fluorescent material, C3 accumulation in the neurosensory retina, BM thinning, alteration in RPE organelle distribution, and rod photoreceptor cell outer segment disruption. This was also associated with significantly attenuated electroretinogram response.[125] Lundh von Leithner et al also demonstrated the importance of CFH in retinal perfusion in *CFH* gene double knockout (-/-) mice. Deposition of C3 and C3b on the endothelium of retinal vessels was seen, which subsequently became atrophic, reducing retinal perfusion.[126] Faber et al found a loss of upregulation of CD59a (a regulator of TCC) in *CFH* double knockout (-/-) mice.[127] Therefore loss of function of the *CFH* gene in mice appears to lead to development of significant retinal changes reminiscent of AMD, associated with decreased regulation and increased activation of the alternative complement pathway, with consequent local damage from presence of TCC. This suggests that CFH has a vital protective role in the human eye.

1.4 The role of the complement system in AMD

(Please also refer to the published review article written by the author regarding the role of complement in AMD – see appendix).

The significance of the role played by the complement system, in particular the alternative pathway regulator CFH, in AMD pathogenesis is based on histological, genetic and biochemical evidence, as described below. Based on this body of evidence, several clinical trials are currently under way investigating the effect of complement inhibition in patients with AMD.

1.4.1 Increased complement expression and deposition with age

Complement gene expression within the retina is up-regulated with increasing age, as demonstrated by several studies on mice of differing ages. Chen et al showed an increase in expression of the complement genes encoding for C1q, C1r, C1s, C3 and CFB protein in RPE / choroid tissue taken from ageing compared to young mice.[121] Faber et al replicated these results, demonstrating increased expression of genes encoding for C1q, C1 inhibitor, C3, and CFB in the RPE/choroid with increased expression of regulators CFH and Cd59a in the neuroretina in old versus young mice.[127] Mandal et al also found expression of CFH increased with age within mouse eyes.[124]

Histological evidence of increased complement deposition in the retina with age also has been shown. Chen et al found increased levels of C1q and C3 deposition in the RPE/choroid complex and BM in aged compared to younger mice.[121] Hageman et al found TCC deposition was minimal or absent in the RPE/choroid from human donors under 50 years old, but was more apparent for older donors.[9]

The increase in complement expression and deposition within the retina with advancing age suggests an increase in complement activation and compensatory regulatory activity in the older eye, which in turn may predispose to the development of AMD.

1.4.2 Complement deposition and AMD

Complement deposition also occurs to a greater extent in eyes with AMD compared to controls, especially at the macula. Hageman et al found increased CFH deposition within the RPE/choroid complex in donor eyes with AMD compared to non-AMD donor eyes, especially in the macular compared to extramacular areas.[9]

Complement deposition has also been found in two characteristic lesions of AMD, namely drusen and CNV. Drusen, one of the early hallmarks of AMD, contain many constituents associated with the inflammatory system, including complement pathway proteins. Johnson et al reported that TCC was present in drusen, and additionally in the cytoplasm of adjacent RPE cells.[79] In a later paper Johnson et al also demonstrated the presence of fluid-phase complement regulators (vitronectin and clusterin), and membrane-bound complement inhibitors (complement receptor 1 and membrane cofactor protein) in drusen. They also reported the presence of small spherical structures in drusen, which appear to contain C3 proteolytic fragments, characteristically found in areas of complement activation.[80] Mullins et al found drusen from histological specimens from donor eyes contained, amongst other inflammatory-related constituents, complement proteins C5 and TCC.[81] Hageman et al also found high levels of CFH in drusen, which in addition appeared to co-localise with C3 fragments, known ligands of CFH.[9] A druse may act as a nidus of on-going inflammation within the retina, with resulting accumulation of complement and other inflammatory proteins.[80;81] The high lipid content of drusen may also induce inflammatory changes, similar to that occurring in atherosclerosis.[29]

Complement deposition has also been observed in CNV secondary to nvAMD. Baudouin et al reported significant amounts of complement proteins, including C1q, C3c and C3d in surgically removed CNV from human patients.[88] Lommatzsch et al also found complement-associated proteins within surgically-extracted CNV, including C3, TCC, CFH and vitronectin.[128;129] Complement activation within drusen and may be associated with VEGF-induced CNV formation. Nozaki et al showed C3a and C5a in drusen could stimulate VEGF production in RPE cells in culture *in vitro* and via intravitreal injection of C3a and C5a *in vivo*. Furthermore, inhibition of C3a and C5a action, either by pharmacological blockade or genetic

elimination of their respective receptors, reduced VEGF production, leucocyte chemotaxis, and CNV formation after laser trauma.[130]

Complement gene variations are associated with AMD

Many of the genes encoding for proteins involved in the complement pathway have been associated with AMD. Most of these associations are related to the finding of both exonic and intronic SNPs being more common in patients with AMD compared to patients without AMD (controls).

The *CFH* gene was the first complement gene to be associated with AMD. In 2005 in the journal Science, three separate groups simultaneously reported an increase in frequency of the non-synonymous rs1061170 T→C allele variation in the *CFH* gene in patients with AMD compared to controls.[4-6] SNPs throughout the *CFH* gene have subsequently been associated with AMD.[4;9;66;131-135] By investigating SNPs in other complement genes in case/control studies, further associations with AMD were discovered. These include *C2*/*CFB* (in close proximity to each other)[61], *C3*[136], *C7*[137], *CFI*[63] and *SERPING1*[64]. A large deletion (almost 85,000 bases) in *CFH*-related genes (*CFHR1* and *CFHR3*) has also been associated with AMD.[62] It should be noted that all these studies restrict analysis to patients of European-origin, to avoid confounding results with varying gene pools. Furthermore definitions and classification of AMD and controls tend to vary between studies, depending on the grading system used.[25;26;138;139] However certain genes have shown strong associations with AMD which have been replicated in multiple studies, regardless of AMD definition. A recent large meta-analysis of multiple genome-wide association studies (17000 cases of advanced AMD and >60000 controls) found that even after Bonferroni correction for multiple SNP analysis, significant associations were found with SNPs in the *CFH*, *C2*/*CFB*, *CFI*, and *C3* complement genes. However the initial meta-analysis included patients of both European and African origin. When both ethnicity and variability in effect size between multiple studies was taken into account, only SNPs near the *CFH* gene were still significant.[140] This suggests out of all the genes encoding for complement proteins, the *CFH* gene is most strongly associated with AMD, and therefore probably most likely to influence AMD pathogenesis.

Systemic complement activation, CFH and AMD

Several papers have reported complement proteins / activation products in plasma of AMD patients compared to non-AMD controls. Sivaprasad et al found elevated plasma levels of C3a were associated with early/nvAMD.[141] In a smaller study, Machalinska et al also found elevated levels of C3a-des-Arg were associated with nvAMD.[142] Reynolds et al found the highest quartile plasma levels of activation fragments Bb and C5a were significantly associated with advanced AMD (GA/CNV), and raised C3a levels associated with GA.[143] Hecker et al reported elevated plasma Ba, C3d (markers of chronic activation), and factor D were elevated in patients with moderate / advanced AMD). There was also a trend for a greater increase in plasma levels of these factors in more advanced AMD, suggesting that increased progression of AMD may be associated with greater systemic complement activation.[144] Scholl et al (2008) found plasma levels of a wide range of complement activation products (C3d, Ba, C3a, C5a, SC5b-9) plus CFD were elevated in patients AMD.[145] In summary, there is some evidence of systemic complement activation in association with AMD, although the elevation of specific complement activation products is not always replicated between studies. Also it should be noted that the definitions of AMD are inconsistent between studies, which may influence interpretation of results.

The association of systemic (plasma) CFH protein levels with AMD is inconsistent. Reynolds et al found reduced plasma CFH protein levels in patients with advanced AMD compared to controls.[143] However Hakobyan et al reported elevated plasma CFH levels in AMD patients versus controls (although no p value is mentioned in the paper).[146] Other studies have found no difference.[144;145] The lack of consistency between reports of systemic CFH levels in AMD could reflect a difference in how AMD was defined, since more pronounced results could be expected when comparing more advanced AMD against controls. Alternately these may suggest systemic CFH levels do not have a bearing on AMD pathogenesis.

1.4.3 Clinical trials of complement-based therapies for AMD

A range of complement inhibitors are currently being evaluated in clinical trials for the treatment of early and advanced AMD (Table 1.2 – information obtained from www.clinicaltrials.gov). The complement cascade is targeted at several

levels, through C3, C5 and CFD inhibition. Most are administered by intravitreal injection, which is the method of administration of anti-VEGF agents, the currently-approved treatment of nvAMD. Eculizumab is an exception, needing to be given as an intravenous infusion.

POT-4 is a C3 inhibitor being investigated for nvAMD. Although phase 1 studies have reported no drug-toxicity, phase 2 studies have been suspended for unknown reasons. Eculizumab marketed as "Soliris", is a recombinant humanized monoclonal immunoglobulin G antibody against C5, licenced for use in paroxysmal nocturnal haemoglobinuria. However its chronic systemic use is associated with increased risk of *Neisseria meningitides* infection.[147] This highlights the dangers of immune suppression during chronic complement inhibition. The systemic effects of intravitreal injections are considered less significant.[148] ARC1905 is an anti-C5 aptamer. Aptamers are non-immunogenic single-stranded oligonucleotides which, due to particular three-dimensional folding characteristics, bind to specific protein targets and inhibit protein-protein interactions.[149] FCFD4515S is a recombinant humanised monoclonal antibody fragment which binds to the C-terminal portion of CFD, in turn preventing binding to C3Bb pro-convertase, and reducing formation of active C3bBb convertase.[150]

There are no reported clinical trials investigating the effect of CFH administered either locally or systemically. However a recent study by Kim et al described reduced development and progression of laser-induced CNV in rat eyes injected with intravitreal human-derived CFH protein, together with reduction in C3a and TCC deposition. As a result they suggested a possible role for intravitreal CFH in treating nvAMD in humans.[151]

The ideal complement inhibitor would have beneficial improvement in vision and disease pathogenesis, with minimal systemic effects whilst preserving the more proximal part of the complement pathway to allow local clearance of pathogens. The results of clinical trials are awaited.

Table 1.2: Complement-targeting investigational medicinal products undergoing clinical trials for AMD

Investigative product	Pharmaceutical company	Mechanism of Action	Mode of delivery	AMD group targeted	Phase	Status	Clinicaltrials.gov reference	Results of clinical trial
POT-4 (AL-78898A)	Potentia Pharmaceuticals / Alcon	C3 inhibitor	Intravitreal injection	NvAMD	1	Ended	NCT00473928[152]	No drug-related toxicity in patients treated with up to 450 µg POT-4[153]
					2	Suspended	NCT01157065[154]	N/A
Eculizumab	University of Miami / Alexion Pharmaceuticals	C5 inhibitor	Intravenous infusion	Dry AMD (drusen and GA)	2	Ongoing	NCT00935883[155]	N/A
ARC1905	Ophthotech Corporation	Anti-C5 aptamer	Intravitreal injection	NvAMD	1	Completed	NCT00709527[156]	58 patients given 3-6 monthly ARC1905 (0.03, 0.3, 1, or 2 mg) with ranibizumab(0.5mg). No dose-limitingtoxicity seen.Mean visual acuity (VA) change +10.2letters at week 8. 35% gained ≥3lines of VA. Mean macular central thickness change = -124µm.[157]
				Dry AMD (GA)	1	Ongoing	NCT00950638[158]	N/A
LFG316	Novartis Pharmaceuticals	?C5 inhibitor*	Intravitreal injection	Advanced AMD (nvAMD or GA)	1	Completed	NCT01255462[159]	N/A
FCFD4514S	Genentech	Anti-CFD	Intravitreal injection	GA	1	Completed	NCT00973011[160]	N/A
					2	Ongoing	NCT01229215[161]	N/A

AMD: Age-related Macular Degeneration, nvAMD: Neovascular AMD, GA: Geographic Atrophy, N/A: Not available, *Details unclear from clinicaltrials.gov website and Medline search

1.4.4 The importance of CFH in AMD

As described above, CFH protein is produced at high levels within the retina and choroid, and has a vital role in protecting the eye from indiscriminate complement activation. The expression and deposition of CFH increases with age, and in AMD patients compared to controls, especially at the macula. In addition, CFH deposition has been found in drusen. Furthermore, SNPs in the *CFH* gene are amongst the most strongly associated with AMD. Although systemic levels of CFH have not been consistently associated with AMD, there appears to be sufficient evidence to suggest that CFH has a key role to play in the pathogenesis of AMD.

The relevance to AMD of the alternative complement pathway, of which CFH is a key regulator, was shown by an elegant experiment by Bora et al. They used short interfering RNA (siRNA) to demonstrate the importance of the alternative complement pathway in AMD pathogenesis (siRNA against a particular gene silences local expression of that gene). Blocking the alternative complement system in mice by giving intravenous CFB siRNA significantly reduced the incidence of laser-induced CNV. However blocking the classical and lectin complement pathways with intravenous C1q siRNA had no effect on laser-induced CNV.[162]

1.4.5 *CFH* Y402H and AMD

As mentioned previously, complement factor H (CFH) was the first complement protein to be implicated in the pathogenesis of AMD, following reports of a strong association of the rs1061170 SNP in the *CFH* gene and AMD.[4-6] The rs1061170 SNP, also called the *CFH* Y402H sequence variation, will be discussed in more detail in this section.

Rs1061170 and AMD

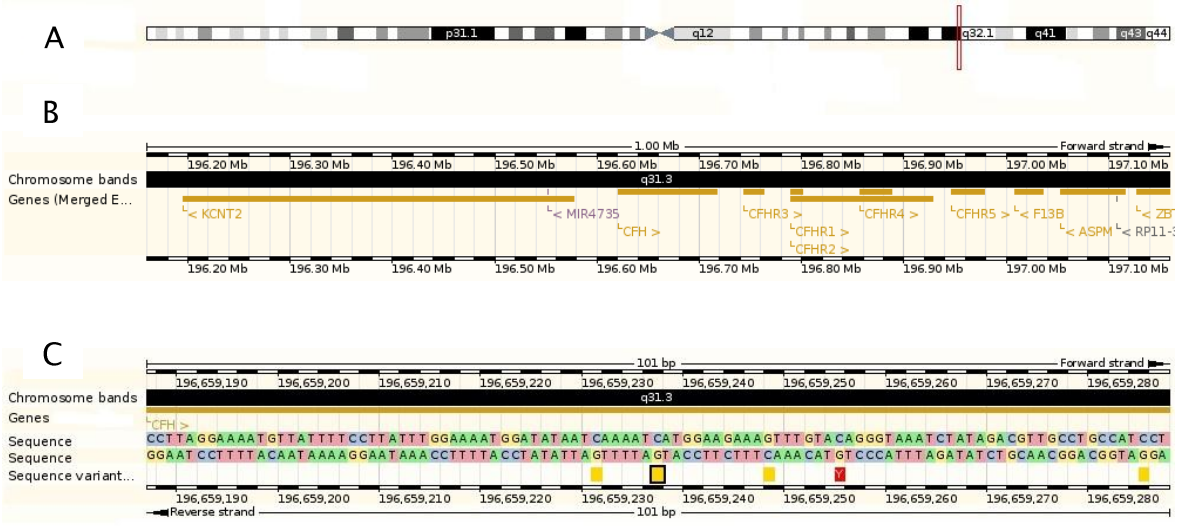
The *CFH* gene is located at chromosome one on the long arm, at position 31.3 (chromosome position 1q31.3), from base pair 196,621,007 to base pair 196,716,633 (Figure 1.19A+B). In 1998 Klein et al first reported linkage to markers in this region in a large family in which AMD appeared to be segregating as an autosomal dominant Mendelian disorder.[163] However it was not till 2005 when large case-control studies demonstrated a specific genetic sequence

variation in this region, the non-synonymous rs1061170 SNP in the *CFH* gene, was significantly associated with AMD.[4-6] The rs1061170 SNP results in a base substitution of thymine (T) with cytosine (C) at position 196659237 within an intron of the *CFH* gene (Figure 1.19C). This in turn results in altered CFH polypeptide production due to an amino acid substitution. Histidine (H) replaces tyrosine (Y) at position 402 of the polypeptide, resulting in a CFH H402 protein instead of the CFH Y402 protein. The rs1061170 polymorphism is therefore also referred to as the "*CFH* Y402H" variation.

It should also be emphasised that that AMD is currently not regarded as a simple Mendelian disorder, but a complex multifactorial disease with both genetic and environmental risk factors.[164]

(NB: For purposes of this thesis, the following terms will be used. The rs1061170 SNP will be referred to as "*CFH* Y402H". The low risk *CFH* Y402H homozygous genotype (TT) will be referred to as "*CFH* YY402", high risk homozygous genotype (CC) as "*CFH* HH402", and heterozygous genotype (CT) as "*CFH* YH402".)

Figure 1.19: Location of rs1061170 sequence variation



A: The complement factor H gene is located at position 31.3 on the long arm (q) of chromosome 1.

B: Position 1q31.3 magnified (to 1 million base pairs shown) to show the location of *CFH* and its neighbouring genes (base pair number listed at top and bottom of diagram). Several *CFH*–related genes (*CFHR1-5*) are in close proximity to the *CFH* gene.

C: Position 1q31.3 magnified (to 101 base pairs) to show the position of the rs1061170 sequence variation. The 9 digit numbers above and below the DNA sequence indicate the base pair location. The next line indicates the gene (*CFH*). The bases of both the forward ("sequence" – upper line) and reverse ("sequence" – lower line) DNA strands are shown. The next line indicates the sequence variations present in this region. The yellow squares indicate a missense variant is present. Rs1061170 is indicated at position 196659237 by a yellow square with a black outline. Other missense sequence variations are present but are rare (less than 0.1% in a European population according to Ensembl, therefore are not classified as SNPs). The red square refers to a stop variant (which would stop DNA synthesis), however no data was available regarding population genetics for this variation using Ensembl.

Images and information from Ensembl release 70 – January 2013. Accessed 21/3/13.[165]

CFH Y402H is a common polymorphism. In a European epidemiological study (Rotterdam Study), the prevalence of high risk *CFH* HH402 homozygotes was 13.7%, and *CFH* YH402 heterozygotes 45.1%, with a minor allele frequency of 36.2%.[166] *CFH* HH402 homozygotes are approximately six times, and *CFH* YH402H heterozygotes are 2.5 times more likely to have AMD compared to those with the low risk *CFH* YY402 genotype.[167] Table 1.3 summarises the *CFH* Y402H genotype variations, the associated AMD risk, and the effect on CFH protein production.

The prevalence of the *CFH* Y402H risk allele (C) varies according to ethnic groups, being most prevalent in those of European and African descent. Hageman et al looked at a set of 1064 DNA samples from 47 populations (Human Genome Diversity Panel), and found the *CFH* Y402H C allele frequency was 35% in both Europeans and Africans, 25–30% in both North Africans and Middle Easterns, 10–15% in Asians, and 0–5% in Native Americans.[168]

Table 1.3: *CFH* Y402H genotype and protein production

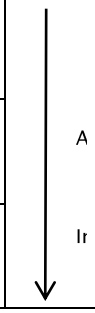
***CFH* Y402H status**	**AMD risk**	**Genotype**	**CFH protein produced**
Low risk homozygous genotype "*CFH* YY402"		TT	CFH Y402 only
Heterozygous genotype "*CFH* YH402"		CT	CFH Y402 + CFH H402
High risk homozygous genotype *"*CFH* HH402"		CC	CFH H402 only

Table showing *CFH* Y402H genotype with associated AMD risk, and consequent CFH protein production. Low/high risk refers to the associated reported age-related macular degeneration risk.[167]

The *CFH* Y402H SNP is also associated with type 2 membranoproliferative glomerulonephritis, also called "dense deposit disease" due to the appearance of drusenoid deposits in the glomerular basement membrane. Complement dysregulation is a feature of this disease.[169] Many patients develop early onset retinal drusen, of similar structure and composition to that found in AMD.[170]

Other sequence variations in the CFH gene have also been associated with AMD.[4;9;66;131–135;171] For this project, the focus was on the *CFH* Y402H SNP, since at

the time of project initiation, this was the most reported and investigated SNP associated with AMD. The functional consequences of the *CFH* Y402H polymorphism, and how this may influence AMD pathogenesis, will be discussed next.

How does the *CFH* Y402H sequence variation alter CFH protein function?

The *CFH* Y402H polymorphism leads to a replacement of tyrosine with a histidine amino acid residue at position 402 of the CFH protein, at SCR 7 (Figure 1.17).[114] This affects ligand-binding at this site, including binding with CRP, glycosaminoglycans and M protein.[172] This in turn may lead to reduced binding to cell surfaces, and therefore impaired local regulation of the alternative complement pathway.

-CFH Y402H and binding to the retina

Several studies have shown that the CFH H402 protein binds less well to RPE cells than the Y402 protein[173;174] but this has not been replicated by other studies.[175;176] Reduced binding of the CFH H402 protein to Bruch's membrane has been also demonstrated in one study, but again not replicated.[176;177]

-CFH Y402H and alteration of binding to CRP

CRP is an acute phase protein, which increases substantially as part of the innate immune response to tissue injury and infection. It binds to polysaccharides on pathogens, and damaged cell surfaces. Its presence is thought to provide secondary sites for CFH binding, therefore increasing CFH presence on cell surfaces[178]. Lauer et al reported that CRP particularly assists recruitment of CFH to necrotic RPE cell surfaces, such as that found in AMD.[174]

Several biochemical studies have shown that the CFH H402 variant protein has reduced affinity for CRP compared to Y402 protein.[173;174;179-183] Johnson et al found raised CRP levels in the RPE and choroid in *CFH* HH402 (high risk) donor eyes compared to *CFH* YY402 donors. The authors also found no evidence of local CRP production from RPE and choroid. They suggested that the increased local CRP deposition in *CFH* HH402 eyes was a result of either chronic low-grade inflammation in the presence of dysfunctional CFH protein, or due to inadequate binding of CRP to the CFH H402 protein.[184]

-*CFH* Y402H and alteration of binding to other ligands

Bruch's membrane (BM) is particularly rich in glycosaminoglycans (GAGs), including heparan sulphate and dermatan sulphate. CFH activity at the level of the retina may occur via binding to GAGs within BM. The importance of GAGs within the BM in CFH activity was demonstrated by Kelly et al, who found that heparan sulphate in human donor BM/choroid tissue played a key role in CFH-mediated inactivation of C3b, as measured by functional assays.[177]

Clark et al found the CFH H402 protein bound less well to BM, and that this was due to impaired affinity to both heparan and dermatan sulphate.[176] However Kelly et al found no difference in the binding of CFH Y402 or H402 protein to BM/choroid tissue from elderly non-AMD donor eyes, although specific GAG binding was not investigated.[177]

Histological evidence of intraocular complement activation and *CFH* Y402H

Does complement activation in the eye correlate with *CFH* Y402H genotype? Mullins et al found the levels of TCC level in the RPE/choroid from human donor eyes were higher in *CFH* HH402 versus YY402 donors.[185] Loyet et al found elevated levels of activated factor B (Bb) in the vitreous of patients with advanced AMD versus no controls, largely explained by a combination of the *CFH* Y402H, *C2*, *CFB* and *C3* gene sequence variations.[186]

However this was not replicated by Johnson et al, who found similar levels of TCC within drusen and choroidal vessels between *CFH* YY402 and HH402 donors. They did find elevated CRP levels in the choroid of *CFH* HH402 compared to YY402 donor eyes, suggesting this could be a result of impaired binding between the CFH H402 protein variant and CRP.[184] Furthermore disputing the importance of the *CFH* Y402H polymorphism within the retina is a study by Ufret-Vincenty et al. They created chimeric mice carrying either human Y402 or H402 sequence in the SCR6-8 region within the mouse *CFH* gene (SCR6-8 was selected since the rs1061170 sequence variation affects SCR7). They found no difference in clinical or histological findings within the retina between Y402 or H402 chimeric mice. This may reflect the greater importance of other SCR sites on CFH protein function. However extrapolating findings from this study are limited due to the chimeric nature of the CFH protein.[187] Therefore the evidence demonstrating

whether complement activation in the eye is associated with *CFH* Y402H genotype is inconclusive.

Is *CFH* Y402H associated with systemic inflammation?

Systemic inflammation can be determined by measuring circulating levels of CRP, complement activation or cytokines. Elevated circulating CRP is a non-specific indicator of systemic inflammation, however systemic CRP levels are not associated with *CFH* Y402H genotype, as shown by studies by Zee et al and Mooijaart et al.[188;189] Despriet et al found that although serum CRP levels were associated with AMD, levels were similar between *CFH* YY402, YH402 and HH402 patients.[190] However Mooijaart et al did find a linear increase in CRP levels with age in patients 85 years or more carrying the *CFH* HH402 genotype, when followed up over five years.[189]

Systemic complement activation is also not consistently associated with the *CFH* Y402H genotype in AMD. Scholl et al reported a risk *CFH* haplotype (including Y402H) was associated with elevated plasma levels of complement activation products (Ba and C3d) in AMD.[145] Other groups however have found the *CFH* Y402H genotype did not influence complement activation in AMD.[141;144] Only one study has investigated the association of systemic cytokines with CFH Y402H genotype, finding elevated levels of plasma interleukin 6 with the *CFH* HH402 genotype.[189] Overall the evidence associating *CFH* Y402H with systemic inflammation seems to be equivocal.

1.4.6 Determining if local or systemic CFH production is more important in AMD pathogenesis

The complement system, in particular CFH appear to be important in AMD pathogenesis. Based on this evidence several clinical trials are under way investigating the effect of intraocular or systemic complement regulation in managing AMD. However what is not known is whether local or systemic complement activity is more important to AMD pathogenesis.

Systemic versus local complement production

As described above, CFH is produced both by the liver and the eye. However it is still unknown whether local intraocular or systemic CFH protein activity is more important in the pathogenesis of AMD. Answers can be provided by observing the effect on AMD by modulating either intraocular or systemic CFH protein activity individually.

Alteration of local CFH production in AMD

Intraocular CFH protein production can be modulated by injecting CFH siRNA, which silences local *CFH* gene expression. Lyzogubov et al gave subretinal injections of CFH siRNA to mice eyes, and found these mice demonstrated greater intensity and quicker onset of laser-induced CNV, together with increased TCC deposition. Hepatic *CFH* gene expression was unchanged. This suggests locally produced CFH protein has an important role in AMD pathogenesis.[191]

Alteration of systemic CFH production in AMD

The effect of selectively modifying hepatic and therefore systemic CFH protein production on AMD pathogenesis has not been studied, and this is the knowledge gap to be filled by this thesis. Modification of hepatic CFH protein production, through liver transplantation (LT), has however been carried out in a different disorder called atypical Haemolytic Uraemic syndrome (aHUS).

What is haemolytic uraemic syndrome

Haemolytic uraemic syndrome (HUS) is the most common cause of acute renal failure in children. In most cases (95% – "typical HUS"), it is caused by *Escherichia coli* (*E. coli*) infection, and usually resolves following diarrhoeal symptoms. The rarer "atypical HUS" (aHUS) has a much poorer prognosis, and is associated with complement-mediated damage to the renal glomerular vascular endothelium, causing a thrombotic microangiopathy within capillaries and arterioles, eventually leading to renal failure potentially requiring renal transplantation.[192] Low levels of circulating C3 (indicating increased C3 consumption) and raised C3d (indicating increased C3 activation) are found in aHUS, reflecting systemic activation of the complement system.[193;194] Reducing complement activation with intravenous Eculizumab, a monoclonal humanised antibody against complement C5, can lead to clinical improvement.[195]

Atypical HUS is associated with underlying sequence variation in genes encoding proteins involved in the alternative complement pathway, including *CFH*, *C3*, *CFI, CFB* and deletions in *CFHR1-3*.[193;194] Over 100 SNPs in the *CFH* gene have been reported in patients with aHUS (including the Y402H SNP), the majority which are nonsense or missense mutations.[196;197]

Remuzzi et al was the first to describe the possibility of modulating hepatic CFH production through LT. They attempted to treat aHUS, a life-threatening condition associated with a mutation in the *CFH* gene, by transplanting a liver with a *CFH* gene without this mutation.[198]

Liver transplantation - background

Approximately 600 LTs are carried out in the UK annually. The European Liver Transplant Registry in 2003 reported the primary indications for over 40,000 LTs performed over a 13 year period. The most common indication is cirrhosis, due to either chronic hepatitis virus infection (especially hepatitis C), or alcohol abuse. Other causes include cholestatic disease (blockage of biliary flow), including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). The most frequent cancer indication is hepatocellular carcinoma (HCC). The most common cause in the UK of acute liver failure requiring LT is paracetamol overdose.[199] The European Liver Transplant Registry of 2003 also reported patient survival rates, which were 80% for one year, 79% after five years, and 62% after ten years. Survival rates are increasing with improved surgical technique and immunosuppression.[200]

How can liver transplantation treat atypical HUS?

In aHUS associated with a *CFH* gene mutation, the existing liver produces dysfunctional CFH protein. This leads to dysregulation and over-activation of the alternative complement pathway, with ensuing renal damage. Renal transplantation on its own is often not successful, due to recurrent disease in the donor kidney. Co-transplanting a new liver carrying a *CFH* gene without the implicated sequence variation aims to restore normal CFH protein production, reducing recurrence of disease in the donor kidney.

The patient treated by Remuzzi et al was an Argentinian two year old boy with life-threatening renal failure secondary to aHUS. Genetic testing revealed a

heterozygous non-synonymous SNP (3620T>A), resulting in an amino acid change (Trp1183Arg) in SCR20 of the CFH molecule. Elevated plasma C3d levels and C3d:C3 ratio indicated a state of increased alternative complement pathway activation, presumably due to dysfunctional CFH protein. Renal transplantation was combined with transplantation of a new liver carrying a *CFH* gene without the Trp1183Arg SNP, to ensure systemic delivery of non-mutant CFH protein. Unfortunately this liver rejected after 26 days, and a second liver was transplanted a few days later. Although the *CFH* genotype of the second donor liver is not mentioned in the paper, both plasma C3d and C3d:C3 ratios returned to normal, and more importantly renal function stabilised, suggesting the new liver also carried a *CFH* gene without the Trp1183Arg SNP.[198] Subsequent case reports have described more successful combined renal and liver transplantation in HUS, with stabilisation of both renal and hepatic function long-term.[201-205] However multiple organ transplantation is fraught with risks, and some fatalities have occurred from graft failure or the effects of immunosuppression.[206;207]

It is also possible to develop aHUS if given a donor liver carrying a *CFH* gene sequence variation, as described by Brown et al.[208] These cases therefore indicate that it is possible to manipulate systemic CFH protein production through alteration of hepatic *CFH* genotype with LT.

Liver transplantation and AMD

LT is of course not a therapeutic option for AMD. However LT patients provide a unique opportunity to investigate whether modulation of systemic CFH protein production by altering hepatic *CFH* genotype through LT, can have a bearing on AMD pathogenesis, without altering local intraocular CFH protein production. This would provide evidence as to whether systemic or intraocular CFH protein is more important in AMD pathogenesis, and suggest whether systemic or intraocular CFH gene / protein therapy would be a better option in AMD management.

Professor Gregory Hageman (Moran Eye Centre, Utah, USA) has previously shown fundus images at several conferences of patients pre and post-LT depicting a reduction in macular drusen following LT, suggesting a change in protein production by the new liver, possibly from dysfunctional to functional CFH, may influence AMD development. However there are no published reports.

Since the *CFH* Y402H genotype is strongly associated with AMD and there is evidence that this SNP can affect complement activation, the effect of modulating this genotype through LT was explored.

1.5 Aim, hypothesis and objectives

The aim of this project was to determine whether local or systemic CFH protein production, through the alteration of hepatic *CFH* Y402H genotype by liver transplantation, was more important in AMD pathogenesis.

Therefore the hypothesis for this thesis was that AMD in LT patients was associated with donor, and not recipient *CFH* Y402H genotype. A secondary hypothesis was that systemic complement activation was associated with donor, and not recipient *CFH* Y402H genotype.

The primary objectives were therefore as follows:
1. Recruitment of LT patients (recipients)
2. Determining recipient AMD status
3. Determining recipient *CFH* Y402H genotype
4. Determining donor *CFH* Y402H genotype (donors referring to the individuals who donated the transplanted liver)
5. Measurement of complement protein levels in the plasma of LT patients to investigate for any associations with *CFH* Y402H genotype
6. Statistical analysis to determine whether donor or recipient *CFH* Y402H genotype is associated with AMD / plasma complement levels

The results of this project will contribute to the understanding of AMD pathogenesis, and also provide some guidance to whether local or systemic complement therapy would be more successful in managing AMD.

1.6 Thesis structure

The remaining thesis is divided into the materials and methods chapter, describing how each of the primary objectives was achieved, followed by the results chapter, describing what was found for each objective. The implications of these results are then described in the discussion.

CHAPTER 2 Materials and Methods

This chapter describes the main theoretical principles behind the methods used, followed by the specific methods chosen for evaluating each objective, including how these methods were chosen.

2.1 Methodology principles

2.1.1 Polymerase chain reaction

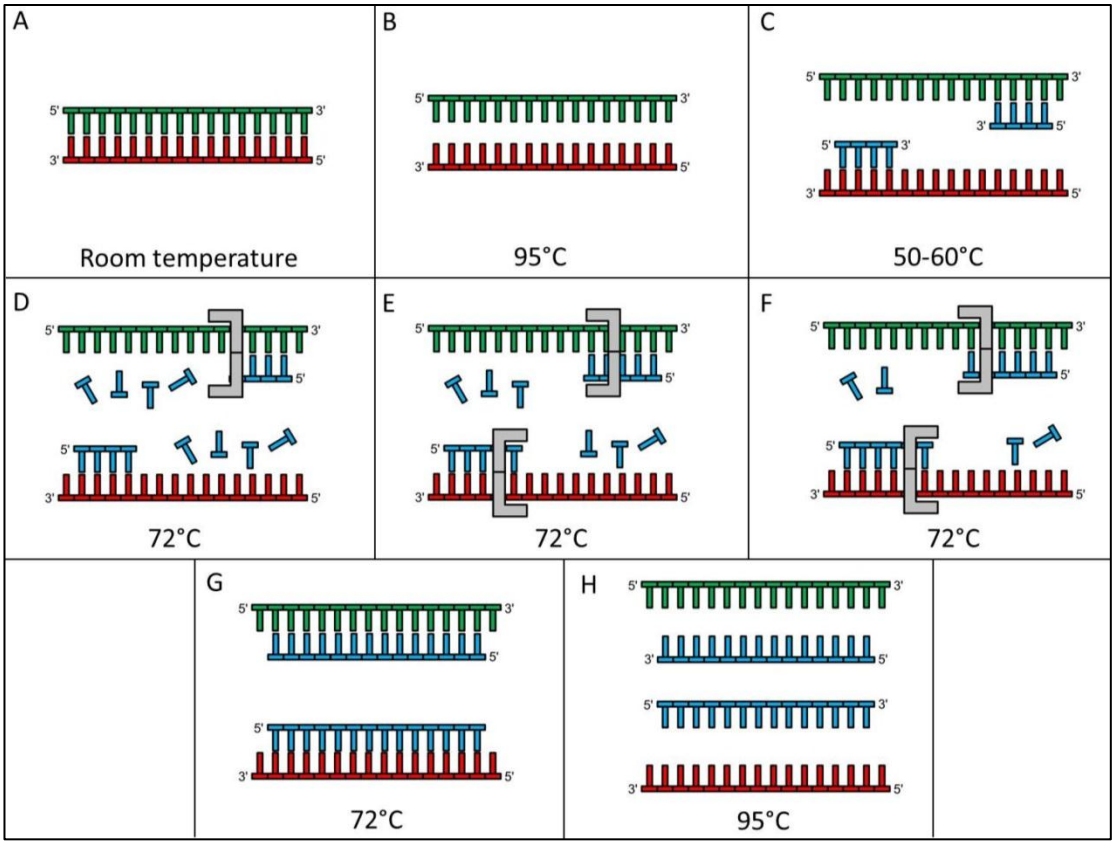
Before DNA can be analysed in the laboratory, the DNA sequence of interest is amplified many times to produce more measurable quantities. This process of *in vitro* DNA replication is known as the Polymerase Chain Reaction (PCR), and utilises changes in DNA characteristics with varying temperature. *Taq* polymerase originally acquired from the bacterium *thermus aquaticus* in hot springs is the DNA polymerase enzyme of choice in PCR, since it is activated only above a certain temperature, and is resistant to high heat. Figure 2.1 summarises the steps of a typical PCR.

The constituents of a typical PCR reaction are as follows:

1. DNA to be targeted
2. Primers – a pair of forward and reverse primers (18–25 nucleotides long) is required to bind to the forward and reverse DNA strand immediately flanking the target sequence.
3. dNTPs (pool of deoxyriboNucleotide TriPhosphates to form new strand)
4. *Taq* polymerase
5. $MgCl_2$ (this is required as a cofactor for binding of the other constituents to each other, and concentrations need to be optimised)
6. Buffer (usually made up of tris, which is tris(hydroxymethyl)aminomethane).

Amplicon concentration usually levels out after 30–40 cycles, due to depletion of reagents and accumulation of inhibitors. Annealing temperatures and magnesium concentrations may need to be optimised to enable successful PCR.

Figure 2.1: PCR cycle



A–H show the steps of a PCR cycle in chronological order. A: At room temperature forward DNA strand (green) binds to its complementary reverse strand (red) to form the double stranded DNA helix. B: The temperature is raised to around 95°C – this denatures the double-stranded DNA two single DNA strands. C: Lowering the temperature (typically around 50–60°C) allows annealing of forward and reverse primers (blue) to their complementary DNA sequence. The primers are designed to border the DNA sequence of interest to be amplified. D: Heating to a higher temperature (72°C) then activates Taq polymerase (grey), and DNA replication is initiated. E–G: Taq polymerase adds free nucleotides (blue) to the primer, based on the complementary strand sequence, to form two new double-stranded molecules bordered by the forward / reverse primers. H: The cycle is then repeated as the temperature is once again raised to 95°C, to denature the new DNA strands. In this way the region of interest is amplified exponentially. Redrawn based on diagram from University of Leicester web site.[209] Information from Strachan T. Human Molecular Genetics, 2011.[10]

Designing the ideal primer

Certain properties of the primer sequence can improve the results of PCR amplification. Primers must have a high number of bases complementary to their target DNA strand to bind effectively. Primers also must be specific to the intended target sequence, to ensure only a single amplicon is produced from PCR. To prevent binding between the forward and reverse primers ("primer dimer"), the 3' end of a primer should not contain a sequence complementary to any region of the other primer. To prevent bases binding to other bases within a single primer ("hairpin"), self-complementary sequences (more than 3bp) are avoided.

To improve specificity, the temperature of the primer annealing step should be as high as possible, since low temperatures will cause primer bonding to non-specific DNA sequences with partly complementary sequences. The temperature at which the primer separates from its template is known as the primer melting temperature (T_M). Therefore the annealing temperature should not exceed the primer T_M, and usually the annealing temperature is set at 5°C below the primer T_M.

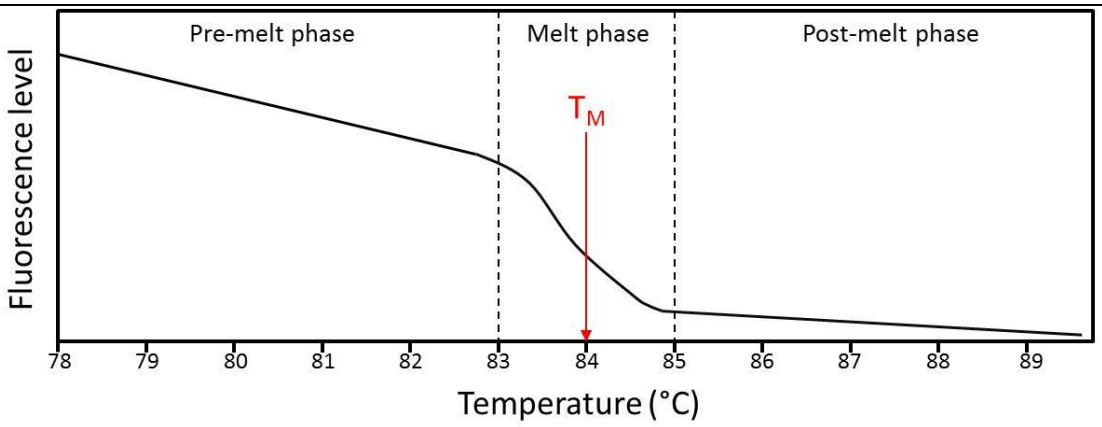
An important determination of primer T_M is the number of G-C base pairs a particular primer will form to its complementary strand. This is because a greater temperature is required to provide the energy to break the three hydrogen bonds between G-C base pairs, compared to the two hydrogen bonds between A-C base pairs. Optimal primers should have a GC content of between 40% and 60%, with an overall even distribution of nucleotides. The T_M of primer pairs should also be similar (not more than 5°C difference). The amplicon T_M should not differ from the primer T_M by more than 10°C.[10]

2.1.2 High resolution melt

High resolution melt (HRM) is a laboratory technique which can be used to discriminate SNPs. Double-stranded DNA (dsDNA) separates ("melts") into single stranded DNA (ssDNA) once the T_M is reached. High resolution melt (HRM) utilises an intercalating fluorescent dye, which has the property of being able to bind selectively to double, but not single-stranded DNA. Therefore fluorescence is only produced in the presence of dsDNA. The amount of fluorescence is

measurable and corresponds to the amount of dsDNA. HRM also requires equipment sensitive enough to alter temperature with small but precise adjustments, in order to precisely plot the melting of dsDNA. This is indicated by a fall in fluorescence, corresponding to gradually decreasing presence of dsDNA.[210;211]

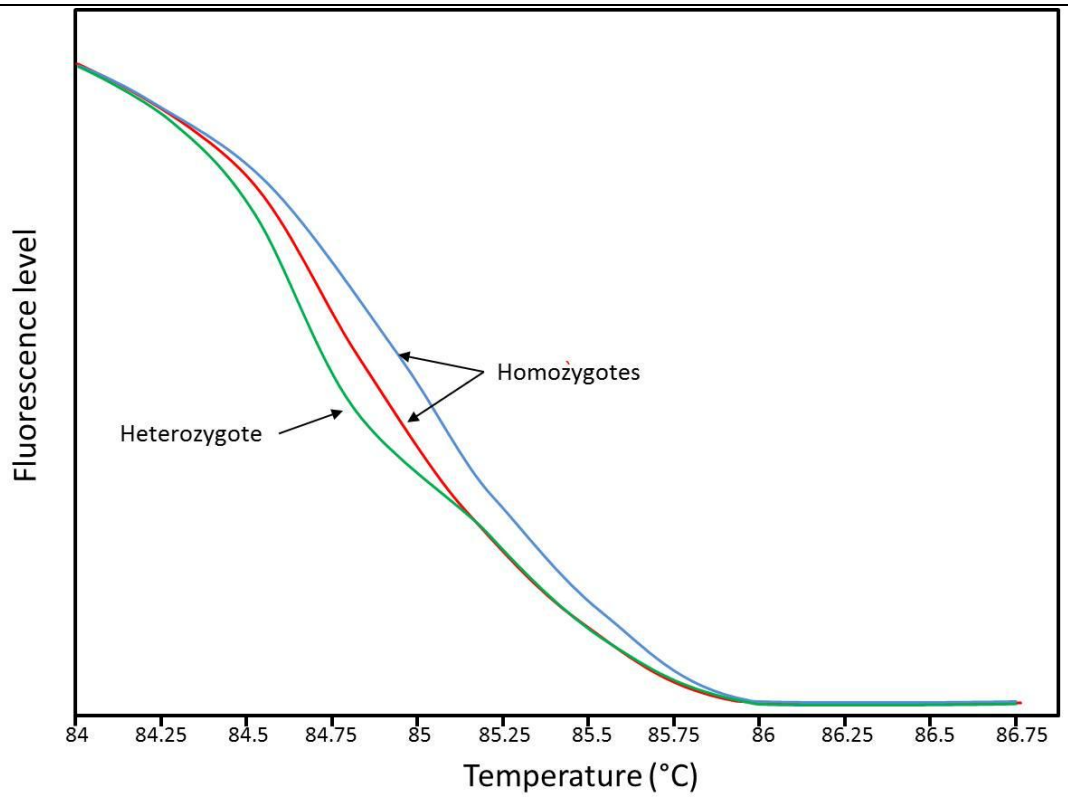
Figure 2.2: DNA melt curve



Graph showing DNA melt curve in the presence of an intercalating fluorescent dye which binds selectively to dsDNA. As the melting temperature is reached (T_M), dsDNA separates into ssDNA. Fluorescence therefore decreases rapidly during the "melt phase". T_M represents the midpoint of the melt phase, where the rate of change of fluorescence is greatest. Redrawn from Rotorgene 6000 Protocol.[210]

HRM can identify SNPs since a single nucleotide change in a sequence of DNA can cause an alteration in the melting temperature profile, allowing identification of homozygous and heterozygous variants of the SNP (Figure 2.3). Melting temperature shifts vary according to the base change (Table 2.1).

Figure 2.3: SNP analysis by HRM



HRM curves showing example of SNP discrimination for one sample. Melt curves for homozygotes (either 2 major alleles or 2 minor alleles) have similar shape to each other, and are discriminated by a shift in the T_M. This occurs due to a variation in binding strength between differing allele types.

The melt curve for the heterozygote genotype is differentiated by a change in shape from the homozygous melt curve. This occurs since as heterozygous dsDNA enters the melting phase, annealing occurs between the ssDNA to create both homozygous and heterozygous dsDNA. As temperature rises, these strands will also melt and an amalgamation of melt curves results. Redrawn from Rotorgene 6000 Protocol.[210]

Table 2.1: SNP classes according to melt curve

SNP class	Alleles (base variation)	Typical melting temperature shift	Prevalence in human genome
1	C/T and G/A	Large (>0.5°C)	64%
2	C/A and G/T	↓	20%
3	C/G	↓	9%
4	A/T	Very small (<0.2°C)	7%

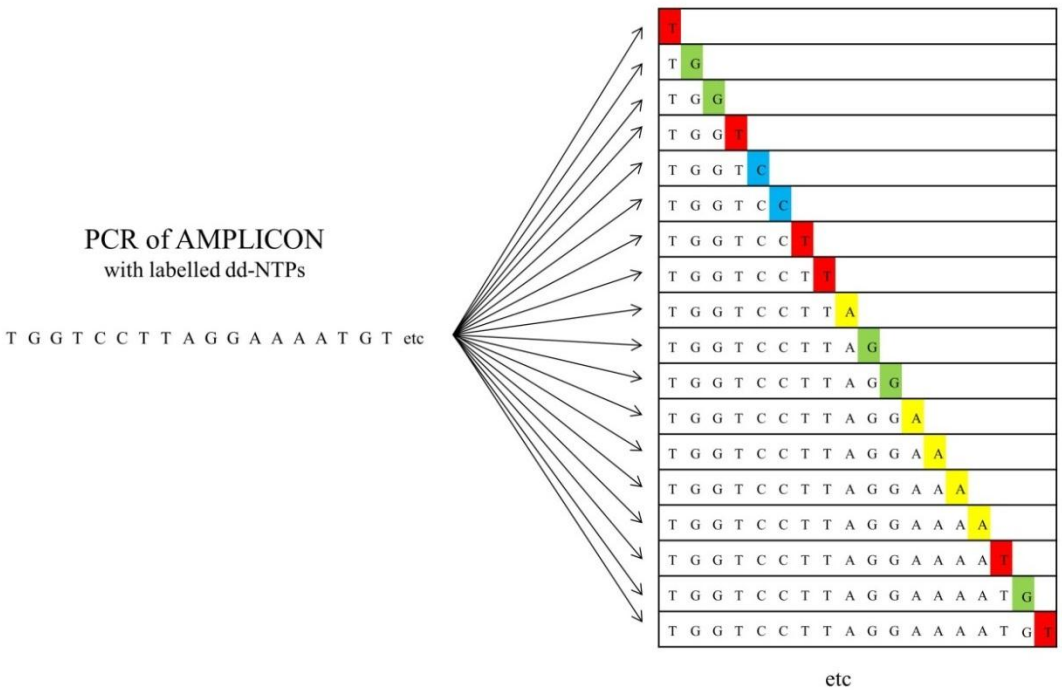
Single nucleotide polymorphisms (SNPs) can be grouped together into 4 classes based on typical melting temperature shift as one base is replaced by another.[211] Eg. C/T represents a SNP with alleles C and T, resulting in a large shift of melting temperature. Prevalence of SNPs in human genome taken from Venter et al, 2001.[212] Table redrawn from Rotorgene 6000 Protocol.[210]

2.1.3 DNA sequencing by dye terminator chemistry

"Dye Terminator Chemistry", a modified form of Sanger sequencing, can determine the sequence of a strand of DNA. Sanger sequencing, the originally described method of DNA sequencing, was first described by Sanger et al in 1977.[213] Dye terminator chemistry utilises modified fluorescent-labelled dideoxynucleotides (ddNTPs) – "dye-terminators". A ddNTP is a nucleotide base analogue which lacks an essential component (3'-hydroxyl group). As a result, the phosphodiester bond, which is required for attachment of one nucleotide to another within a single DNA strand, cannot form.

PCR takes place using either normal nucleotides (deoxynucleotide triphosphates – dNTPs) or ddNTPs. If a ddNTP is added, the sequence extension is terminated. This results in a range of DNA fragments, with differing lengths from one nucleotide to the whole DNA amplicon corresponding to the PCR product. Each fragment ends with a fluorescent-labelled ddNTP detectable by capillary electrophoresis (see below). Each of the four ddNTPs (ddATP, ddTTP, ddGTP, ddCTP) is labelled with a tag which fluoresces at a different wavelength, therefore each ddNTP type can be identified (Figure 2.4). The improvements of dye terminator chemistry over the original Sanger sequencing method included use of dyes instead of radioactive labelling, the absence of requirement for gel electrophoresis, and the ability to perform the entire reaction in a single tube.[214]

Figure 2.4: Cycle sequencing reaction



Each fragment ends with a fluorescent-labelled ddNTP. Each of the four ddNTPs (ddATP, ddTTP, ddGTP, ddCTP) is labelled with a tag which fluoresces at a different wavelength, therefore each ddNTP type can be identified (represented by different colours above). Own diagram.

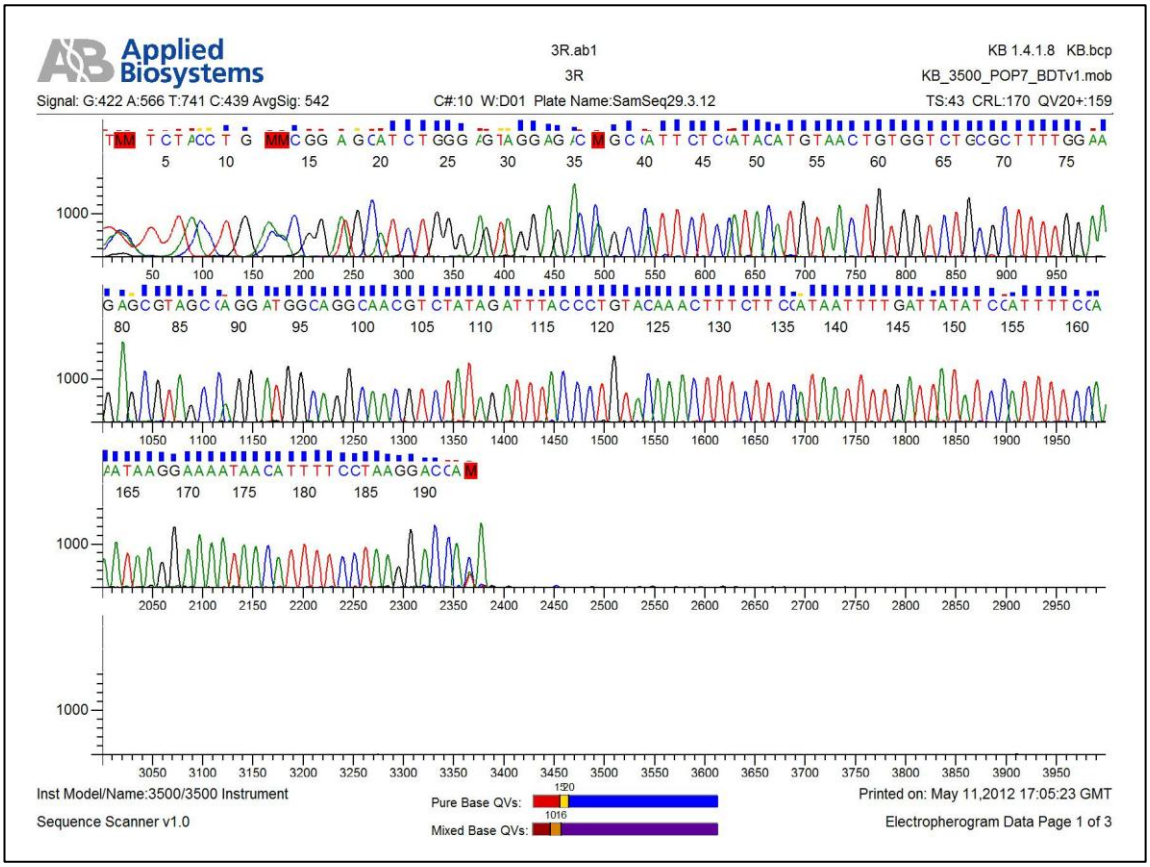
Capillary electrophoresis is then used to determine the DNA sequence. A capillary is inserted into each sample tube, and an electric current applied to allow migration of the negatively charged DNA fragments through the capillary. The smallest DNA fragment migrates the quickest and passes through the capillary first. This is similar to gel electrophoresis, however capillary electrophoresis can separate DNA fragments with a resolution of one base. Activation of a laser beam, present at the other end of the capillary, enables fluorescence of each ddNTP. An optical detection device detects each ddNTP-associated fragment as it passes through the capillary in size order. The wavelength of each of the four types of ddNTP can be differentiated by the detector. An emission spectrum is therefore obtained for each amplicon (the chromatogram), from which the sequence could be determined.

Signs of a reliable chromatogram include evenly-spaced peaks. Variation of peak amplitude up to three-fold is acceptable. Noise (baseline peaks) should be

minimal. The first few bases are usually difficult to read, and there is often overlap initially within the first 50 base pairs. This occurs due to co-migration of the smallest fragments)[214;215], and also due to the poor recovery of very short DNA fragments, during the pre-capillary electrophoresis purification step.

An example of a chromatogram is shown in Figure 2.5.

Figure 2.5: A Chromatogram from DNA sequencing



The chromatogram shows the fluorescence levels detected (y axis) with size of the detected fragment (x axis). The smallest fragment will be detected first, and is shown on the upper left of the chromatogram. The four ddNTPs (corresponding to each base) each have differing fluorescence wavelengths, and therefore can be detected individually. The chromatogram gives each base type a different colour, to allow easy identification. Above each base is a coloured bar – this represents the Quality Value (QV) score. This is a score produced by the DNA analyser to indicate the accuracy of each base call. The QV score is defined as $-10 \log_{10} (P_e)$, where P_e is the probability of error. The size of each bar is proportional to the base QV score. The significance of bar colour is summarised in Table 2.2. Ideally bases should have blue bars indicating a QV 20+, which implies a high accuracy of base call. "M" refers to a mixed base call. According to standardised nomenclature (International Union of Biochemistry – IUB), M="aMino bond" = A + C. Note the first 50 or so bases have reduced QV scores, implying reduced accuracy.

Chromatogram obtained from this study.

Table 2.2: Quality Value (QV) scores and the chromatogram

Colour of bar	QV	Accuracy of base call
Blue	20+	Very good
Yellow	10-20	Possibly correct
Red	0-9	Poor, unreliable

2.1.4 Enzyme-linked immunosorbent assays

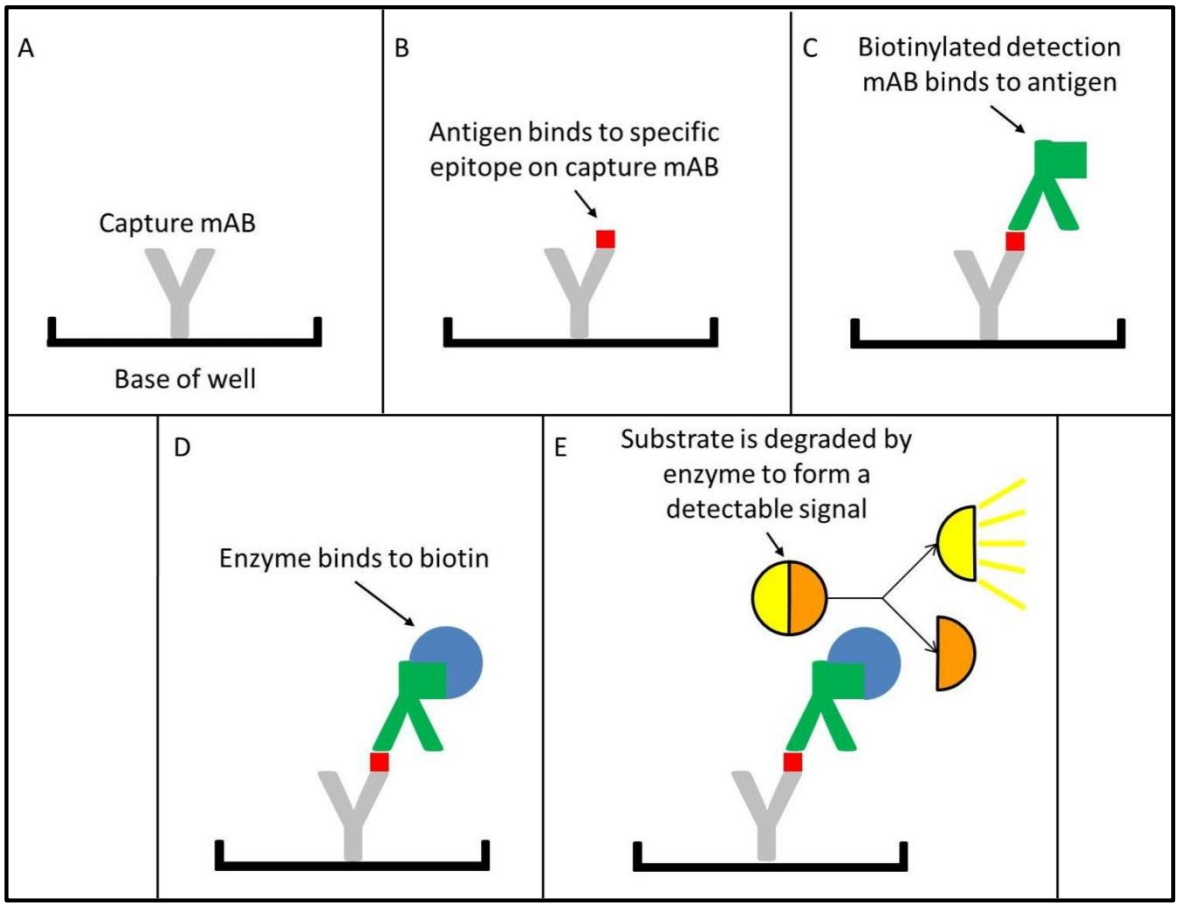
Enzyme-linked immunosorbent assays (ELISAs) are used for the detection and quantification of peptides, proteins, antibodies and hormones. ELISA requires fixation of an antigen to a solid surface ("immunosorbence"). An antibody (see Figure 1.13) attached to an enzyme ("enzyme-linked") is added – this antibody is able to bind specifically to the adsorbed antigen. A substrate is added, which will be converted by the enzyme to a detectable coloured product. The level of colour change will correspond to the amount of antigen, and is measurable quantitatively.

"Sandwich" ELISA is the most common form of ELISA. The antigen measured is bound between two monoclonal antibodies (mAB). The "capture antibody" is attached and immobilised to the plate prior to sample addition, and the "detection antibody" added subsequently. Essential requirements of both the capture and detection antibodies are the need be able to recognise two non-overlapping epitopes on the same antigen, and the absence of interference with each other.[216] ELISA is performed on multi-well plates. Plates are incubated for up to one hour after each step, to allow sufficient binding, usually followed by several washes with a wash buffer to remove any unbound components.[216]

The steps of a sandwich ELISA are as below.
1. The specific capture monoclonal antibody is attached to the base of each well. Plates may already come prepared, alternately this may need to be done manually (Figure 2.6A)
2. Samples are diluted to a specific concentration (determined by the individual protocol) and then added to each well (Figure 2.6B). Positive standards of known concentrations are added to each plate, to allow a standard curve to be plotted.

Plates are incubated to allow the antigen of interest within the samples to bind to the antibody at the Fab site, and washed.
3. A biotinylated detection mAB (attached covalently to a biotin, ie. vitamin B7) is then added to each well (Figure 2.6C). Plates are then incubated and washed.
4. An enzyme (eg. horseradish peroxide HRP or alkaline phosphatase AP) conjugated to streptaviridin is added to each well. Streptaviridin is used due to its ability to bind strongly to biotin, therefore the enzyme is attached indirectly to the detection antibody and therefore the antigen of interest (Figure 2.6D). Plates are then incubated and washed.
5. A substrate (eg. TetraMethylBenzidine, TMB) is then added to each well. The substrate is degraded by the enzyme (HRP/AP). After a brief incubation, the reaction is stopped with an acid, e.g. sulphuric, phosphoric or oxalic acid. The substrate changes colour to form a measurable chromogenic signal, eg. TMB appears blue when oxidised by HRP, and yellow after addition of acid (Figure 2.6E).
6. The plate is then placed in a spectrophotometer set to measure optical density (OD) at a particular wavelength determined by the substrate used (eg. TMB, following stopping of the reaction by acid, has a detectable wavelength of 450nm). For each plate, standards of known concentrations are then plotted against detected OD to obtain a standard curve. The concentration of the antigen of interest can then be determined from the standard curve based on the measured OD.

Figure 2.6: Stages of sandwich ELISA



A–E shows the stages of ELISA in chronological order. Redrawn based on figure from Mabtech Inc. website.[217]

2.2 Recruitment of liver transplant patients

2.2.1 Choosing the methodology

A cross-sectional observational multicentre study was set up, to assess the point-prevalence of AMD in LT patients, and associating this with recipient / donor *CFH* Y402H genotype.

A prospective study would be study design of choice to investigate the influence of modulating liver genotype on the development of AMD, recruiting patients pre-LT. However prospective studies investigating the effect of intervention on AMD progression tend to be of at least five years duration, since AMD tends to progress slowly. For example, the AREDS study examining the effect of oral supplementation on AMD had a follow-up duration of 6.3 years.[218] A prospective study was therefore not considered feasible within the time constraints of a PhD.

Both recipient and donor information was collected - these variables were either known to be associated with AMD (eg. age, gender, smoking status[164]), or collected to explore for novel associations. Variables were used in logistic regression in the final analysis (see section on Statistics page 138).

2.2.2 Sample size

The hypothesis of this project was that in LT patients, presence of age-related macular degeneration (AMD) was associated with *CFH* HH402 and *CFH* YH402 risk genotypes in the donor rather than the recipient liver.

The *CFH* Y402H risk allele is C (cytosine). Therefore sample size depended on the expected prevalence of AMD in LT patients, and the expected prevalence of donor *CFH* Y402H C allele in LT patients with and without AMD.

The expected prevalence of AMD in LT recipients was assumed to be 37.1%, based on reported figures of AMD prevalence in the general population (Rotterdam Study population), with similar age and ethnicity.[26]

Based on reported figures from a case-control study by Hageman et al, the prevalence of donor *CFH* Y402H C allele frequency was expected to be 0.560 in patients with AMD, and 0.341 in patients without AMD (Table 2.3).[9] An assumption was made that donor ethnicity was of Western European origin, although this was unknown due to donor confidentiality. A further assumption was made that a history of LT was not associated with *CFH* Y402H mean allele frequency.

Table 2.3: Expected minor allele frequency of *CFH* Y402H in AMD and non-AMD patients

***CFH* Y402H rs1061170 C allele**	**AMD**	**Controls**
C (risk) allele count	1066	275
T allele count	838	531
Total allele count	1904	806
Risk allele (C) frequency	0.560 (1066/1904)	0.341 (275/806)

Figures derived from Hageman et al[9] (online supplementary information, table 3).

Sample size was calculated assuming final analysis by logistic regression (SPSS SamplePower V3.01, IBM Corporation, New York, USA). The figure calculated was **174**, based on an event rate (presence of *CFH* Y402H C allele in the donor) of 0.560 in the AMD group, and 0.341 in the non-AMD group, assuming 37.1% of LT patients would have AMD. Power was set at 80%, and significance (alpha) at 0.05.

2.2.3 Ethics statement

This study was approved by the National Research Ethics Service Committee South Central – Southampton B, UK (08/H0504/191 – approval letter in appendix). The study was conducted according to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients prior to study recruitment.

2.2.4 Site set up and patient recruitment

Patients were recruited from four LT sites based in the UK. Site principal investigators (one ophthalmologist and one hepatologist / liver surgeon per site) are listed in Table 2.4.

Table 2.4: Sites and principal investigators (PI)

SITE	LIVER UNIT PI	EYE UNIT PI
Queen Elizabeth Hospital Birmingham, UK	Prof David Adams	Prof Jonathan Gibson
Addenbrooke's Hospital Cambridge, UK	Dr Graeme Alexander	Prof Keith Martin
Kings College Hospital London, UK	Prof Nigel Heaton	Ms Sobha Sivaprasad
University Hospital Southampton (UHS), Southampton, UK	Dr Kathryn Nash	Prof Andrew Lotery

Eligible patients were selected from a pre-existing hospital database by a member of the patient's own clinical team (this was required by the ethical committee). These patients were sent invitation letters along with patient information leaflets, and those willing to take part were asked to register their interest by sending a signed reply slip back. These patients were then seen in research clinics conducted on one of the four sites, ideally on the same day of their normal liver clinic follow-up appointment for convenience. Examples of patient invitation letters, patient information sheets, and consent forms are present in the appendix.

2.2.5 Inclusion and exclusion criteria

Ethnicity was restricted to those of Western European origin since as described previously, both minor allele frequency and association of the *CFH* Y402H polymorphism with AMD varies with ethnic background.[49;168]

Patients 55 years or over were selected since AMD prevalence typically increases after this age.[219] The selection of patients a minimum of five years post-LT was to allow adequate time for any modulation of hepatic *CFH* Y402H genotype to influence AMD development. This duration was based on the minimum follow-up period utilised in the Age-Related Eye Disease Study, which successfully demonstrated a change in outcome (AMD progression) based on a particular intervention (oral supplementation).[31] Since survival following LT is good (62% after ten years[200]), recruitment of LT patients aged 55 years and above was presumed feasible.

Patients discovered to have severe ocular disease were excluded, due to the possibility of misdiagnosing AMD. Examples included retinal vein occlusion, severe diabetic retinopathy. Patients were also excluded if fundus views were inadequate (eg. significant cataracts, inability to carry out slit lamp examination / fundus photography).

Liver transplants can fail acutely or chronically, due to amongst other reasons, rejection, hepatic artery thrombosis or resurgence of the original disease. These patients may require re-transplantation with a second or even third liver. This may affect the hypothesis of this study, since each new liver will have its own *CFH* genotype. However the duration between LTs varies. For purpose of this study, patients with a history of re-transplantation were only included if the total number of LTs was ≤2, the current transplant was of duration at least five years and the duration of the current transplant exceeded that of the original transplant.

2.2.6 Recipient and donor information collected

Information was obtained from several sources, including patient interview, medical records and archives. Information was obtained concerning both the recipients and donors. Recipient information included demographics, duration of current LT, underlying cause of LT, concomitant medical conditions, medications, smoking history, blood pressure, body mass index, hepatocellular dysfunction (example of proforma used for collecting information in appendix). Donor information obtained included age and gender. All variables were obtained in

order to investigate associations with the presence of AMD in LT patients. Details of each variable are given below.

Recipient information

Demographics: This included age and gender.

Duration of current LT: For patients with a history of two or more transplants, this was calculated for the current LT only.

Underlying cause of LT: Underlying causes were grouped for purpose of analysis into the following categories.

- Cholestatic disease, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).
- Infection, including the Hepatitis B and C viruses.
- Autoimmune causes, including PBC, PSC and autoimmune hepatitis.
- Inflammatory causes, including autoimmune and infectious causes.

Concomitant medical conditions: Common concomitant conditions were recorded to explore any associations with AMD in LT patients. These included renal impairment, hypercholesterolaemia, diabetes, thromboembolic phenomena (ischaemic heart disease/cerebrovascular accident/transient ischaemic attack), and colitis.

Medications: All LT patients are on life-long immunosuppression, often on more than one agent. The type of agent and number of agents presently used was recorded. Other concomitant medications were also recorded.

Smoking history: Smoking history was indicated by pack-years, a measure of the number of cigarette packs smoked per patient over a lifetime. Pack-years was calculated using the formula (C x Y) / 20, where C = number of cigarettes smoked per day, and Y = number of years smoked. This assumes a single pack contains 20 cigarettes. If the patient had a history of intermittent smoking and abstinence, the total number of years of actual smoking was used. Where cigarette use varied from one period to another, the patient was asked for the average number of cigarettes per day for the whole period.[220] For non-cigarette tobacco use (e.g. cigars), the equivalent cigarette number was determined, in

terms of tobacco weight. One cigar or pipe was considered equivalent to three cigarettes. One roll-up was considered the equivalent of one cigarette. Tobacco smoked in pipes and roll-ups were often quoted in ounces, with 1oz equal to 28.3 grams. Where tobacco amounts were specified by weight, one gram of tobacco was considered equivalent to one cigarette.[220]

Mean arterial blood pressure: Blood pressure was obtained either by direct measurement using a sphygmomanometer, or from medical records. Mean arterial blood pressure (MABP) was calculated (for ease of statistical analysis) using the equation DBP + 1/3 (SBP –DBP), where SBP = systolic BP (mmHg) and DBP = diastolic BP (mmHg).[221]

Body mass index: Weight and height were obtained at the time of recruitment, either by direct measurement or from medical records. Body mass index (BMI) was calculated from the equation $BMI = W / H^2$, where W = weight (in kg) and H = height (in metres).

Hepatocellular dysfunction: Hepatocellular dysfunction is reflected by raised plasma levels of liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Systemic levels of ALT and AST were obtained from routine blood tests. Any results within six months of the time of recruitment were included. Levels of ALT>35iU/L or AST >35 iU/L were interpreted as evidence of hepatocellular dysfunction (iU = international unit).[222]

Renal impairment: Renal impairment was defined as an estimated glomerular filtration rate (eGFR) <60 ml/min.[223] eGFR in turn was calculated from the widely used "Modification in Renal Disease Study Group" (MDRD) equation[224]: $eGFR = 32788 \times (\text{serum creatinine})^{-1.154} \times \text{age}^{-0.203} \times [0.742 \text{ } \textit{if female}]$. Serum creatinine values were obtained from routine blood tests (within six months of the recruitment date).

Donor information

Donor information collected included donor age and gender, obtained from a member of the recipient's clinical team (since identification of the donor remained anonymous to all but the recipient's clinical team).

2.2.7 Bloods collected and immediate processing

20ml blood was collected from the patient in 2x10ml Ethylenediaminetetraacetic acid (EDTA) tubes for DNA extraction and plasma analysis. EDTA is an anticoagulant which prevents clotting of whole blood, therefore enables DNA extraction. EDTA is preferred over the other commonly used anticoagulants (sodium / lithium heparin) due to a relative improvement in DNA preservation and reduced inhibition of DNA polymerase activity downstream.[225;226] Plasma for purpose of complement analysis was also collected in tubes containing EDTA; EDTA has the property of minimising *in vitro* complement activation by binding calcium ions necessary for complement activation.[227]

All blood samples were processed within 1-2 hours (maximum four hours) of acquisition. Care was taken to minimise unnecessary freeze/thaw cycles throughout all stages of blood processing, since this could affect quality of DNA and measurement of plasma complement levels.[226;228]

1x10ml blood in EDTA tubes was immediately frozen at -80°C for DNA extraction at a later stage. For plasma acquisition, 10ml blood in EDTA tubes were centrifuged at 2600 revolutions per minute (rpm) for 10 minutes at room temperature. Supernatant plasma was then divided into pre-labelled polypropylene microcentrifuge tubes, and frozen at -80°C. All samples were transported to the Clinical Neurosciences department, University of Southampton, under ice blocks or dry-ice, and stored at -80°C pending analysis.

2.3 Determining recipient AMD status

AMD status of recruited LT patients ("recipients") was determined from fundus examination and fundus photography, and the prevalence compared to the general population.

2.3.1 Choosing the methodology

Grading systems considered for AMD grading included the AREDS and Rotterdam study classification systems, both used in recent clinical studies.[26;218] Choice of grading system was determined by its use in a previous study of patients representing the general population of similar age and ethnicity as the LT patients in this study, and with available AMD and *CFH* Y402H prevalence data.

Patients recruited in the Rotterdam Study were Western Europeans aged 55 years and older (similar criteria to the LT patients).[26] Patients recruited in the AREDS were "Non-African Americans" aged 55-80 years old; however whereas patients recruited in the AREDS were subject to specific inclusion / exclusion criteria[229], patients in the Rotterdam study were recruited to represent the general population living in Rotterdam at the time. Prevalence of both AMD and *CFH* Y402H sequence variation in patients from the Rotterdam Study has been reported.[26;166] Therefore the Rotterdam Study grading system was chosen to grade AMD in LT patients, to allow greater validity of comparisons of AMD and *CFH* Y402H prevalence between LT patients and age and ethnicity-matched general population.

2.3.2 Examination of patients

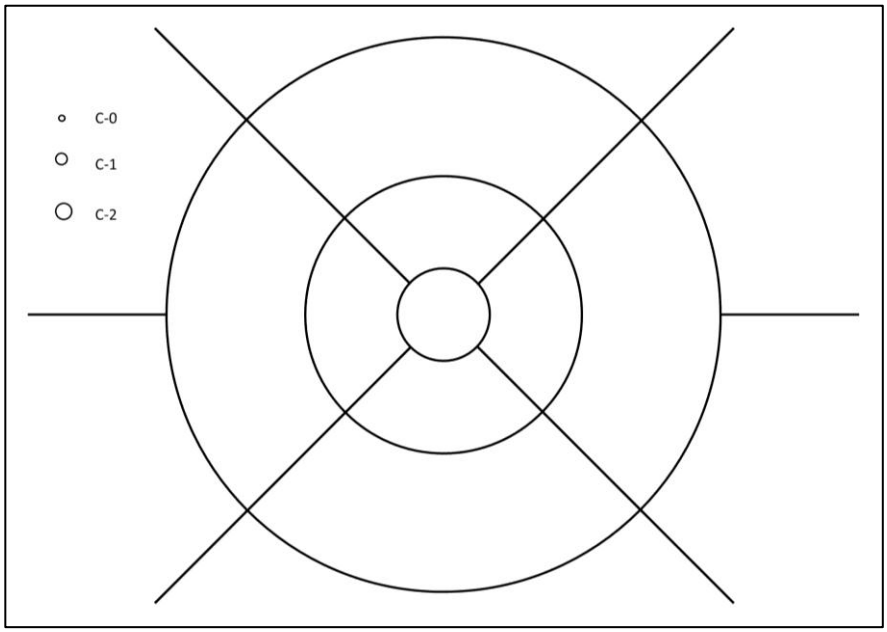
The pupils of all patients were dilated to allow adequate examination and photography of the macula. One drop of tropicamide 1% (Bausch + Lomb, Rochester, New York, USA) was instilled in each eye. Any clinical features of AMD (such as drusen, pigmentary changes, bleeding, fluid, elevation, scarring) were noted during slit lamp biomicroscopy examination. Photographs of the macula of each eye were taken using a digital fundus camera. The Topcon TRC 50DX fundus camera (Topcon Corporation, Tokyo, Japan) was used all patients except for those

recruited from Birmingham, where the portable Nidek NM-200 (Nidek Co. Ltd, Gamagori, Aichi, Japan) was used.

2.3.3 Rotterdam grading system

The Rotterdam Study grading system used a standard grid superimposed onto photographs of the macula, and any AMD lesions (drusen or pigmentary changes) measured using standard circles of known diameter (Figure 2.7).[230]

Figure 2.7: Standard grid for AMD grading



The standard grid consisted of three concentric circles, with diameters defined according to multiples of a normal optic disc diameter (taken as 1500nm). The outer, middle and inner circles had diameters x2, x1 and x1/3 that of a normal optic disc respectively. Four radial lines were positioned at 45°, 135°, 225° and 315°, to form a grid. Two horizontal lines projected from the outer circle at 3 and 9 o'clock, to assist with correct positioning. Standard circles for measuring AMD lesions were created based on defined diameters, as follows. C-0: 0.042 disc diameter (DD), C-1: 0.083DD, C-2: and 0.117DD. (created using Microsoft Powerpoint 2010). Redrawn based on Klein et al (1991) and Klaver et al (2001).

26;230

This grid was superimposed on fundus photographs using Microsoft Powerpoint software. The fundus photograph was adjusted with respects to size and rotation, to allow correct grid positioning. The outer circle of the grid was sited at the temporal edge of the optic disc, and the central circle positioned at the macular centre. The normal macular centre (fovea) was identified visually as the area devoid of blood vessels and with greater levels of pigmentation. Red-free images were also viewed. These were produced using photo-editing software (Corel

Photo-Paint X5, Ottawa, Canada). This assisted in identifying otherwise invisible drusen.

Eyes were allocated a grade from 0-4, in increasing order of AMD severity (Table 2.5). Non-AMD causes of pigmentary irregularities and GA causes were NOT included. These included retinal vascular disease (diabetic retinopathy, retinal vein occlusion), chorioretinitis, high myopia, trauma, congenital disease, and previous photocoagulation.

All grading was carried out within 3000μm radius of macular centre (within the large circle of the grid). The worse eye defined the overall AMD grade allocated to a particular patient. Grades 1-4 represented a diagnosis of AMD. Primary grading was carried out separately by two graders, who were blinded to each other's grading. Any discrepancies in primary grading were arbitrated by a senior grader (Professor Andrew Lotery), who provided the final grading.

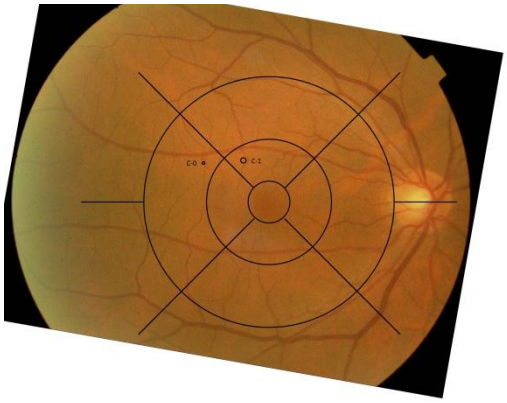
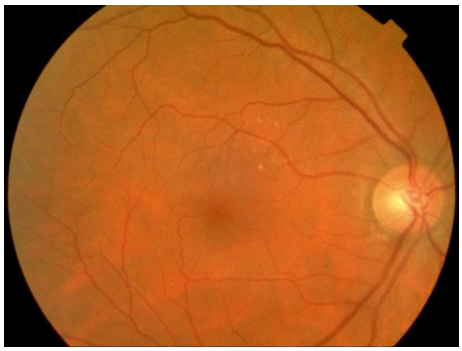
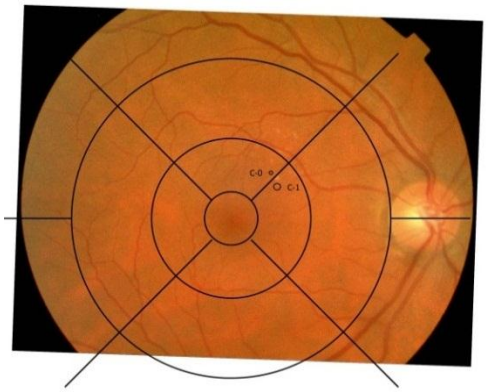
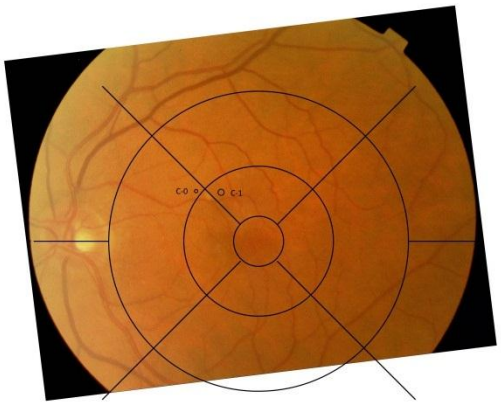
Table 2.5: Rotterdam classification of AMD

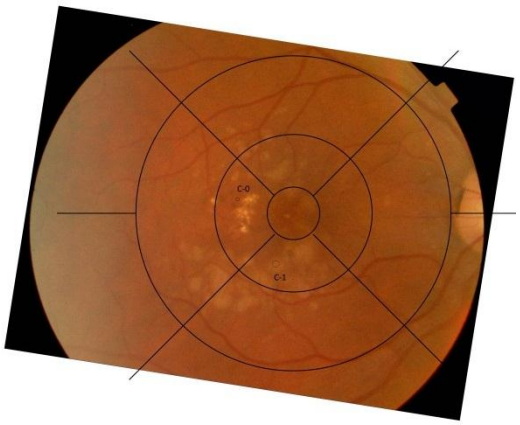
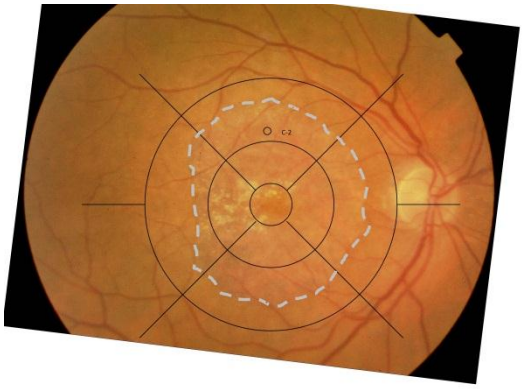
ARM grade	**Features**
0	Drusen <63µm
1a	Drusen ≥63µm and <125µm, no pigmentary irregularities
1b	Pigmentary irregularities only
2a	Drusen ≥125µm or reticular drusen
2b	Drusen ≥63µm and <125µm, with pigmentary irregularities
3	Drusen ≥125µm or reticular drusen + pigmentary irregularities
4	GA: Minimum area ≥ C2 (175µm) anywhere within grid Any CNV (including fibrosis from previous CNV)

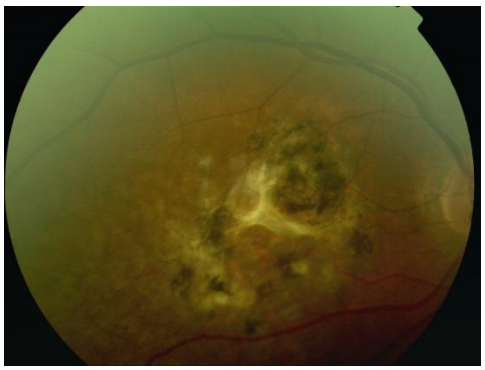
Rotterdam Study grading system[26]. Pigmentary irregularities = Hypo/hyperpigmentation ≥ 63µm. GA = geographic atrophy, defined as a well-demarcated area of RPE atrophy with sharp edges and visible choroidal vessels.

Figure 2.8 shows examples of how patients recruited from this study were graded using the Rotterdam grading system.

Figure 2.8: Examples of fundus photographs and Rotterdam grading

GRADE	FUNDUS PHOTO	ANALYSIS
0		
	Fundus photo rotated and resized to fit grid. No AMD seen within grid	
1a		
	Fundus photo rotated and resized to fit grid. Small drusen within grid, with greatest linear dimension (GLD) greater than circle C0, but less than circle C1 (ie. between 65 and 125μm). No pigmentary changes	
2a		
	Fundus photo rotated and resized to fit grid. Large drusen within grid, with GLD greater than circle C1 (>125μm). No pigmentary changes	

3		
	Fundus photo rotated and resized to fit grid. Multiple large drusen within grid, each with GLD greater than circle C1 (>125µm diameter). Pigmentary changes at centre of macula, with atrophy and hyperpigmentation greater than circle C0 (>63µm diameter)	
4		
	Fundus photo rotated and adjusted to fit grid. Large area of geographic atrophy (GA, marked in grey hashed lines) at macular centre, with underlying choroidal vessels visible. Area of GA easily larger than circle C2 (>175µm diameter)	
4		(no analysis required since diagnosis clear)
	Subretinal haemorrhage secondary to active choroidal	

	neovascularisation	
4		(no analysis required since diagnosis clear)
	Significant subretinal fibrosis from previous nvAMD	

NB all photos from patients recruited from this study.

2.4 Determining recipient *CFH* Y402H status

Recipient *CFH* Y402H genotype was determined from DNA extracted from whole blood collected from recruited LT patients.

2.4.1 Choosing the methodology

Whole blood was collected in EDTA tubes (see page 96 for further information on blood collection and initial processing). DNA extraction was carried out using a modified "salting-out" method was used (based on Miller 1988[231]), developed and verified in-house at the Clinical Neurosciences Department, University of Southampton. DNA extracted using this modified method has been successfully genotyped and the results reported in several publications.[64;232;233]

The initial step in the genotyping for a particular SNP involved PCR amplification of a short sequence of DNA including the SNP of interest (page 78). A method of allelic discrimination was then required to determine the particular base variation at the SNP. The options considered included Taqman assays, DNA sequencing, high resolution melt (HRM), or KBioscience KASP genotyping.

Taqman assays use allele-specific probes (short DNA sequences) which can bind to the DNA sequence depending on the base variation. Perfect complementary binding between the probe and the complementary sequence on the DNA strand results in detectable fluorescence from the probe, whereas imperfect probe binding shows no fluorescence.[14] DNA sequencing with dye-terminator chemistry in conjunction with capillary electrophoresis has the ability to determine DNA sequence of up to 1000 nucleotides with a resolution of one base. This method was used for the human genome project, and until the recent development of next generation sequencing, was considered the gold-standard.[234] *CFH* Y402H genotyping has previously been successfully accomplished by both Taqman assays (Goverdhan et al[233]), and DNA sequencing (McKibbin et al[235]). However genotyping all samples using either Taqman assays or DNA sequencing was considered excessive in terms of cost for this project.

HRM was available in-house and was of more reasonable cost. However no previous studies exist reporting the use of HRM in *CFH* Y402H genotyping.

Therefore prior to using HRM in this study, the accuracy of HRM genotyping of *CFH* Y402H was investigated in stored DNA samples from a previous study, with known *CFH* Y402H status genotyped by other means.

Since HRM was not able to allocate *CFH* Y402H genotype successfully to all tested samples, all samples were outsourced to KBioscience Ltd (Cambridge, UK) for *CFH* Y402H genotyping. Selected samples successfully undergoing *CFH* Y402H genotyping by KBioscience were verified using DNA sequencing.

2.4.2 DNA extraction from whole blood using a modified salting-out method

The steps in DNA extraction were as follows:

1. Preparation of blood samples
2. Removing red blood cells
3. Protein digestion
4. Isolating DNA
5. Resuspension of DNA

These steps are described in more detail below. The method described by Miller[231] used buffy coats to obtain white cells by centrifuging tubes with anticoagulated whole blood, whereas the modified method used erythrocyte lysis buffer (ELB) to obtain these cells. Otherwise reagents used and quantities were similar. Quantities of reagents / chemicals specified below are for 10ml whole blood samples.

1. Preparation of blood samples

Whole blood samples were defrosted in a water incubator at 37°C for 20 minutes, then mixed at 4°C (rotator speed 11rpm). The low temperature of 4°C limits the degradation of DNA.

2. Removing red blood cells

35ml ELB was added to each blood tube. This was mixed for 30 minutes at 4°C (rotator speed 11rpm), and centrifuged at 1500rpm. This allowed the haemoglobin to rise, leaving white cells and protein to settle at the bottom of tube. The haemoglobin-containing supernatant was then discarded. This step was repeated two more times (with 30ml ELB), to “wash” the sample thoroughly,

eventually leaving a white-grey pellet. After pouring away the supernatant the third time, the tubes were then carefully placed upside-down on paper towels to drain the remaining supernatant, whilst leaving the pellet undisturbed

3. Protein digestion

150µl 40mg/ml protease was added to each pellet for overnight digestion of protein. This facilitated the removal of histone proteins to allow release of DNA, and degraded other proteins to allow DNA isolation. 3ml nuclear lysis buffer (NLB) was added to this mixture to release the nuclear contents. 250µl 10% sodium dodecyl sulphate (SDS) was also added. Also known as "sodium lauryl sulphate", SDS is a common detergent, and acts to disrupt lipids in cell and nuclear membranes, so cells burst open and release DNA. This mixture was left in a heated rocker (speed 22 rocks per minute) to mix overnight at 37°C.

4. Isolating DNA

1ml saturated sodium chloride (NaCl) salt was added to the mixture. The salt clumped together any protein and other debris, helping DNA to precipitate out later. This was centrifuged at high speed (14000rpm) for 5 minutes, at room temperature. The supernatant, containing DNA in solution, was pipetted into a 15ml conical tube, taking care not to include any solid matter at the base of the tube. Absolute (100%) ethanol at twice the volume of the supernatant was added. DNA, being insoluble in ethanol, precipitated out as a white strand. This was transferred to a microcentrifuge tube with 1ml 70% ethanol, and left at room temperature for at least 2 hours to "wash" the DNA.

5. Resuspension OF DNA

The microcentrifuge tubes were pulse-spun, and the supernatant ethanol carefully poured off without disturbing the settled DNA. The microcentrifuge tubes were then left open on their sides on the bench at room temperature for 15 minutes to allow the DNA to dry. DNA was then resuspended in 500µl TE (Tris and EDTA) buffer. The DNA was left to dissolve at room temperature overnight.

Potentially infected samples

There was an increased likelihood that blood samples would be infected with hepatitis B or C virus. Therefore the following precautions were undertaken, with the assumption that all samples were potentially infected. All blood samples were

initially processed in a ventilation cabinet until the final ELB wash. All emptied blood tubes were placed and sealed in 30ml Falcon conical tubes containing Virkon® (a disinfectant); these were then disposed via incineration. No glass equipment was used – all blood collection tubes were plastic. This was to reduce risk of breakage and consequent infection.

Reagent composition

Table 2.6 lists the reagents used during the DNA extraction process, along with instructions for preparation.

Table 2.6: Reagents used for DNA extraction

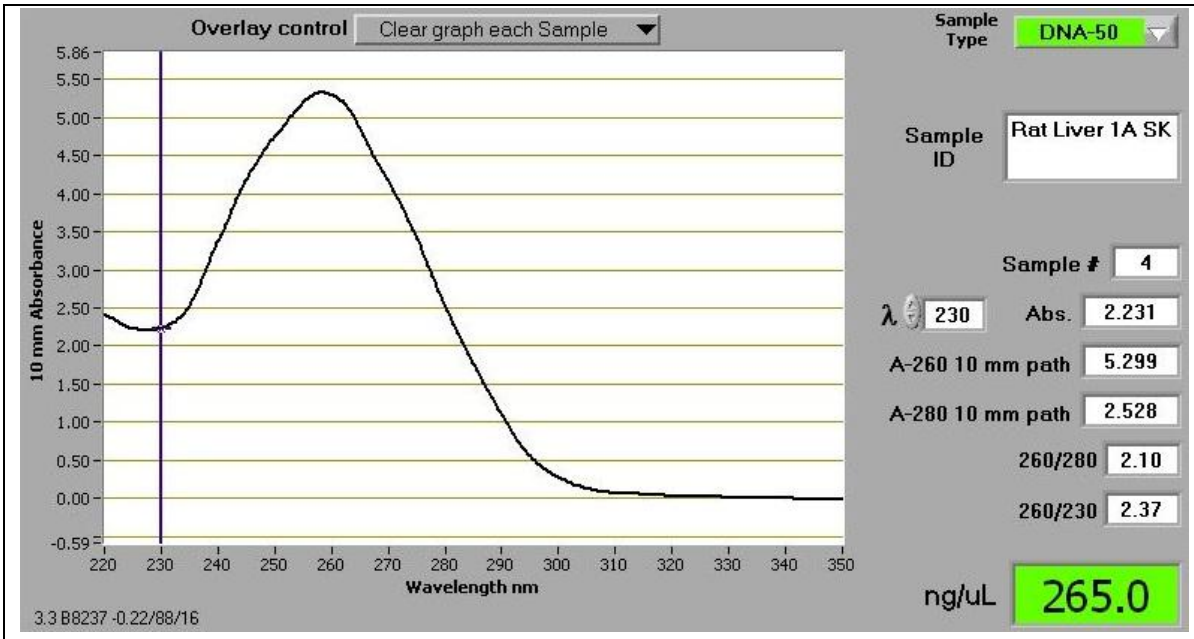
Reagent	Composition	Preparation
ELB	10.1g Potassium bicarbonate 82g Ammonium chloride 2.0ml EDTA (0.5M, ph8)	Make up to one litre with purified water. Then dilute 1 in 10 with purified water. Autoclave for 15 minutes at 121°C, then place in fridge ready for use.
NLB	40ml NaCl (5.0M) 5ml Tris (1.0M, ph8) 2ml EDTA (0.5M, ph8)	Make up to 500ml with purified water. Autoclave.
SDS	50g SDS powder	Make up to 500ml with purified water. Place on stirrer till fully dissolved.
Protease (40mg/ml)	800mg protease	Make up to 20ml with purified water. Dissolve and aliquot into 1.5ml tubes. Store at -20°C.
Saturated NaCl	35mg NaCl	Add to 100ml sterile water.
70% ethanol	350ml Absolute Ethanol	Add to 150ml sterile water to make 500ml.
TE Buffer	5ml Tris (1.0M, pH 7.5) 1ml EDTA (0.5M, ph 8.0)	Make up to 500ml with purified water. Autoclave in small (approx. 80ml) bottles

2.4.3 Standardising and storage of DNA

For DNA analysis, all stock DNA was diluted to 50ng/µl to standardise DNA concentrations. Stock DNA concentration was measured using a spectrophotometer (Nanodrop ND1000, Thermo Scientific, Wilmington, USA). A spectrophotometer measures the absorbance of light as it passes through a drop of the sample containing DNA. The samples were then diluted in a separate microcentrifuge tube using DNAse-free, RNAse-free and pyrogen-free water, to the required concentration.

Spectrophotometry also enables the quality of DNA to be assessed, through measurement of the "260:280 ratio". This is based on the absorbance levels (corrected for a 10mm path-length) for light with wavelength of either 260 or 280 nm passing through the sample. The 260.280 ratio varies for individual nucleotides – Guanine is 1.15, Adenine 4.50, Cytosine 1.51, Thymine 1.47, and Uracil 4.00. Since Uracil is found exclusively in RNA, a higher 260:280 ratio is typically found with RNA. Therefore, a higher 260:280 ratio for a particular sample indicates higher RNA content, and therefore lower DNA "purity". Generally acceptable ratios for DNA are 1.8-2.0, as suggested in the Nanodrop ND100 manual.[236] Figure 2.9 shows a typical sample reading of DNA concentration and quality from the Nanodrop ND100 spectrophotometer.

Figure 2.9: DNA quantity and quality analysis by the Nanodrop ND1000 Spectrophotometer



Analysis of a sample of DNA by the Nanodrop ND1000 Spectophotometer. The graph shows the absorbance of light by the sample (y axis) at different wavelengths of the xenon light (x axis). DNA concentration is indicated in the green box. The 260:280 ratio is quoted just above the green box.

Both undiluted and diluted DNA was stored at -20°C at the Clinical Neurosciences Department, University of Southampton, until required for analysis.

2.4.4 *CFH* Y402H genotyping by high resolution melt

The DNA sequence of interest was first amplified using PCR (page 78), and followed by HRM (page 80).

Protocol for *CFH* Y402H SNP analysis by HRM

Eight DNA samples were selected from a pre-existing database, with known *CFH* Y402H genotype. Three positive controls were also selected, corresponding to the three *CFH* Y402H genotypes (YY, YH and HH402). All genotyping of samples from the pre-existing database was previously carried out by KBioscience Ltd (page 115). Duplicates of all samples were also created for simultaneous analysis.

PCR was set up to create multiple copies of an amplicon containing the *CFH* Y402H SNP. Three pairs of primers (forward/reverse) were created flanking the *CFH* Y402H SNP (Table 2.7) using the web-based software Primer3 (http://frodo.wi.mit.edu).[237] The genomic sequence flanking the *CFH* Y402H SNP was determined using www.ensembl.org (version 58, accessed July 2010).[238] Neighbouring SNPs were identified (www.ncbi.nlm.nih.gov/snp/ accessed July 2010)[239] and avoided in both primer creation and product assessment, to limit interference with primer binding. Specificity of primers was tested using *in silico* PCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start. Assembly Feb 2009, GRCh37/hg19). Any primers not specific to the particular genomic region containing the *CFH* Y402H SNP were discarded. Primers were designed to conform as close as possible to the optimal primer criteria described before (page 80). Primers were manufactured by Invitrogen (Life Technologies Ltd, Paisley, UK).

Table 2.7: *CFH* Y402H HRM primers and resulting amplicons

PRIMER PAIR	DIRECTION	PRIMER SEQUENCE (5' to 3')	GC%	T_M (°C)	SIZE (bp)	FORWARD AMPLICON	T_M (°C)
1	Forward	TTTCCTTATTTGGAAA ATGGATATAA	23%	58.5	74	TTTCCTTATTTGGAAAATGGATATA Atcaaaat**C/T**atggaagaaagtttgtACAG GGTAAATCTATAGACGTTGC	80.5
	Reverse	GCAACGTCTATAGATT TACCCTGT	42%	57.9			
2	Forward	TTCCTTATTTGGAAAA TGGATATAA	24%	57.3	73	TTCCTTATTTGGAAAATGGATATAAt caaaat**C/T**atggaagaaagtttgtACAGG GTAAATCTATAGACGTTGC	80.8
	Reverse	GCAACGTCTATAGATT TACCCTGT	42%	57.9			
3	Forward	TTTCCTTATTTGGAAA ATGGATATAA	23%	58.5	82	TTTCCTTATTTGGAAAATGGATATA Atcaaaat**C/T**atggaagaaagtttgtacagg gtaaatctATAGACGTTGCCTGCCATC	82.8
	Reverse	GATGGCAGGCAACGT CTAT	53%	58.7			

HRM = High resolution melt, GC% = Percentage of G:C base pairing between primer or amplicon and complementary DNA sequence, T_M = Melting temperature (°C), Size = Amplicon size (base pairs). Position of the forward primer within the amplicon is shown (blue), and the sequence on the amplicon which is complementary to the reverse primer (which binds to the complementary strand in the 5'-3' direction -in green). The allele variation resulting from the *CFH* Y402H SNP is in red (either C or T). Only the forward amplicon fragment is shown - the reverse amplicon fragment is also generated by the PCR reaction, and is complementary to the forward amplicon. Primer GC%, melting temperature and size from UCSC in-silico PCR (assembly Feb 09)[240], amplicon melting temperature and size from Oligocalc (www.basic.northwestern.edu/biotools/OligoCalc.html)[241], using default settings of 50 mM salt and 50 nM primer concentration.

The PCR mix (Table 2.8) contained the intercalating fluorescent dye from the start to allow immediate post-PCR HRM analysis. The intercalating fluorescent dye used was Syto 9, (products.invitrogen.com/ivgn/product/S34854), diluted to 1%. A pre-optimised reaction mix was used (Sensimix™, Quantace Ltd, now Bioline Ltd, London, UK), which included reaction buffer, Hot start Taq DNA polymerase, dNTPs, and $MgCl_2$ (6mM). "Hot start" Taq DNA polymerase has the advantageous properties of being stabile at room temperature, and is activated at raised temperatures only (95°C). Hot-start PCR reduces the possibility of more non-specific primer binding which can happen when all reagents are pre-mixed with an active DNA polymerase.

PCR and HRM analysis was carried out in the Rotorgene 6000 (2 plex, Corbett Research Ltd, now Qiagen Ltd, Hilden, Germany). This is a rotary system in which multiple reactions in closed tubes can be carried out simultaneously. The tubes were rotated in a centrifuge, and heated to required temperatures with dry heat. Emitted fluorescence was measured from each reaction tube by a detector. PCR/HRM conditions used are shown in Table 2.9.

Table 2.8: PCR reaction mix

PCR REACTION MIX	**VOLUMES (ul)**
2x Sensimix™	5
Forward primer (20uM)	0.15
Reverse primer (20uM)	0.15
Syto® 9 dye (1%)	1
Sterile DNAse / RNAse / pyrogen-free water	2.7
DNA sample (10ng/ul)	1
TOTAL	**10**

Table 2.9: PCR/HRM conditions:

STEP		TEMP	DURATION	STEP	PROCESS
PCR	Hold	95°C	10	Activation	Hot start for Taq DNA polymerase.
	40 cycles	95°C	20s	Melting / denaturing	dsDNA is divided into 2 separate strands of single-stranded DNA (ssDNA).
		60°C	15s	Annealing	Binding of primers and Taq DNA polymerase. The chosen temperature depends on the T_M of the primers.
		72°C	20s	Extension	Creation of complementary DNA strands.
	Hold	65°C	90s	Pre-melt	Preparation for melt.
HRM	Conditions: 1. Ramp temperature from 65°C to 94.5°C, rising by 0.05°C per 2 seconds 2. Optimise gain before melt on all tubes (the gain giving highest fluorescence below 70°C will be selected). 3. Fluorescence detection on HRM channel: Source: 460nm, detector 510nm				

Normalisation of HRM curves was performed to allow for variation in pre-melt and post-melt fluorescence levels. This allowed differences in melt profiles to be highlighted more clearly. Pre-melt normalisation was set at 66-67°C, and post-melt normalisation at 75-76°C.

HRM of all samples was run blinded to known *CFH* Y402H genotype. HRM was run separately for each sample using each of three primer pairs previously manufactured for HRM analysis (Table 2.7).

Rotorgene Q Series Software (V2.1.0) (Qiagen, Hilden, Germany) was used to predict genotype, by comparison of HRM curves between samples of unknown genotype to known positive controls. Confidence threshold of the Rotorgene software for genotype allocation was set at 90%.

2.4.5 KBioscience genotyping

Recipient DNA samples were sent externally to KBioscience Ltd (Cambridge, UK) for *CFH* Y402H genotyping using KASP (KBioscience competitive Allele-Specific PCR") technology. This used PCR and end-point fluorescence for allele discrimination, with a reported >99% accuracy rate, and <0.3% error rate.[236] KBioscience results were verified in a small number of samples using DNA sequencing.

KBioscience Ltd required 300ng DNA per sample for genotyping. Therefore 30ul of 10ng/ul DNA per sample was plated out in standard PCR 96 well plates. 24ul of PCR-quality DNAse / RNAse-free water was added to each well, followed by 6ul of 50ng/ul sample DNA. Plates were centrifuged at 1000rpm and covered with PCR sealing film to prevent spillage. Plates were frozen at -80°C, and then shipped overnight on dry ice to KBioscience Ltd.

2.4.6 Calculating minor allele frequency and Hardy-Weinberg Equilibrium

Minor allele frequency

Mean allele frequency (MAF) was calculated as follows. For a given population, a particular allele frequency at a given locus is calculated using the equation:

MAF = [(2 x H) + h] / (2 x n)

Where:

H = number of individuals homozygous for this allele ("homozygotes")
h = number of individuals heterozygous for this allele ("heterozygotes")
n = Total number of individuals

Hardy-Weinberg equilibrium:
In the absence of migration, mutation, natural selection and consanguineous mating, the expected genotype frequency at a particular gene locus can be calculated mathematically from the frequency of the alleles.[242;243] This is known as the Hardy-Weinberg equilibrium (HWE). This can be illustrated using the *CFH* Y402H SNP as an example. The alleles of the *CFH* Y402H are a single base variation at position C and T. The genotypes are CC, CT or TT. If the frequency of allele C = p, and the frequency of allele T = q, then the frequency of the genotypes can be calculated as in Table 2.10.

Table 2.10: Calculation of genotype from allele frequency

Genotype	**CC**	**CT**	**TC**	**TT**
Frequency	p x p	p x q	p x q	q x q
or…	p^2	2pq		q^2

The results of genotyping were compared to that expected from HWE in the control (non-AMD) population using statistical testing (the Exact test).[244] Any significant deviation from this suggested irregularities with genotyping or the population tested.

2.4.7 DNA sequencing

DNA sequencing was carried out to verify KBioscience Ltd *CFH* Y402H genotyping in eight randomly selected samples from this study. DNA sequencing was carried out blinded to known genotype.

PCR (page 78) was first used to amplify the DNA sequence containing the *CFH* Y402H SNP. Production of a single amplicon was verified using gel electrophoresis. Purified amplicons then underwent cycle sequencing using dye terminator chemistry (page 83). Purified cycle sequencing products were read by a sequencing analyser using capillary electrophoresis.

Step 1: PCR amplification

The section of DNA containing the *CFH* Y402H SNP was amplified using PCR (Rotorgene 6000 2 plex, Corbett Research Ltd, now Qiagen Ltd, Hilden, Germany).

CFH Y402H primers previously designed for HRM analysis were found to be unsuitable for DNA sequencing through previous testing. Therefore new primers were selected, based on *CFH* Y402H sequencing primers described in the literature by McKibbin et al, and manufactured by Invitrogen (Life Technologies Ltd, Paisley, UK).[235] Primers used, together with the resulting amplicon, are shown in Table 2.11 and Table 2.12.

Table 2.11: *CFH* Y402H primers used for DNA sequencing reaction

Direction	**Sequence (5' to 3')**	**GC%**	**T_M (°c)**
Forward	TGGTCCTTAGGAAAATGTTA	35%	52.5
Reverse	GAACATGCTAGGATTTCAGAGTAGTG	42%	59.8

Table shows *CFH* Y402H sequencing primers described by McKibbin et al.[235] GC% = Percentage of G:C base pairing through complementary binding between primer and target DNA strand. T_M=melting temperature (°C) of primer. GC% and Tm data from UCSC *in silico* PCR (assembly Feb 09, using default settings of 50 mM salt and 50 nM primer concentration).[240]

Table 2.12: Resulting amplicons using *CFH* Y402H sequencing primers

Amplicon	Size (bp)	Amplicon sequence	T_M (°C)
Forward	220	TGGTCCTTAGGAAAATGTTAttttccttatttggaaaatggatataatcaaaatC/Tatggaagaaagtttgtacagggtaaatctatagacgttgcctgc catcctggctacgctcttccaaaagcgcagaccacagttacatgtatggagaatggctggtctcctactcccagatgcatccgtgtcagtaagtaCACTACTC TGAAATCCTAGCATGTTC	92.4%
Reverse	220	GAACATGCTAGGATTTCAGAGTAGTGtacttactgacacggatgcatctgggagtaggagaccagccattctccatacatgtaactgtggtctgcgc ttttggaagagcgtagccaggatggcaggcaacgtctatagatttaccctgtacaaactttcttccatG/AattttgattatatccattttccaaataaggaaaaTA ACATTTTCCTAAGGACCA	

Table shows resulting forward and reverse amplicon fragments after PCR using *CFH* Y402H sequencing primers described by McKibbin et al.[235] Size = Amplicon size (base pairs). Forward amplicon: The position of the forward primer is highlighted in blue, the sequence complementary to the reverse primer is highlighted in green, and the position of the *CFH* Y402H SNP (either C or T) is highlighted in red. Reverse amplicon: The sequence of the reverse amplicon is complementary to the forward amplicon. The position of the reverse primer is highlighted in yellow, the sequence complementary to the forward primer is highlighted in purple, and the position of the *CFH* Y402H SNP (either G or A) is highlighted in grey. T_M=melting temperature (°C) of double-stranded amplicon. Amplicon sequence from UCSC in-silico PCR (assembly Feb 09)[240], amplicon melting temperature and size from Oligocalc (www.basic.northwestern.edu/biotools/OligoCalc.html)[241], using default settings of 50 mM salt and 50 nM primer concentration. DNA sequence of reverse amplicon generated using www.bioinformatics.org, accessed 25 Marcy 2013.[245]

The reaction mix used is shown in Table 2.13, and the PCR conditions in Table 2.14. The Bioline SensiMix™ SYBR No-ROX Kit was used (this is a ready-to-use pre-mixed kit containing hot-start Taq DNA polymerase, SYBR® Green I intercalating dye, dNTPs, $MgCl_2$ (3mM), and PCR stabilisers/enhancers).[246]

Table 2.13: Reaction mix for amplification PCR (10µl)

REACTION MIX	**VOLUME (per 10µl reaction)**
Sensimix x2	5µl
Forward (25µM)	0.1µl
Reverse primer (25µM)	0.1µl
Water	3.8µl
DNA template	1µl (concentration: 10ng/µl)

Table 2.14: PCR conditions

CYCLES	**STAGE**	**TEMP**	**TIME**	**NOTE**
1	Hold	95°C	10 mins	Activation of "hot-start" DNA polymerase
40	Denaturation	95°C	15 s	Separation of dsDNA into ssDNA
	Annealing	58°C	15 s	Melting temperature of primers[235]
	Extension	72°C	15 s	Acquisition of fluorescence levels at end of step

Step 2: Gel electrophoresis

Electrophoresis was carried out to confirm a single PCR product. PCR products were placed in wells within agarose gel. When a current is applied across the gel, the negatively charged DNA fragments migrate through the gel towards the positive anode. Smaller fragments move faster and therefore a greater distance. A higher percentage of agarose enables better resolution of smaller sized DNA fragments. The gels contained ethidium bromide, a nucleic acid stain which binds to DNA though intercalation (fits between base pairs of DNA), and fluoresces under UV light. Due to its mutagenic risk, all experiments with ethidium bromide were carried out with care to avoid any excess exposure.

1.5% agarose gel (large tray) was made up as follows. Combs were first placed into a gel tray. 10g agarose was dissolved by heating in 500ml 1x TBE (Tris Borate EDTA) buffer. Once cooled to 60°C, 27.5µl ethidium bromide (10mg/ml) was added. This gel was poured into the gel tray, and any air bubbles were removed. The gel was left to set for 30-40 minutes. The set gel was then placed in a gel tank, with the loading wells placed closest to the negative (black) electrode. The combs were then removed, and the whole gel covered with 1x TBE buffer.

4µl of each PCR product was added and mixed with 2µl loading buffer (1 % Bromophenol Blue). The loading buffer adds colour and density to the sample, which makes loading into the gel easier. Additionally the dye has a slightly negative charge, therefore migrates through the gel at the same rate as a 200-400bp DNA fragment, and can be used to approximate the migration of PCR samples. 6ul PCR products + loading buffer were added to the loading wells of the gel. The gel was run at 150V for 40 minutes, and visualised under UV light. Successful samples generating a single amplicon were selected for the next step.

Five of the eight samples successfully amplified with PCR were chosen at random for DNA sequencing of the *CFH* Y402H SNP.

Step 3: Clean-up of PCR product

Purified DNA amplicons were obtained by removing primers, nucleotides, polymerases, and salts using QiaQuick Spin Columns (Qiagen, Hilden, Germany).

These columns use a special silica membrane to which DNA binds, whilst other substances pass through and are discarded. The kit protocol was followed.[247]

Step 4: Cycle sequencing reaction

The sequencing reaction was carried out using BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Paisley, UK).[248] This kit contains a DNA polymerase, normal nucleotides (dNTPs), and "dye terminators" (ddNTPs).

The cycle sequencing reaction was carried out in a thermal cycler (Tetrad 2 Peltier Thermal Cycler, Bio-Rad Laboratories Ltd, Hercules, California, USA). All reactions were carried out in accordance with the BigDye® Terminator v1.1 Cycle Sequencing Kit protocol (Applied Biosystems, Paisley, UK).[248] A separate forward and reverse reaction was set up for each PCR product (Table 2.15 and Table 2.16). Primers used were similar to that used in the initial PCR amplification step (Table 2.11). Cycle sequencing reaction conditions are listed in Table 2.17.

Table 2.15: Forward reaction mix

REACTION MIX	**VOLUME (per 20µl reaction)**
Big Dye reaction mix (1/2 strength)	4µl
5x Buffer	2µl
Forward primer (1µM)	3.2µl
PCR product (10ng)	2µl
Sterile water	8.8µl

Table 2.16: Reverse reaction mix

REACTION MIX	**VOLUME (per 20µl reaction)**
Big Dye reaction mix (1/2 strength)	4µl
5x Buffer	2µl
Reverse primer (1µM)	3.2µl
PCR product (10ng)	2µl
Sterile water	8.8µl

Table 2.17: Cycle sequencing reaction conditions

CYCLES	STAGE	TEMPERATURE	TIME
1	Initial denaturation	96°C	1 min
25	Denaturation	96°C	10 s
	Annealing	50°C	5 s
	Extension	60°C	4 min
End	Cooling	10°C	Indefinitely

Step 5. Dye terminator removal

The cycle sequencing reaction product from step 4 contained excess unincorporated dye-terminators, which could interfere with DNA sequencing by competing for capillary injection during capillary electrophoresis (step 6). These excess dye-terminators were therefore removed using the Dye-Ex 2.0 Spin Kit (Qiagen, Hilden, Germany).[247] The spin kits consisted of prehydrated gel-filtration resin (composed of spheres with uniform pores) in spin columns. The resin used the principles of gel-filtration chromatography, separating molecules based on size. Smaller dye terminator molecules diffused into the pores, and therefore were retained by the resin. Larger DNA fragments passed through the resin, and were collected after centrifuging. 20µl reaction product from each cycle sequencing reaction was added to individual spin columns, centrifuged at 3000rpm, and the elute collected for the next step. The percentage of DNA recovery was dependent on the size of DNA fragment, therefore very small fragments may not have been recovered sufficiently. This in turn may have had an impact on base-calling early on in the amplicon (see page 83).[214]

Step 6: Capillary electrophoresis

Samples were prepared for capillary electrophoresis as below.

1. 1µl of each sample (from step 5 – separate forward and reverse reactions per sample) was added to each well of a 96-well optical plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems, Paisley, UK).
2. 9µl "Hi-Di" (highly deionised) Formamide (Applied Biosystems, Paisley, UK) was placed into each well containing sample. This is a loading buffer used to re-suspend samples prior to capillary electrophoresis.
3. The plate was sealed with a heat resistant sealer and briefly spun to ensure all samples settled to the bottom of each well.

4. The samples were denatured using a thermal cycler (Tetrad 2 Peltier Thermal Cycler, Bio-Rad Laboratories Ltd, Hercules, California, USA) at 94°C for five minutes
5. Plates were loaded into the Applied Biosystems 3500XL 24 capillary DNA analyser (Applied Biosystems, Paisley, UK). The plate information was entered and DNA analysis initiated.

Step 7: Chromatogram analysis

Chromatograms ("electropherograms") were analysed both visually and using Sequence Scanner V1.0 software (Applied Biosystems, Paisley, UK) to identify the base present at the SNP position of interest. Both forward and reverse DNA strand chromatograms were obtained for each sample.

Since base-calling was inaccurate for the first few bases, analysis of the *CFH* Y402H SNP was carried out using the reverse amplicon. The *CFH* Y402H SNP was further away from the start of the reverse amplicon, compared to the forward amplicon (Table 2.12). Therefore *CFH* Y402H genotype was interpreted from chromatograms by base identification at the SNP position within the reverse amplicon (Table 2.18).

Table 2.18: Interpretation of *CFH* Y402H SNP from chromatogram

Chromatogram findings at *CFH* Y402H SNP position	***CFH* Y402H genotype**	***CFH* Y402H protein**
Forward amplicon: T only (1 peak) Reverse amplicon: A only (1 peak)	TT	*CFH* YY402
Forward amplicon: C only (1 peak) Reverse amplicon: G only (1 peak)	CC	*CFH* HH402
Forward amplicon: T and C (2 peaks) Reverse amplicon: A and G (2 peaks)	TC	*CFH* YH402

Table showing the base call on the forward and reverse amplicon, with the *CFH* Y402H genotype/protein.

2.5 Determining donor CFH Y402H genotype

The initial plan was to determine donor *CFH* Y402H genotype directly from DNA extracted from donor tissue. However due to both a lack of donor tissue availability and difficulty with genotyping, donor *CFH* Y402H genotype was determined indirectly from plasma CFH Y402 and H402 protein levels of LT patients.

2.5.1 Choosing the methodology

NB: The term “Donor” in this section refers to the individual who donated their liver to a patient recruited in this study.

Donor tissue samples were retrieved wherever possible from archived tissue stored from the donor at the time of organ donation, in order to obtain maximum tissue for DNA extraction. Where this was not possible, the only other source of donor material available was from stored biopsy material taken from the donor liver after transplantation into the recipient, although the tissue obtained from this method was far smaller in quantity.

DNA was extracted from donor tissue (spleen/lymph node) using a newly-customised version of the salting-out procedure previously described for DNA extraction from whole blood (page 106). All reagents used were proportionally adjusted to match tissue volumes. The number of ELB washes was reduced according to the amount of red blood cells present, therefore only one ELB wash was carried out for lymph node tissue. This customised DNA extraction protocol was verified first in a small number (n=8) of donor tissue samples.

Liver biopsy samples were only available in previously stored form within formalin-fixed and paraffin-embedded (FFPE) blocks. A review of six different commercial kits for DNA extraction methods from FFPE tissue indicated a kit from Qiagen yielded the highest quantity of DNA, with similarly good levels of purity for all kits.[249] Therefore FFPE samples from this study were extracted using the Qiagen QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) was used.[250] The

ability of this kit to extract DNA from FFPE samples was first verified in FFPE rat liver tissue prior to use in samples from this study. Following variable results of DNA extraction from the initial batch of FFPE samples, a modified protocol was designed after consultation with Qiagen Ltd to maximise DNA yield. DNA was amplified using whole genome amplification (Qiagen kit).

All donor DNA was sent to KBioscience Ltd for *CFH* Y402H genotyping as previously described (page 115).

Since it became apparent during the course of the study that donor tissue DNA was not available for all recruited patients, an alternative method for donor *CFH* Y402H genotyping was sought. Hakobyan et al described how *CFH* Y402H genotype could be deduced indirectly by measuring plasma CFH Y402 and H402 protein levels individually using enzyme-linked immunosorbent assays (ELISA, page 87).[251] Therefore this method was used to measure recipient plasma CFH Y402 and H402 levels, in order determine indirectly the donor *CFH* Y402H genotype.

2.5.2 Obtaining tissue from liver donors

Donor DNA was obtained from one of the two following sources:
1. Stored tissue (spleen / lymph node) from the donor at the time of organ donation.
2. Recipient liver biopsies.
All donors were identified from pre-existing medical records by the site clinical or pathology department.

Use of donor tissue was strictly regulated. Clarification and permission was received from the Human Tissue Authority regarding use of donor tissue, as follows.

"If you will be accessing anonymous donor tissue which was collected before the Human Tissue Act came into force; this tissue is considered as an existing holding. An existing holding is material from the living or deceased that was already held at the time the HT Act came into force on 1 September 2006. You have indicated you will be using this tissue for DNA analysis as part of a REC

approved project. You do not require consent from the donor's family to do this as you will be using the tissue for an 'excepted purpose' which is 'use of bodily material, which is an existing holding, for the purpose of research in connection with disorders or functioning of the human body and transplantation.' As you are conducting this research under REC approval; a licence for the storage of this material is not required. However, if the REC approval expires and no further approval is sought for a project using this tissue, the tissue must either be disposed of or stored on a HTA licensed premises"

(Email received from Miss Stacey Last, Regulation Officer, Human Tissue Authority on the 4th February 2011).

For patients with a history of re-transplantation fulfilling the recruitment criteria (page 92), the DNA of the donor of the **current** LT only was required for genotyping. Therefore it was important to ensure the correct donor was selected.

Retrieval of stored donor tissue samples

Tissue from the donor previously retrieved at the time of organ donation was stored at the study site at -20°C in archives, for purpose of future clinical diagnostic reasons. Samples obtained included donor spleen or lymph node tissue, both of which were rich sources of cells, especially lymphocytes.

Obtaining spleen sample

The following protocol was used (derived from Tissue Typing laboratory, Addenbrooke's Hospital, Cambridge).

1. Samples were defrosted in a water bath at 37°C for 20 minutes
2. A small section (about 5mm square) was dissected from each sample of spleen using a pair of scissors. This was placed in a separate microcentrifuge tube (labelled in such a way as to enable accurate association with the correct recipient)
3. The retrieved sample was stored at -20°C pending transport
4. Samples were transported on dry ice to the Clinical Neurosciences Department, University of Southampton, and stored at -80̊C pending DNA extraction.
5. Original samples were refrozen and replaced in their previous location.

Obtaining lymph node sample

The following protocol was used (derived from Tissue Typing laboratory, Addenbrooke's Hospital, Cambridge).

1. Samples were defrosted in a water bath at 37°C for 20 minutes, and then transferred to a labelled microcentrifuge tube.
2. Samples were previously stored in Dimethyl sulphoxide (DMSO) – this is a storage medium and is toxic to thawed cells. DMSO was therefore removed as soon as samples thawed. Samples were centrifuged at 13000rpm for two minutes in a microfuge, and the supernatant DSMO pipetted off and discarded
3. 2ml of phosphate buffered saline (PBS) 0.01M was added to each pellet.
4. The pellet was broken up with a mini pestle to allow suspension of white cells.
5. 1ml of suspension was pipetted into separate microcentrifuge tubes (labelled in such a way as to enable accurate association with the correct recipient), which were then stored at -20°C pending transport.
6. Samples were transported on dry ice to the Clinical Neurosciences Department, University of Southampton, and stored at -80°C pending DNA extraction.
7. Original samples were replaced in previous location.

Obtaining recipient liver biopsies

Where donor spleen or lymph node tissue was not available, donor DNA was obtained from recipient liver biopsies. LT recipients often have biopsies of their new liver for diagnostic reasons. However, the new liver can be infiltrated by recipient cells, which can cause chimerism and confuse genetic testing. This can occur as early as one week following transplantation, peaking at around 30-40 weeks post-transplant. Up to 50% of the cells found in the donor liver can be recipient-derived. These cells are mostly macrophages.[252] Biopsies from the new liver are often taken at "day 0" - the day of LT, and these were retrieved where possible.

All liver biopsies are routinely stored as formalin-fixed and paraffin-embedded (FFPE) blocks. Formalin (formaldehyde) causes reversible cross-linkage of amino acid groups in protein with neighbouring nitrogen atoms in DNA, or with other

proteins. This causes fixation of the tissue, and stops further decay by arresting any biochemical reactions, preserving the tissue. Paraffin is then used to surround the tissue to create blocks. Sections are cut from the block for analysis.

A technician in the histopathology department of each site identified each sample, and cut 5 x 5 micrometre sections ("curls") into a microcentrifuge tube. Samples were sent to the Clinical Neurosciences Department, University of Southampton at room temperature.

2.5.3 DNA extraction from spleen samples

DNA was extracted from spleen samples using a newly customised salting-out method.[231] The following protocol was devised and implemented (please refer to page 108 for further information re. reagents).

1. Defrosting. Samples (within tubes) were defrosted in a water bath at 37°C for 20 minutes.
2. Homogenisation. 0.5 ml PBS (0.01M) was placed in each tube. A hand-held homogeniser (Pellet Pestle Motor, Kontes/Kimble Chase, Vineland, USA) was used to mash up the tissue for 1 minute. The supernatant was pipetted into a 15ml tube, leaving solid matter behind.
3. ELB wash x3 (In 15ml conical tube). Cold ELB was added up to the15ml mark, mixed at 4°C for at least 15 minutes. Tubes were centrifuged at 2000rpm for ten minutes at 4°C. Supernatant was discarded by pouring into 1% Virkon, leaving a pellet at bottom undisturbed. 14ml cold ELB was added, the tube shaken to break up and disperse the pellet, and mixed at 4°C for 15 minutes. Tubes were centrifuged at 2000rpm for ten minutes at 4°C. Supernatant was poured off leaving the pellet at bottom undisturbed. A further 14ml cold ELB was added, the tube shaken to break up and disperse the pellet, and mixed at 4°C for 15 minutes. Tubes were centrifuged at 2000rpm for 10 minutes at 4°C. All supernatant was poured off. The tubes were then carefully placed upside down on paper towels to drain remaining supernatant, whilst leaving the pellet undisturbed.
4. Overnight Protein Digestion. 1.5ml NLB, 150µl 10% SDS and 75µl 40mg/ml Protease was added to all tubes. This mixture was left in a heated rocker (speed 22 rocks per minute) to mix overnight at 37°C.

5. DNA precipitation. The next morning, all tubes were cooled to room temperature. 500µl saturated NaCl solution was added to each sample, shaken for 15 seconds, and then centrifuged at 4000rpm for 20mins at room temperature. The supernatant was pipetted into fresh tubes, taking care not to include any solid matter at the base of the tube. Cold absolute ethanol (twice the volume of the pipetted supernatant) was added to each sample, and the tube inverted gently several times until DNA precipitated out to form a fluffy pellet.
6. DNA wash. Clumped DNA was removed using a 200µl pipette and placed in labelled microcentrifuge tube containing 1ml of 70% Ethanol for at least 2 hours. Tubes were centrifuged in a microfuge for 10-15 seconds at 14000rpm. The ethanol was poured off, the tops of the tubes blotted, and the tubes left open to allow DNA to air-dry (~15 minutes).
7. DNA storage and standardisation. 100-500µl TE Buffer was added to each DNA sample (the volume depending on the amount of DNA obtained as determined visually), and left overnight at room temperature to allow DNA to dissolve. DNA concentration and quality was measured the next morning using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA), and concentrations standardised to 50ng/ul (see page 109). DNA was stored at -20°C pending analysis.

2.5.4 DNA extraction from lymph nodes

DNA was extracted from lymph node samples using a customised salting-out method.[231] The following single-day protocol was devised and implemented (please refer to page 108 for further information regarding reagents).

1. Defrosting. Samples were placed (within microcentrifuge tubes) in a water bath at 37°C for 20 minutes, vortexed and centrifuged at 4000rpm for five minutes.
2. ELB wash (x1). 1ml ELB was added, tubes vortex thoroughly, then centrifuged at 4000rpm for five minutes. Supernatant was discarded by pouring into 1% Virkon, leaving a pellet at the bottom undisturbed. Tubes were then carefully placed upside down on paper towels to drain remaining supernatant, whilst leaving the pellet undisturbed. (Only one ELB wash was

carried out, since lymph node tissue, being predominantly composed of white blood cells, does not require substantial lysis of erythrocytes).

3. Protein digestion. 1ml NLB, 100µl (10%) SDS and 50µl Protease (40mg/ml) was added, and tubes vortexed thoroughly to ensure the pellet was suspended. Tubes were placed in a waterbath at 55°C for 40 minutes, and contents mixed after 20 minutes by inverting. Samples were then allowed to cool to room temperature.
4. DNA precipitation. 330ul saturated NaCl was added, tubes vortexed for at least 15 seconds, and centrifuged at 4000rpm for 5 minutes. The supernatant was pipetted into a clean labelled microcentrifuge tube. Cold absolute ethanol (twice the volume of the pipetted supernatant) was added to each sample. Tubes were inverted gently to precipitate DNA.
5. DNA wash. Clumped DNA was removed using a 200µl pipette and placed in labelled microcentrifuge tube containing 1ml of 70% Ethanol. This was left for at least 2 hours. Tubes were centrifuged in a microfuge for 10-15 seconds at 14000rpm. The ethanol was poured off, the tops of the tubes blotted, and the tubes left open to allow DNA to air-dry (~15 minutes).
6. DNA storage and standardisation. 100-500µl TE Buffer was added to each DNA sample (the volume depending on the amount of DNA obtained as determined visually), and left overnight at room temperature to allow DNA to dissolve. Resulting DNA concentration and quality were measured the next morning using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA), and concentrations standardised to 50ng/ul (page 109). DNA was stored at -20°C pending analysis.

2.5.5 DNA extraction from FFPE samples

Original protocol for DNA extraction from FFPE samples

DNA was extracted from FFPE samples using the QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany).[250] Paraffin was first removed by dissolution in xylene. The sample was lysed using proteinase K, and heated at 90°C in a water bath to remove some of the formalin-induced cross-linkage. The resulting solution was added to a tube containing a silica-based membrane with selective binding properties. DNA binds to this membrane, allowing proteins, nucleases and other impurities to be washed away. Pure DNA was then eluted from the membrane. All

steps involving xylene was carried out in a fume cupboard (since inhalation of xylene may depress the central nervous system).

Resulting DNA concentration and quality was measured using the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA (Page 109).

Verification of Qiagen QIAmp DNA FFPE Tissue kit using rat liver

DNA extraction from FFPE tissue using the Qiagen QIAamp® DNA FFPE Tissue kit was first verified using a specimen of FFPE rat liver. Three sections of FFPE rat liver were cut at different thickness from the block using a microtome, firstly to test whether DNA could be successfully extracted, and secondly to test whether thickness affected quantity and quality of DNA. Each sample of extracted DNA was dissolved in 50 µl of TE buffer.

Modified protocol for DNA extraction from FFPE samples

A modified protocol based on the QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) was designed to maximise the quantity and quality of DNA extracted from FFPE samples.

The changes made in the modified protocol over the original protocol were as follows.

1. Deparaffinisation solution: Instead of using xylene to remove paraffin, 320µl of Deparaffinisation solution (Hexadecane from Qiagen, Hilden, Germany)[253] was added per sample. This modification enabled removal of paraffin without the need to open the reaction tube, which was required with xylene and could potentially cause loss of valuable sample.

2. Heating block: A water bath originally used to heat samples to the required temperature, was recognised as being inefficient in being able to reach the high temperatures (90°C) essential for sufficient reversal of cross-linkage. A heating block verified with a mercury thermometer was therefore used instead to ensure the correct temperature was reached. The block was in turn placed on a rocker to allow sufficient mixing of the samples.

3. Buffer: To ensure adequate dissolution, buffers (AT and ATL) were heated to 70°C prior to use. In addition the volume of buffer used to elute DNA from the

membrane within the QiaAMP tube was reduced (to 30µl) to increase the concentration of eluted DNA. Furthermore the elution time was extended to 30 minutes (rather than the specified one minute) to maximise DNA yield.

DNA was extracted from 15 samples of liver biopsy FFPE tissue using both the original and modified QIAamp® DNA FFPE Tissue kit protocol, as described above. The choice of protocol used for DNA extraction from the rest of the FFPE samples was based on the results of this preliminary comparison.

2.5.6 Donor *CFH* Y402H genotyping

All donor DNA samples extracted from FFPE tissue were sent for *CFH* Y402H genotyping to KBioscience Ltd (Cambridge, UK) (page 115).

2.5.7 Whole genome amplification

To improve the success of KBioscience *CFH* Y402H genotyping, each DNA sample extracted from FFPE tissue was amplified using whole genome amplification (WGA), with the Qiagen REPLI-g Mini Kit (Qiagen, Hilden, Germany).[254] The kit protocol was followed. Briefly, the DNA sample was first denatured (separated into single stranded DNA), and a specific polymerase enzyme ("phi29 DNA polymerase") was used to replicate the DNA. Phi29 polymerase has 3'→5' exonuclease proofreading capability and therefore up to 100 times higher fidelity of replication compared to Taq polymerase (phi29 polymerase has a reported error rate of 9.5×10^{-6}, compared to 1 in 9000 for Taq polymerase).[255;256] After WGA, each sample was "cleaned up" to remove unused primers, nucleotides and buffers using a modified Qiagen DNA Mini Kit protocol.[257]

2.5.8 Determining donor *CFH* Y402H genotype indirectly from recipient plasma *CFH* Y402 and H402 levels

Due to the difficulty in obtaining donor tissue, donor *CFH* Y402H genotype was determined by measuring plasma CFH Y402 and H402 protein levels in recipients, using "sandwich" ELISA (page 87), based on a previously published protocol by Hakobyan et al.[146]

Measurement of recipient CFH Y402 and H402 protein levels

Blood for plasma acquisition was collected in EDTA tubes and processed as described previously (page 96). Recipient plasma samples stored at the University of Southampton were transported frozen under dry ice to the complement laboratory at Cardiff University, Cardiff, UK. Levels of plasma CFH Y402 and H402 proteins were measured by ELISA using monoclonal antibodies against CFH Y402 and H402, based on a previously published protocol.[146] Details are as below.

100µl 5mg/l affinity-purified polyclonal rabbit anti-CFH antibody (the "capture" antibody) was added to each well of a 96 well-plate (Nunc MaxiSorp® flat-bottom 96 well plates, Nalge Nunc International, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Affinity purification uses chromatography to enable isolation of the protein of interest by passing it through a solid substance to which it binds. This was incubated at 4°C overnight. 1% bovine serum albumin (BSA) in PBS was then added to block the wells. A "blocking" agent improves the sensitivity of a particular assay and reduces background noise by binding to all sites of non-specific interaction. Plasma samples (diluted 1:3000 in BSA/PBS) were added in duplicates. Purified protein standards were also included on each plate. Plates were incubated for 1 hour at 37°C. Wells were washed and incubated with 1mg/l CFH Y402 or H402 antibodies ("detection" antibodies) labelled with the enzyme horse-radish peroxide (HRP), for 1 hour at 37°C. Wells were washed. OPD (o-phenylenediamine dihydrochloride) substrate was added, and absorbance of the resulting yellow-orange product was measured at 492nm using the FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany). Fluorescence levels of standards were plotted to create a standard curve, from which sample CFH Y402 and H402 protein levels could be ascertained.

Determining donor CFH Y402H genotype from recipient CFH Y402 and H402 protein levels

A patient carrying the low-risk homozygous *CFH* YY402 genotype (with two T alleles) will demonstrate presence of the CFH Y402 protein, but complete absence of the CFH H402 protein in the systemic circulation. In contrast, a patient carrying the high-risk homozygous *CFH* HH402 genotype (with two C alleles) will demonstrate presence of the CFH H402 protein, but complete absence of the CFH Y402 protein in the systemic circulation. A patient carrying the heterozygous *CFH* YH402 genotype (with one C and one T allele) will demonstrate presence of both

CFH Y402 and H402 proteins in the systemic circulation. This is summarised in Table 2.19.

Table 2.19: CFH Y402 and H402 proteins according to genotype

***CFH* Y402H status**	**Genotype**	**Protein manufactured**
Homozygous low risk ("*CFH* YY402")	TT	CFH Y402 only
Homozygous high risk ("*CFH* HH402")	CC	CFH H402 only
Heterozygous ("*CFH* YH402")	CT	Both CFH Y402 and CFH H402

Table showing CFH protein production based on *CFH* Y402H genotype. "Risk" refers to the reported AMD risk.[4–6]

Levels of circulating CFH Y402 or H402 protein were determined by the *CFH* Y402H genotype of the liver, since as described before, the liver is the primary producer of circulating CFH protein. Therefore in LT patients, plasma CFH Y402 and H402 protein levels were determined by the donor liver *CFH* Y402H genotype. It followed that measurement of circulating CFH Y402 and H402 proteins in LT recipients could indirectly determine the *CFH* Y402H genotype of the donor liver. This was verified in the subgroup of patients in whom donor tissue DNA and *CFH* Y402H genotyping was available.

2.6 Measurement of plasma complement protein levels in liver transplant patients

Systemic complement protein levels in recruited LT patients were measured to determine any association with recipient or donor *CFH* Y402H genotype. Furthermore, any association of systemic complement protein levels with AMD in LT patients was also determined.

2.6.1 Choosing the methodology

Plasma complement analysis was undertaken at the complement laboratory at Cardiff University, Cardiff, UK, under the guidance of Professor BP Morgan, Head Dean of Medical School, Cardiff University.

The complement proteins chosen for plasma analysis were based around those measured by Scholl et al, as reported in a paper demonstrating systemic complement activation was present in AMD.[145] The complement proteins chosen were C3, C3a, C4, C4a, C5a, TCC and CFH. Plasma levels of C1 inhibitor was also measured since a paper published by Professor Lotery at University of Southampton had shown an association with the SERPING1 gene (which codes for C1 inhibitor) and AMD.[64] Plasma C1 inhibitor has also been shown to be associated with AMD.[258]

Plasma C3a, C4a, C5a, TCC and total CFH levels were measured using ELISA (page 87), available in kit form. The kits chosen were based on advice from Professor Morgan.

Plasma C3, C4 and C1inhibitor levels were measured by the Clinical Biochemistry department at the University Hospital of Wales, Cardiff, UK, using rate nephelometric assays (Dade Behring, Milton Keynes). Nephelometry measures the degree of light scatter by immune complex formation through addition of monoclonal antibodies specific to the protein being measured. This method was used due to its ready availability, which meant time and cost-savings. The assays were previously clinically validated and externally quality controlled (United Kingdom National External Quality Assessment Service - UK NEQUAS).

2.6.2 Plasma complement protein analysis

Recipient plasma samples were collected as previously described (page 96), and transported frozen under dry ice to the complement laboratory at University Hospital of Wales, UK, where samples were analysed for complement levels under the guidance of the local research team. All complement analysis was done blinded to patient genotype.

Plasma C3a, C4a, C5a and TCC were measured with ELISA kits (C3a, C5a, TCC: Hycult Biotech Inc., The Netherlands; C4a: Beckton, Dickinson and Co., USA).[259–262] C3a, C4a and C5a are very short-lived in the circulation and are practically immeasurable, therefore the more stable "-des-Arg" forms are measured by the kits. C3a-des-Arg, C4a-des-Arg and C5a-des-Arg are formed in vivo by the removal of an arginine group by endogenous serum carboxypeptidase N from C3a, C4a and C5a respectively.[263]

Plasma total CFH was measured using ELISA based on a previously published protocol.[146] The steps taken for plasma total CFH measurement were similar to the previously described protocol for measurement of CFH Y402 and H402 protein (page 132), the only difference being anti-CFH (total) detection antibodies labelled with HRP were used. Plasma was diluted as specified for each kit prior to assaying. The FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure OD values for all samples after production of the chromogenic signal. OD values were plotted on a standard curve (obtained using samples of known concentration supplied with each kit), to obtain plasma concentration values for tested samples.

Plasma was also analysed at the Clinical Biochemistry department (University Hospital of Wales, Cardiff, UK) for C3, C4 and C1inhibitor levels using rate Nephelometry. Samples were diluted appropriately beforehand to the pre-specified level.

A summary of the complement proteins measured in plasma and interpretation of abnormal levels is shown in Table 2.20.

Table 2.20: Systemic complement proteins measured

Complement protein category	**Complement protein**	**Interpretation of systemic levels**
Complement substrate proteins	C3	Reduced levels indicate consumption and activation of alternative and terminal complement pathway
	C4	Reduced levels indicate consumption and activation of classical complement pathway
Complement activation products	C3a	Elevated levels indicate activation of the alternative and terminal complement pathway
	C4a	Elevated levels indicate activation of the classical complement pathway
	C5a	Elevated levels indicate activation of terminal complement pathway
	TCC	Elevated levels indicate activation of the terminal complement pathway
Complement regulators	C1inh	Altered levels indicate possible dysregulation of classical complement pathway
	CFH (total)	Altered levels indicate possible dysregulation of alternative complement pathway (NB. total plasma CFH = both CFH Y402 and H402 proteins).

Table shows the complement proteins measured in plasma, and the interpretation of altered levels.

2.7 Statistics

Inter-rater agreement of AMD grading was calculated using the kappa statistic.[264]

The proportion of LT patients with AMD and the *CFH* Y402H rs1061170 risk C allele were compared to a reference population (Rotterdam study baseline population[166], of similar age and ethnicity as this study) using the chi square goodness of fit test.

Conformation of *CFH* Y402H genotyping results to HWE was carried out using the Exact test.

Plasma complement levels were measured between groups using non-parametric analysis with the Mann-Whitney-U test.

Binary logistic regression was used to determine whether recipient/donor CFH Y402H genotype was associated with AMD, using AMD as the dependent variable and other covariates added in stepwise blocks using the forced entry method.

Statistical significance was defined as $p \leq 0.05$, with Bonferroni correction for multiple testing. All statistics were carried out using SPSS version 19 (IBM, New York, USA), except calculation of HWE, for which PLINK software was used (Centre for Human Genetic Research, Massachusetts General Hospital, USA[244;265]).

Reference for statistics: Field et al, 2009[266]

CHAPTER 3 Results

This chapter describes the outcomes of pursuing each objective followed by a summary.

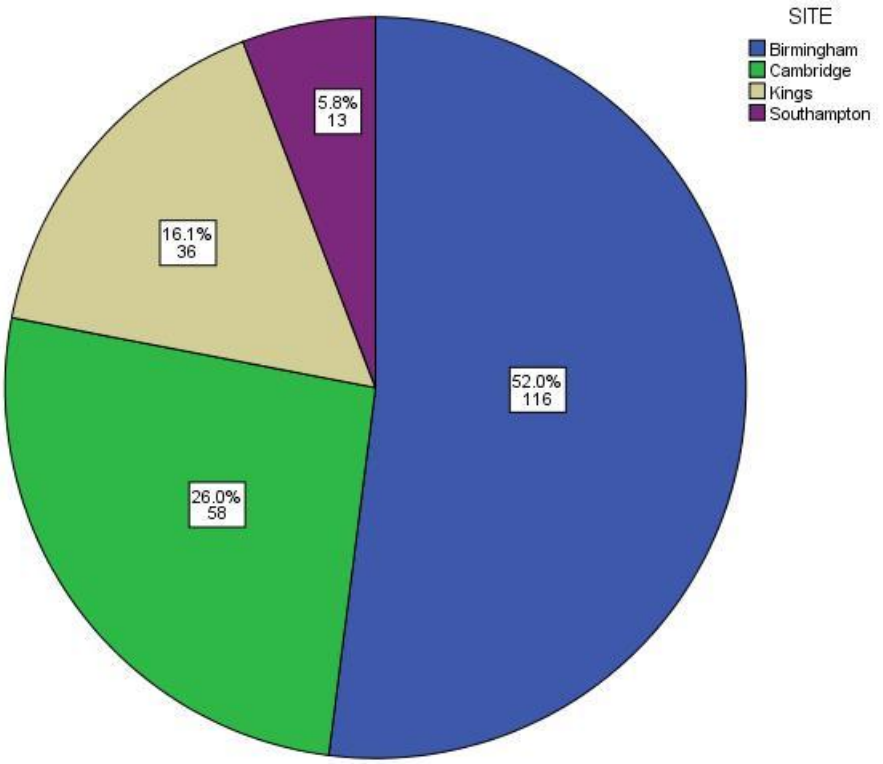
3.1 Recruitment of liver transplant patients

The timeline from setting up the project to patient recruitment is shown in Table 3.1. In total, 223 patients were recruited – most patients were from Birmingham (Figure 3.1).Recruitment was spread over a 15 month period (Figure 3.2). Overall 29% of patients initially approached were recruited (Figure 3.3).

Table 3.1: Timeline of project

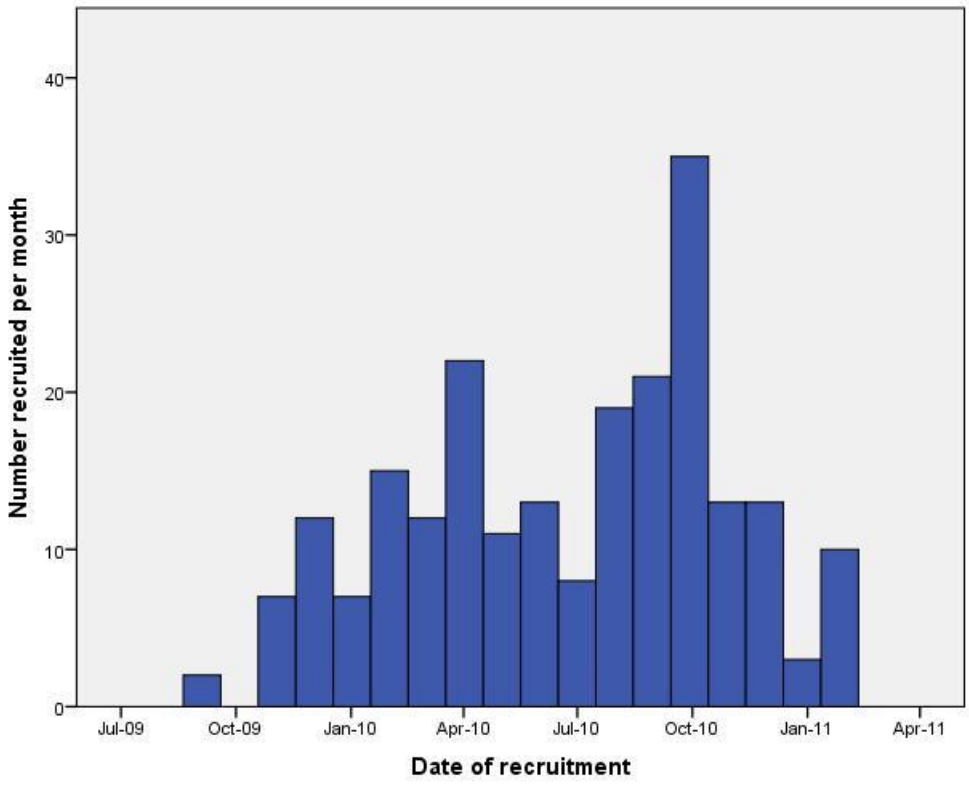
DATE	PROCESS
1/10/08	Beginning of PhD studentship
21/10/08	REC application sent
2/1/09	Full REC approval
27/3/09	Southampton Hospital (host) R+D application
1/5/09	Cambridge site approval
8/5/09	Birmingham site approval
8/6/09	Southampton Hospital full R+D approval given
8/9/09	First patient recruited at Southampton
9/11/09	Kings site approval
10/11/09	First patient recruited at Birmingham
16/11/09	First patient recruited at Cambridge
12/8/10	First patient recruited at Kings
31/1/11	Last patient recruited

Figure 3.1: Total number of patients recruited according to site (n=223)



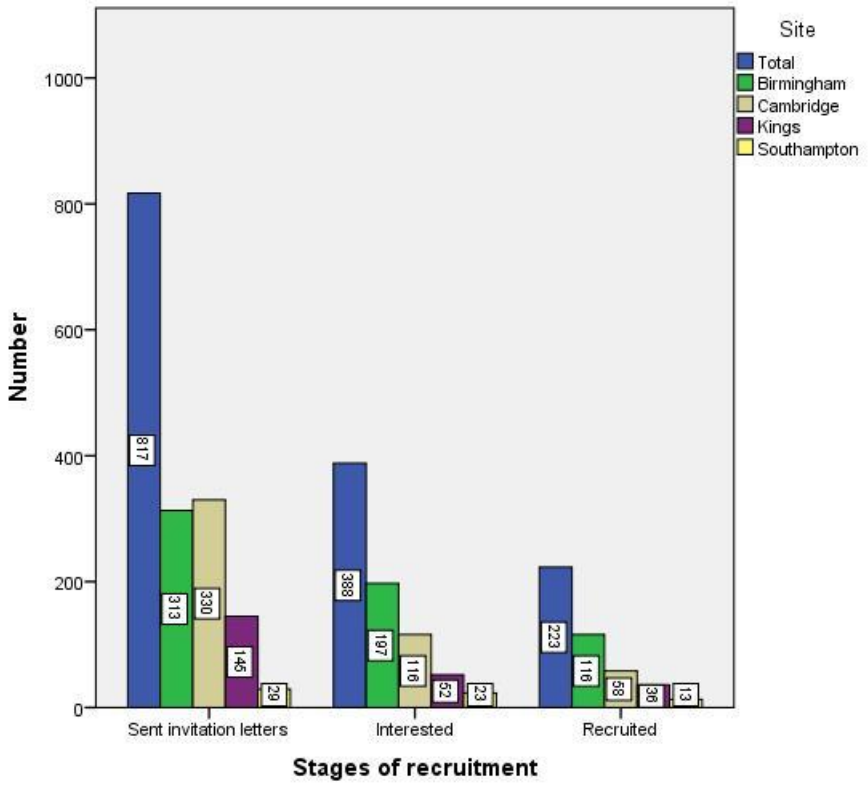
Number of patients recruited according to site (percentage of total, followed by numbers in each category).

Figure 3.2: Pattern of recruitment over time



Bar chart showing number of patients recruited each month from start of project.

Figure 3.3: Pattern of patient recruitment at all sites



817 patients from hospital databases were deemed eligible and were sent invitation letters. 388 (47%) expressed an interest in taking part. 223 in total were recruited (57% of those expressing an interest, and 27% of those initially sent invitation letters).

Characteristics for all patients (n=223), and patients with / without AMD are summarised in Table 3.2. The mean recipient age was 66.9 ± 6.1 years and mean duration post-LT was 11.8 ± 4.4 years. There was a marginal preponderance of males (55.2%, n=123). The mean donor age was younger at 41.4 ± 14.9 years, and 58.3% (n=130) of donors were male. The most common indication for LT was PBC, followed by PSC.

Hypertension, renal impairment and hypercholesterolaemia were common concomitant diseases (>50%). The majority of patients were on a statin. Most patients were on at least two immunosuppression agents (74.4%). Tacrolimus (a calcineurin inhibitor) was the most used immunosuppression, followed by oral steroids (prednisolone / hydrocortisone). Most patients (>75%) did not have evidence of hepatocellular dysfunction as determined by raised AST or ALT levels.

Table 3.2: Characteristics for all subjects, and those with/without AMD.

VARIABLES		UNIT	ALL	AMD	NON-AMD
RECIPIENT	DEMOGRAPHIC INFORMATION				
	Number	Number	223	144	79
	Gender	Females (%)	100 (44.8%)	69 (47.9%)	31 (39.2%)
	Age	Years (Mean ± 1SD)	66.9 ± 6.1	67.7 ± 6.2	65.5 ± 5.8
	Smoking	Pack-years (Median, IQR)	1.5 (0-15)	0.4 (0-14.9)	6 (0-18)
	LT DETAILS				
	Duration of current LT	Years (Mean ± 1SD)	11.8 ± 4.4	11.7 ± 4.4	11.9 ± 4.4
	PRIMARY CAUSE OF LT				
	Primary biliary cirrhosis (PBC)	Number (%)	70 (31.4%)	51 (35.4%)	19 (24.1%)
	Primary sclerosing cholangitis (PSC)	Number (%)	31 (13.9%)	17 (11.8%)	14 (17.7%)
	Alcohol-related	Number (%)	21 (9.4%)	16 (11.1%)	5 (6.3%)
	Autoimmune hepatitis	Number (%)	17 (7.6%)	9 (6.3%)	8 (10.1%)
	Seronegative hepatitis	Number (%)	16 (7.2%)	8 (5.6%)	8 (10.1%)
	Hepatitis B virus	Number (%)	14 (6.3%)	9 (6.3%)	5 (6.3%)
	Cryptogenic cirrhosis	Number (%)	13 (5.8%)	6 (4.2%)	7 (8.9%)
	Hepatitis C virus	Number (%)	13 (5.8%)	8 (5.6%)	5 (6.3%)
	Non-alcoholic steatohepatitis	Number (%)	5 (2.2%)	2 (1.4%)	3 (3.8%)
	Alpha antitrypsin-1 deficiency	Number (%)	3 (1.3%)	3 (2.1%)	0 (0%)
	Amyloidosis	Number (%)	3 (1.3%)	3 (2.1%)	0 (0%)
	Haemochromatosis	Number (%)	3 (1.3%)	3 (2.1%)	0 (0%)

	Budd-Chiari syndrome	Number (%)	2 (0.9%)	1 (0.7%)	1 (1.3%)
	Congenital hepatic fibrosis	Number (%)	2 (0.9%)	1 (0.7%)	1 (1.3%)
	Polycystic liver and kidney disease	Number (%)	2 (0.9%)	2 (1.4%)	0 (0%)
	Wilson's disease	Number (%)	2 (0.9%)	1 (0.7%)	1 (1.3%)
	Azathioprine toxicity	Number (%)	1 (0.4%)	0 (0%)	1 (1.3%)
	Cirrhosis secondary to cholecystectomy complication	Number (%)	1 (0.4%)	1 (0.7%)	0 (0%)
	Epithelioid Haemangio-Endothelioma	Number (%)	1 (0.4%)	0 (0%)	1 (1.3%)
	Erythropoetic protoporphyria	Number (%)	1 (0.4%)	1 (0.7%)	0 (0%)
	Langerhan's Cell Histiocytosis	Number (%)	1 (0.4%)	1 (0.7%)	0 (0%)
	Paracetamol overdose	Number (%)	1 (0.4%)	1 (0.7%)	0 (0%)
	PRIMARY CAUSE OF LT (GROUPED)				
	Cholestatic disease (PBC + PSC)	Number (%)	101 (45.3%)	68 (47.2%)	33 (41.8%)
	Infection (Hepatitis B and C virus)	Number (%)	27 (12.1%)	17 (11.8%)	10 (12.7%)
	Autoimmune causes (PBC, PSC and autoimmune hepatitis)	Number (%)	117 (52.5%)	77 (53.5%)	40 (50.6%)
	Inflammatory causes (autoimmune + infectious causes)	Number (%)	160 (71.7%)	102 (70.8%)	58 (73.4%)
	CONCOMITANT MEDICAL CONDITIONS				
	Hypertension	Number (%)	158 (70.9%)	103 (71.5%)	55 (69.6%)
	Renal impairment	Number (%)	139 (62.3%)	92 (63.9%)	47 (59.5%)
	Hypercholesterolaemia	Number (%)	115 (51.6%)	71 (49.3%)	44 (55.7%)

	Diabetes	Number (%)	54 (24.2%)	32 (22.2%)	22 (27.8%)
	Ischaemic heart disease / cerebrovascular accident	Number (%)	33 (14.8%)	18 (12.5%)	15 (19.0%)
	Colitis	Number (%)	31 (13.9%)	15 (10.4%)	16 (20.3%)
	IMMUNOSUPPRESSION				
	Tacrolimus	Number (%)	113 (50.7%)	70 (48.6%)	43 (54.4%)
	Steroids	Number (%)	100 (44.8%)	66 (45.8%)	34 (43%)
	Mycophenolate	Number (%)	81 (36.3%)	54 (37.5%)	27 (34.2%)
	Azathioprine	Number (%)	59 (26.5%)	36 (25%)	23 (29.1%)
	Cyclosporin	Number (%)	54 (24.2%)	32 (22.2%)	22 (27.8%)
	Sirolimus	Number (%)	23 (10.3%)	17 (11.8%)	6 (7.6%)
	Total number of agents	Mean ± SD	1.9 ± 0.7	1.9 ± 0.6	2.0 ± 0.7
	OTHER MEDICATION				
	Statin	Number (%)	114 (51.1%)	75 (52.1%)	39 (49.4%)
	Bisphosphonate	Number (%)	56 (25.1%)	44 (30.6%)	12 (15.2%)
	Beta blockers	Number (%)	51 (22.9%)	34 (23.6%)	17 (21.5%)
	Aspirin	Number (%)	46 (20.6%)	25 (17.4%)	21 (26.6%)
	Ursodeoxycholic acid	Number (%)	40 (17.9%)	27 (18.8%)	13 (16.5%)
	Diuretics (loop or thiazide)	Number (%)	29 (13.0%)	22 (15.3%)	7 (8.9%)
	CLINICAL				
	Mean arterial blood pressure	mmHg (Mean ± 1SD)	97 ± 11	96 ± 11	99 ± 11
	Body mass index	kg/m^2 (Mean ± 1SD)	27.4 ± 4.4	27.3 ± 4.4	27.6 ± 4.5
	Current hepatocellular dysfunction (AST or ALT >35iU/L)	Number (%)	53 (23.8%)	36 (25.0%)	17 (21.5%)

DONOR	**DEMOGRAPHIC INFORMATION**				
	Donor gender	Females (%)	93 (41.7%)	59 (41.0%)	34 (43.0%)
	Donor age	Years (Mean ± 1SD)	41.4 ± 14.9	41.3 ± 15.2	41.7 ± 14.5
	Donor age (55 years and over)	Number (%)	41 (18.4%)	26 (18.1%)	15 (19.0%)

ABBREVIATIONS

AMD = Age-related macular degeneration, ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, IQR = Interquartile range (25th - 75th percentiles), LT = Liver transplant, PBC = Primary biliary cirrhosis, PSC = Primary sclerosing cholangitis, SD = Standard deviation

3.1.1 Summary

A total of 223 patients were recruited over a 15 month period from four liver transplantation sites. Around 60% of patients who initially expressed an interest were recruited.

Demographic information was obtained from patient interview, medical records and archives. The mean age of the 223 recruited patients was 67 years, and mean duration post-LT 12 years, with a preponderance of males (55%). The mean donor age was younger at 41 years, again the majority (58%) being male. The most common indications for LT were the autoimmune cholestatic diseases (PBC and PSC).

Author contribution
All necessary work to set up the study and run the research clinics at each site was carried out by the author, with the help of the individuals listed below.

Queen Elizabeth Hospital, Birmingham: Ms Bridget Gunson (Centre for Liver Research Manager) selected eligible patients initially from the hospital database, sent invitation letters and provided anonymous donor information. Blood pressure, height and weight were measured by the liver outpatient nurses. Bloods were taken by outpatient and Wellcome Trust Clinical Research Facility (WTCRF) nurses. Ms Davina Scott (research assistant, WTCRF) processed most bloods. Donor information was provided by Bridget Gunson. Linda Ridge and Stella Thomas (Wellcome Trust receptionists) provided administrative support. Overall supervision was provided by Professor David Adams (PI), and Ms Joanna Gray (Wellcome Trust Research Sister).

Addenbrooke's Hospital, Cambridge: Ms Wendy Smeeton (secretary to Dr Graeme Alexander) selected eligible patients initially from the hospital database and sent invitation letters. Blood pressure, height and weight were taken by the liver outpatient and WTCRF nurses. Blood samples were taken by the hospital phlebotomy service and WTCRF nurses. Anonymous donor information was provided by Dr Craig Taylor and Dr Helen Morgan (Histopathology Lab). Ms Faith Pogge Von Strandmann (WTCRF administrator) provided administration support. Overall supervision was provided by Mr Graeme Alexander (PI), Professor Keith

Martin (PI), Mr Stewart Fuller (WTCRF head nurse), and Ms Daisy Appanah (WTCRF manager).

Kings College Hospital, London: Mr Akintunde Afolabi (Liver Department research assistant) selected eligible patients initially from the hospital database and sent invitation letters. Blood pressure, height and weight were taken by the liver outpatient nurses. Fundus photos were taken by Mr Richard Leung and Mr Matthew Richardson (Ophthalmology photographers). Blood samples were taken by the hospital phlebotomy service. Anonymous donor information was provided by Lisa Gifford (Clinical Trials Manager, Institute of Liver Studies). Ms Maureen Tuohy (PA to Professor Nigel Heaton) provided administrative support. Overall supervision was provided by Ms Sobha Sivaprasad (PI) and Lisa Gifford.

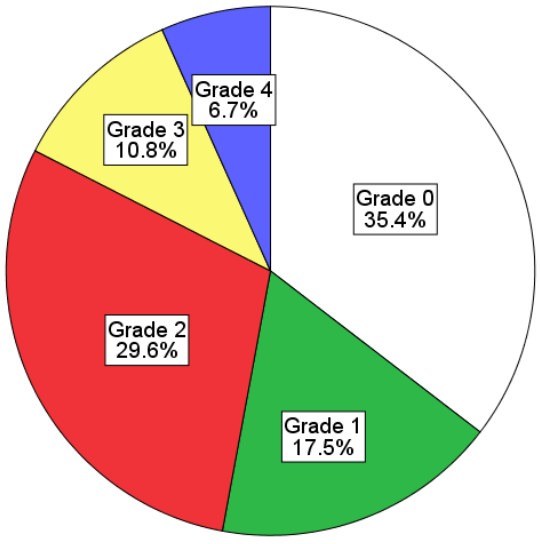
University Hospital Southampton, Southampton: Dr Katheryn Nash (consultant hepatologist) selected eligible patients initially from the hospital database and sent invitation letters. Blood pressure, height and weight were taken by the WTCRF nurses. Blood samples were taken by the hospital phlebotomy service and by WTCRF nurses. Fundus photographs were taken by Mr Tim Moles and Ms Clare O'Brien (Ophthalmology photographers). Supervision was provided by Professor Andrew Lotery and Dr Kathryn Nash (the site PIs).

3.2 Determining recipient AMD status

223 patients were included in the final analysis. The distribution of AMD according to AMD grades in LT group is shown in Figure 3.4. The overall prevalence of AMD (Grades 1-4) in LT patients was 64.6% (n=144). Inter-rater agreement (for 2 sets of primary grading) was moderate for both AMD grade (kappa statistic $\kappa = 0.484$, $p<0.001$) and for overall AMD status ($\kappa= 0.579$, $p<0.001$).

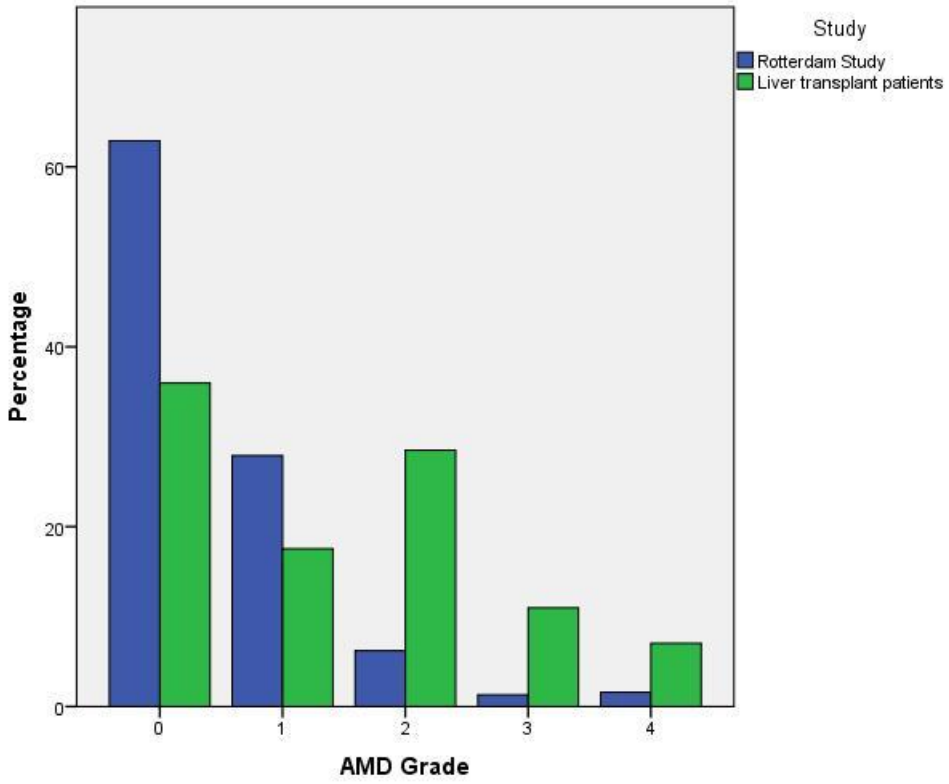
The prevalence of AMD in LT patients was higher compared to the reference population (Rotterdam Study population - a general population of similar ethnicity and age[166]) (64.6% vs 37.1%, chi-square test: $p<0.001$, OR 3.09, 95% CI 2.34-4.09).[26] This increase in AMD prevalence was composed of a greater proportion of AMD grades 2-4 in LT patients compared to the Rotterdam Study (Figure 3.5). The prevalence of AMD in LT patients increased (as expected) with age (Figure 3.6).

Figure 3.4: Distribution of AMD grading in UK liver transplant patients



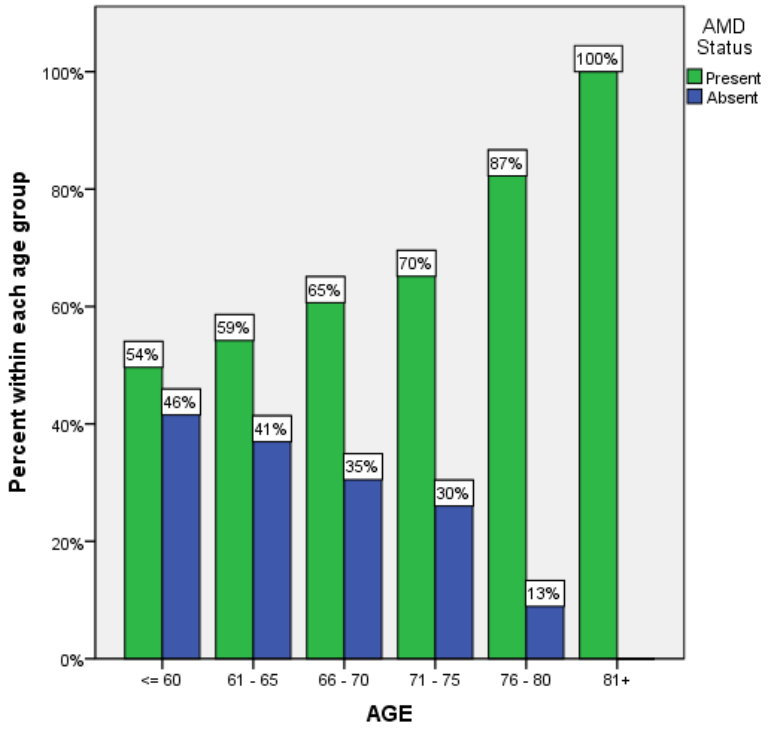
Results from AMD grading of all patients using the Rotterdam system [26] are shown. Grades 1-4 = AMD (early AMD = grades 1-3, late AMD = grade 4).

Figure 3.5: AMD grades: Liver transplant patients versus Rotterdam study population



Bar chart comparing distribution of AMD grades in LT patients (blue) with Rotterdam Study patients (green). There is a greater proportion of AMD grades 2-4 in LT patients.

Figure 3.6: Prevalence of AMD according to age



Bar chart showing the percentage of LT patients with AMD (green) increased with age.

3.2.1 Summary

The overall prevalence of AMD in 223 LT patients was 64.6%. This was significantly higher compared to a general population of similar ethnicity and age. This was accounted for by a greater proportion of AMD grades 2-4 observed in the LT group.

Author contribution

All procedures were carried out by the author except the following. Photographs were taken by ophthalmic photographers at Southampton (Tim Moles, Clare O'Brien), and at Kings College Hospital (Richard Leung, Matthew Richardson). Ling Z Heng (Retinal fellow, Kings College Hospital London) carried out repeat primary grading of images for AMD, and Professor Andrew Lotery arbitrated any discrepancies.

3.3 Determining recipient *CFH* Y402H genotype

3.3.1 Is high resolution melt accurate enough to genotype *CFH* Y402H?

Out of the three primer pairs designed for HRM *CFH* Y402H genotyping, only primer pair number 2 showed sufficient HRM curve separation between samples to allow genotype discrimination.

PCR results are shown in Figure 3.7. The absence of contamination was indicated by the absence of amplification of the negative control (water only – no DNA template). Out of 16 samples (including duplicates), *CFH* Y402H genotype was correctly predicted by HRM analysis for 12 samples (75%), but was either incorrect or unknown for 4 samples (25%). Genotype allocation was unknown for 3 of these samples since the Rotorgene software confidence level for allocating genotype to these samples fell below 90%. Unexpectedly, HRM analysis was able to correctly allocate *CFH* Y402H genotype in the single sample which failed to amplify sufficiently.

HRM curves grouped by previously known KBioscience *CFH* Y402H genotyping are shown in Figure 3.8. As described previously (page 82), HRM curves for homozygous genotypes (CC and TT) were similar in shape to each other with a shift in melting temperature, whereas the HRM curve for the heterozygous genotype (CT) were of a different shape.

Figure 3.7: Pre-HRM PCR

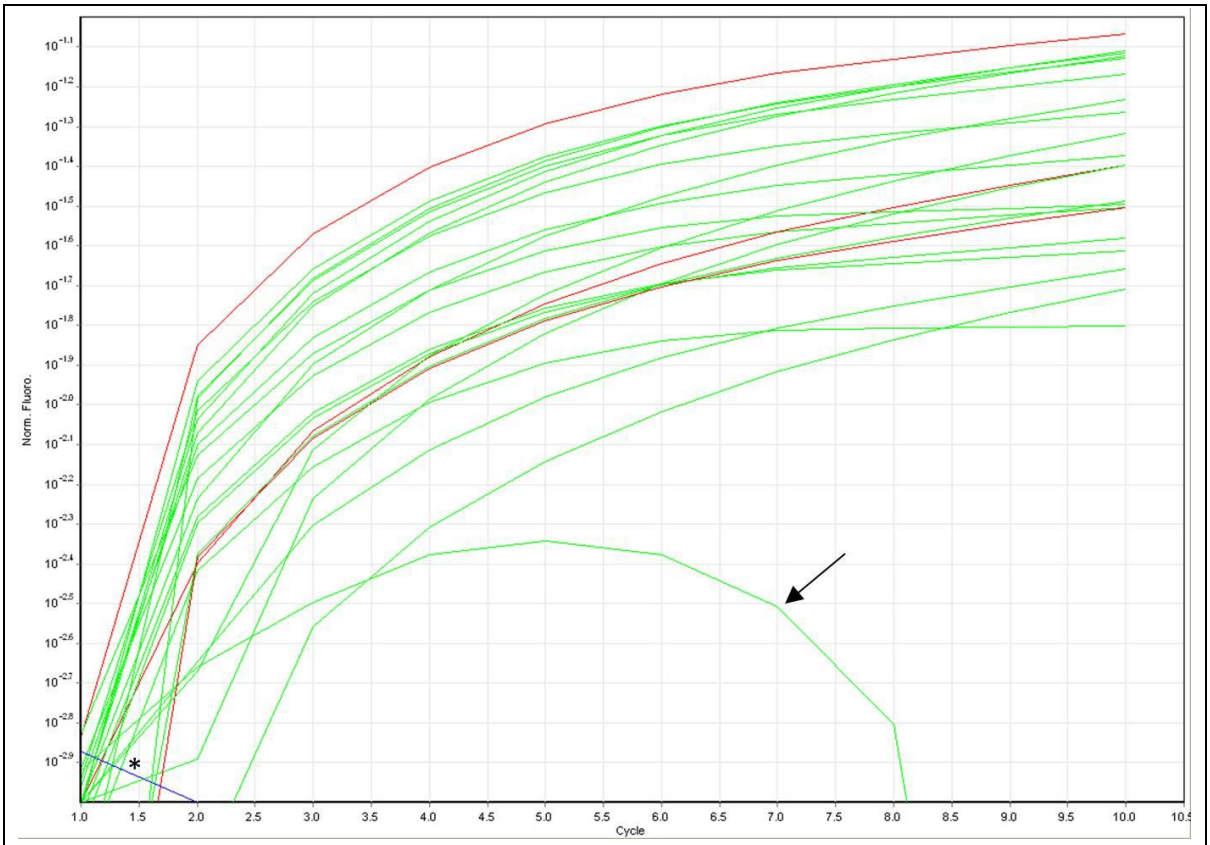
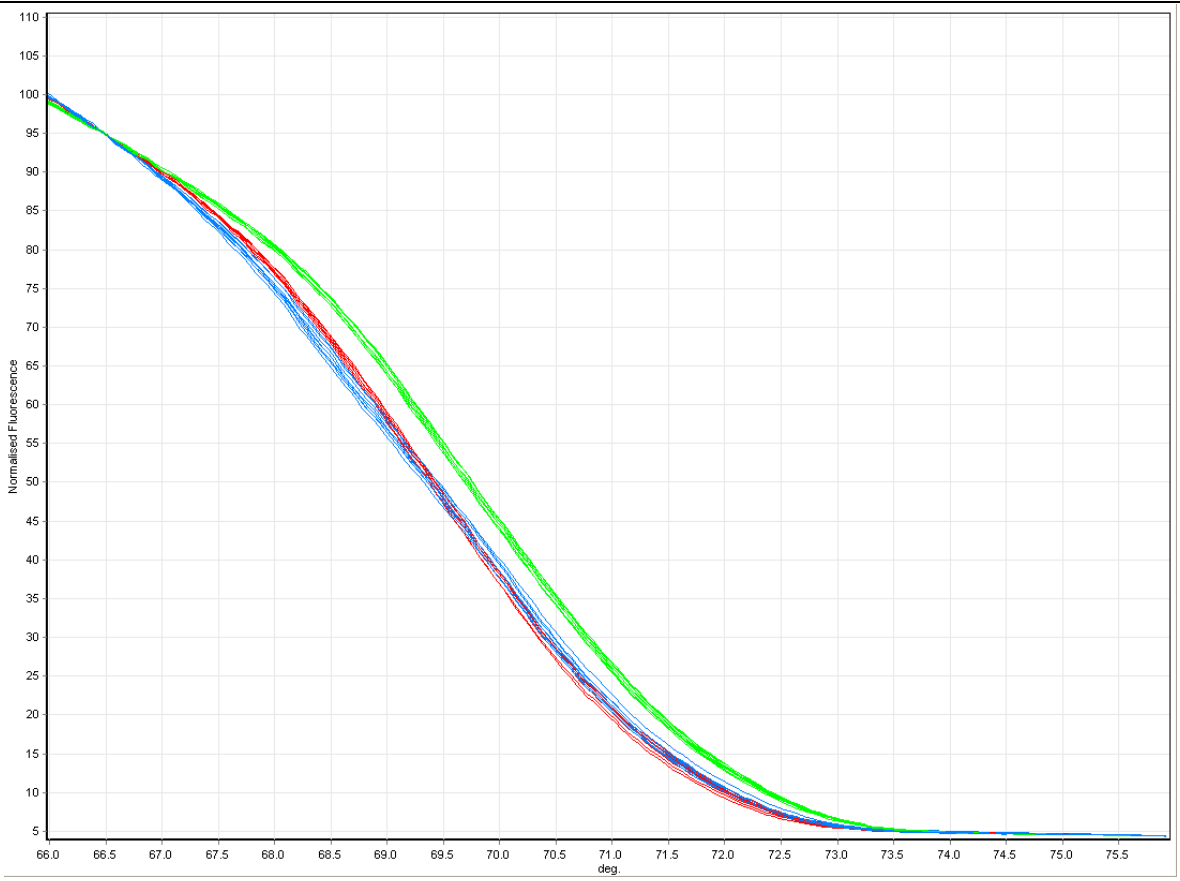


Figure shows results of pre-HRM PCR for 16 samples. X axis indicates number of PCR cycles, y axis the amount of detectable fluorescence, a marker of the quantity of PCR amplicon produced. Green curves: Samples with unknown *CFH* Y402H genotype (n=16). Red curves: Positive controls (n=3). Blue line with asterisk: Negative control (water only). All samples amplified successfully after 10 cycles (bar one – black arrow). There was no amplification of the negative water-only control.

Figure 3.8. Normalised HRM curve with known *CFH* Y402H genotype



Graph showing HRM profile for all samples, including positive controls. Samples are colour-coded for known *CFH* Y402H genotype: CC = green, CT = blue, TT = red.

Section summary

HRM was not able to correctly allocate correct *CFH* Y402H genotype in all samples, therefore was considered unacceptable for the purpose of accurately genotyping samples for this study. Therefore HRM analysis was not carried out on DNA from LT patients for purpose of this study.

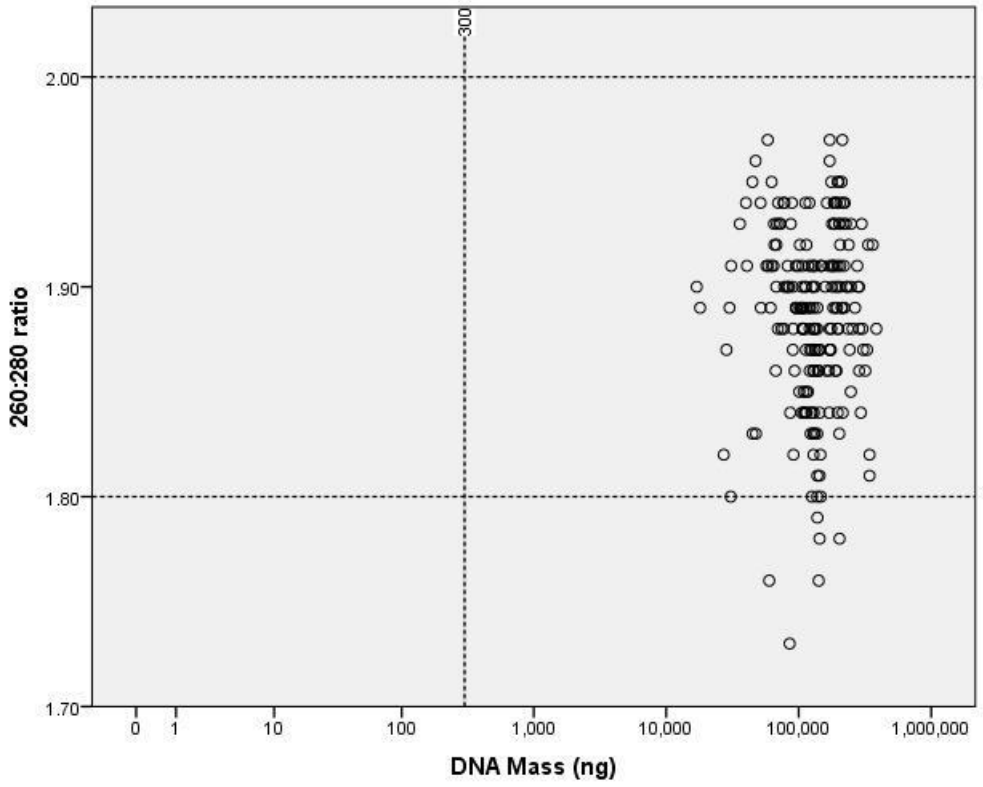
3.3.2 KBioscience genotyping of *CFH* Y402H

CFH Y402H genotyping of recipient DNA from all samples was outsourced to KBioscience (page 115). Duplicates were sent wherever possible.

The quantity and quality of DNA derived from blood samples from 223 patients included in this study are shown in Figure 3.9. Most DNA was of good quantity and quality (n=217, 97%). Results of recipient *CFH* Y402H genotyping are shown

in Table 3.3. All duplicates matched with the original *CFH* Y402H genotype with 100% concordance. Recipient *CFH* Y402H genotype was found to conform to HWE (p=0.088 in controls, ie. non-AMD LT patients). Recipient *CFH* Y402H MAF (C allele) was significantly higher than expected, when compared to the reference population (Rotterdam Study[166]) (0.419 vs 0.362, Chi square test: p=0.014, OR 1.27, 95% CI 1.05-1.54).

Figure 3.9: Recipient DNA characteristics (n=223)



Scatter plot showing DNA quantity (x axis, nanograms – log scale) and DNA quality (y axis, 260:280 ratio) for all DNA samples extracted from whole blood (n=223). DNA quantity was calculated by multiplying concentration with volume of buffer added during DNA extraction. The vertical dashed line represents the minimum amount required for DNA analysis by KBioscience (300ng). The horizontal dashed lines enclose an area representing acceptable DNA quality (260:280 ratio 1.80-2.00), as suggested in the Nanodrop ND100 manual.[236]

Table 3.3: Summary of recipient *CFH* Y402H genotype in LT recipients

***CFH* Y402H Genotype**	**Alleles**	**Frequency**	**Percent**
YY402	TT	77	34.5
YH402	CT	105	47.1
HH402	CC	41	18.4
Total		223	100.0

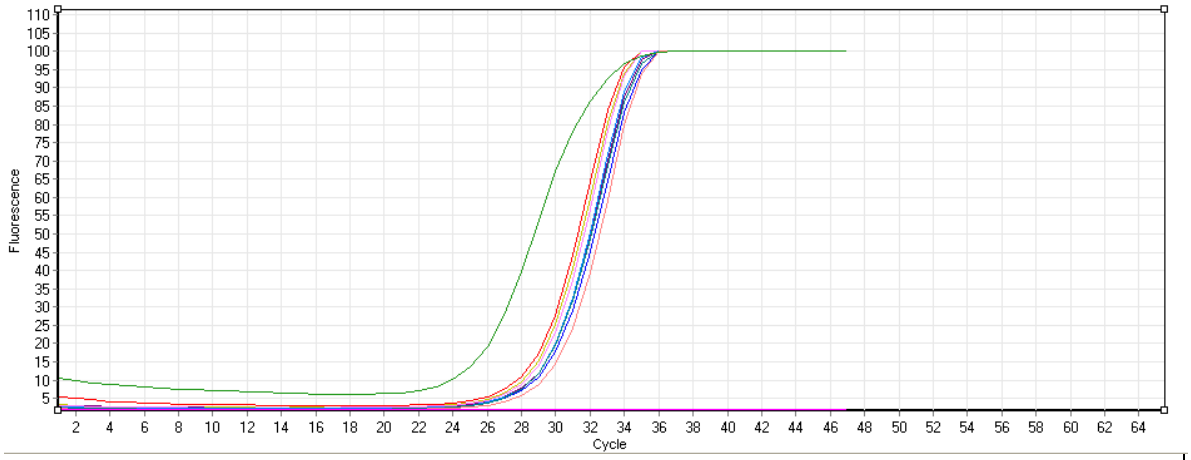
Table showing distribution of recipient *CFH* Y402H genotype based on DNA extracted from recipient peripheral blood cells.

Abbreviations: T = Low risk allele. C = High risk allele. TT = Homozygous for low-risk allele. CT = Heterozygous. CC = Homozygous for high risk allele[4–6].

3.3.3 Verification of *CFH* Y402H genotyping by DNA sequencing

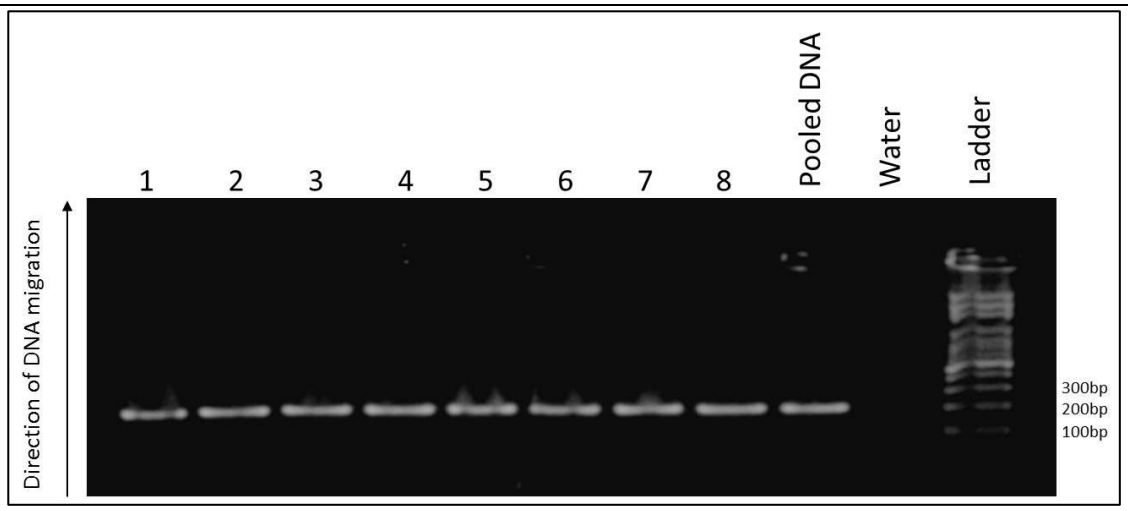
8 samples successfully underwent amplification with PCR (Figure 3.10). Production of a single amplicon was verified by running post-PCR samples on an agarose gel. The results of gel electrophoresis are shown in Figure 3.11.

Figure 3.10: PCR of samples on Rotorgene 6000



X axis indicates number of PCR cycles, y axis the amount of detectable fluorescence, a marker of the quantity of PCR amplicon produced. Green curve: Pooled DNA (+ve control). Pink line along x axis: Negative control (water only) – no amplification indicates absence of contaminants from samples, PCR products for selected samples amplified a little later than compared to positive control (perhaps due to degraded DNA due to repeat freeze/thaw cycles) therefore to ensure amplification of all samples, a total of 50 PCR cycles were run.

Figure 3.11: Agarose gel

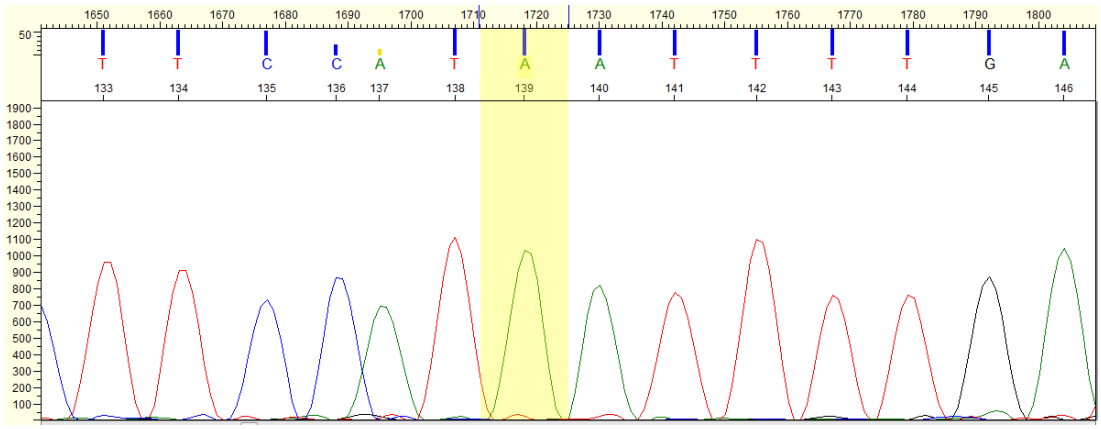


Agarose gel run of PCR products of eight samples (lanes 1-8). Pooled DNA was used as a positive control. Water (i.e. no DNA) was used as a negative control. A 100bp DNA ladder was used (this contains pre-determined DNA fragments differing by 100bp). A single band was obtained at around 200bp for all eight samples, indicating the presence of a single PCR product. This corresponds to the expected amplicon size of 220bp (Table 2.12). A similar band was obtained in the positive control (pooled DNA). No band is seen in the negative control, indicating no contamination.

Five of these eight samples were selected randomly to undergo DNA sequencing for the *CFH* Y402H SNP. Examples of homozygous and heterozygous *CFH* Y402H genotypes identified by DNA sequencing are shown in Figure 3.12 and Figure 3.13.

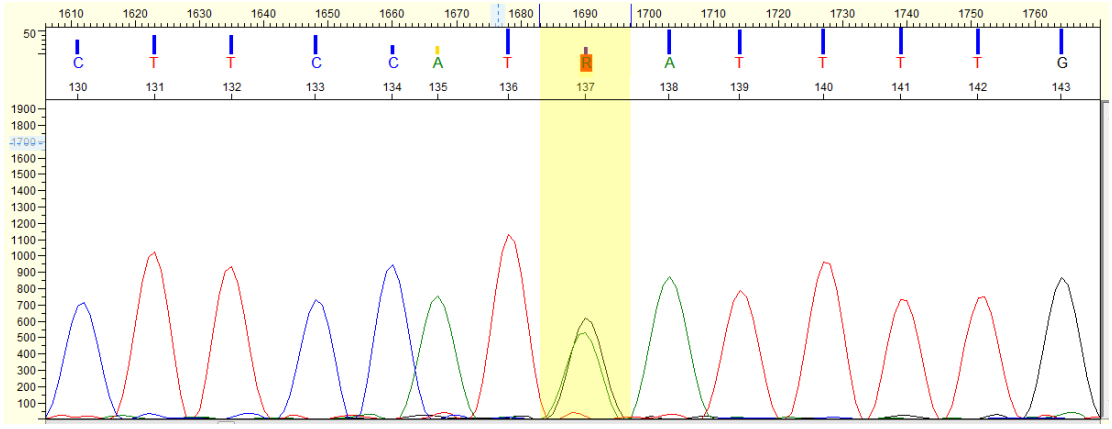
A 100% concordance was found between KBioscience genotyping and DNA sequencing of the *CFH* Y402H SNP for the 5 chosen samples.

Figure 3.12: Example of *CFH* Y402H homozygote



A chromatogram is shown of the reverse strand from sample 3, enlarged to highlight the *CFH* Y402H SNP position (yellow). There is a single green peak at this position, indicating the presence the Adenine (A) base. The complementary base on the forward strand is Thymine. This indicates this sample is homozygous for the low risk *CFH* Y402H SNP (T).

Figure 3.13: Example of *CFH* Y402H heterozygote



This shows a chromatogram of the reverse strand from sample 2, enlarged to highlight the *CFH* Y402H SNP position (yellow). There are two peaks present at this position, indicating the presence of two bases. The black peak indicates the presence of a guanine (G) base, and the green peak indicates presence of the Adenine (A) base. The complementary bases on the forward strand will be Cytosine (C), and Thymine (T). This indicates this sample is heterozygous for the CFH Y402H SNP.

3.3.4 Summary

HRM analysis was not able to accurately discriminate *CFH* Y402H genotype, therefore genotyping was outsourced to KBioscience. The minor allele (C) frequency of the *CFH* Y402H rs1061170 SNP in LT patients was 0.419. There was a significant increase in frequency of recipient *CFH* Y402H risk allele in LT recipients compared to that expected (MAF 0.419 vs 0.362 in general population). The use of KBioscience genotyping of the *CFH* Y402H SNP was verified by the 100% concordance between duplicates genotyped by KBioscience, and also the 100% concordance with DNA sequencing in 5 samples.

Author contribution

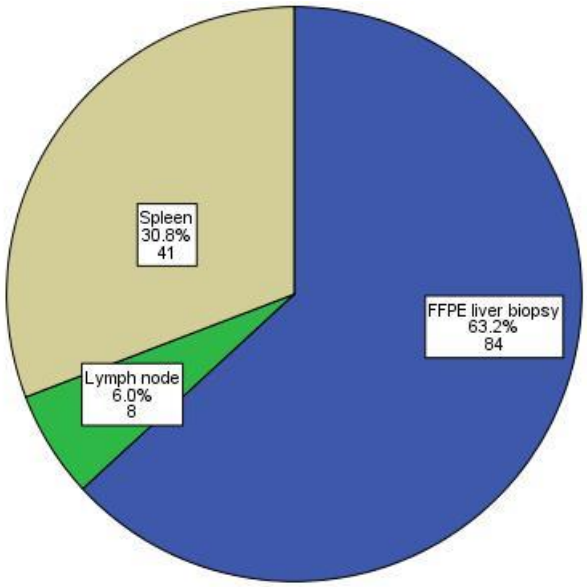
All procedures were carried out by the author except for the following: KBioscience genotyping was outsourced to KBioscience Ltd, Cambridge, UK. Loading and execution of the Applied Biosystems DNA analyser for DNA sequencing was carried out by Steve Doherty (Molecular Pathology, University Hospital Southampton). Helen Griffiths (Senior Research Technician at University of Southampton) and Xiaoli Chen (PhD student, University of Southampton) provided advice and assistance.

3.4 Determining donor *CFH* Y402H genotype

3.4.1 Genotyping donor *CFH* Y402H from retrieved donor tissue

Samples of donor tissue were obtained from a total of 133 patients (59.6%). The majority were in the form of FFPE liver biopsies (Figure 3.14). Donor samples were not available for 90 recipients in any form.

Figure 3.14: Type of donor tissue obtained (n=133)

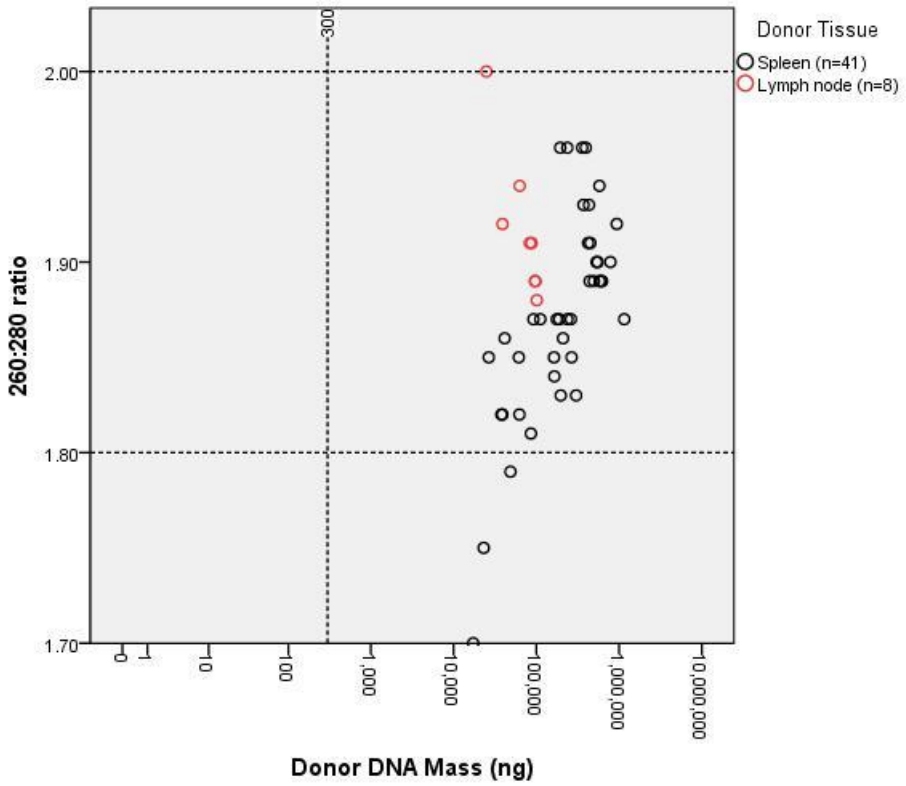


Pie chart showing the source of all donor tissue obtained (type, percentage, number of samples)

DNA extraction from donor spleen / lymph node tissue

DNA was extracted from spleen and lymph node tissue using a modified salting-out method (section 2.5.3 and 2.5.4). DNA from most samples of spleen / lymph node tissue (n=38, 93%) was of good quantity and quality (Figure 3.15), as measured by the Nanodrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The amount of DNA varied, but even for the lowest yields there was sufficient DNA to allow genotyping. The DNA yield from lymph node tissue was generally lower than for spleen tissue

Figure 3.15. Characteristics of donor DNA from spleen / lymph node



Scatter plot showing DNA quantity (x axis, nanograms – log scale) and DNA quality (y axis, 260:280 ratio) for all DNA samples extracted from donor spleen (n=41, black circles) and lymph node (n=8, red circles) tissue. The vertical dashed line represents the minimum amount required for DNA analysis by KBioscience (300ng). The horizontal dashed lines enclose an area representing acceptable DNA quality (260:280 ratio 1.80–2.00).

All DNA samples extracted from donor spleen / lymph node tissue (n=49) were successfully genotyped for the *CFH* Y402H SNP. The results are listed in Table 3.4. The minor allele (C) frequency was 0.429.

Table 3.4: Results of donor spleen / lymph node tissue *CFH* Y402H genotyping

***CFH* Y402H status**	**Genotype**	**Frequency**
YY402	TT	12
YH402	CT	32
HH402	CC	5
Total		49

Table showing distribution of donor *CFH* Y402H genotype obtained from DNA extracted from donor spleen / lymph node.
Abbreviations: T = Low risk allele. C = High risk allele. TT = Homozygous for low-risk allele. CT = Heterozygous. CC = Homozygous for high risk allele[4-6].

DNA extraction from FFPE tissue

Verification of the Qiagen QIAmp DNA FFPE Tissue kit (page 130) was first carried out using three slices of FFPE rat liver, of variable thickness. Good quantity and reasonable quality DNA was obtained (Table 3.5). There was a trend for reduced DNA concentration with thicker slices – this may have been due to excess levels of paraffin interfering with the DNA extraction process.

Table 3.5: Results from DNA extraction from FFPE rat liver

Thickness	DNA conc (ng/µl)	260/280 ratio
10µm	264.95	2.10
20µm	218	2.11
30µm	187.04	2.20

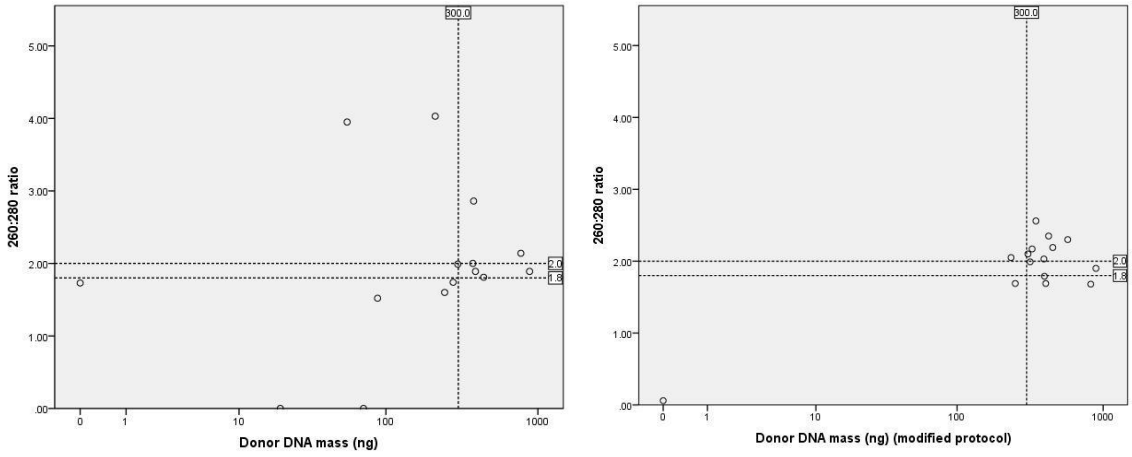
Table showing improved concentration of DNA extracted from thinner slices of FFPE rat liver tissue.

DNA was extracted from 15 samples of donor liver biopsy FFPE tissue (chosen at random) using the original QIAamp® DNA FFPE Tissue kit protocol (Qiagen, Hilden, Germany), and also with the modified protocol (page 131).

There was an improvement in both quantity and quality of DNA extracted from 15 FFPE samples using the modified versus original Qiagen FFPE DNA extraction protocol (Figure 3.16). Therefore the modified protocol was used for DNA extraction from all remaining FFPE samples – results for all samples (n=84) are shown in Figure 3.17. However all DNA samples extracted from FFPE tissue failed

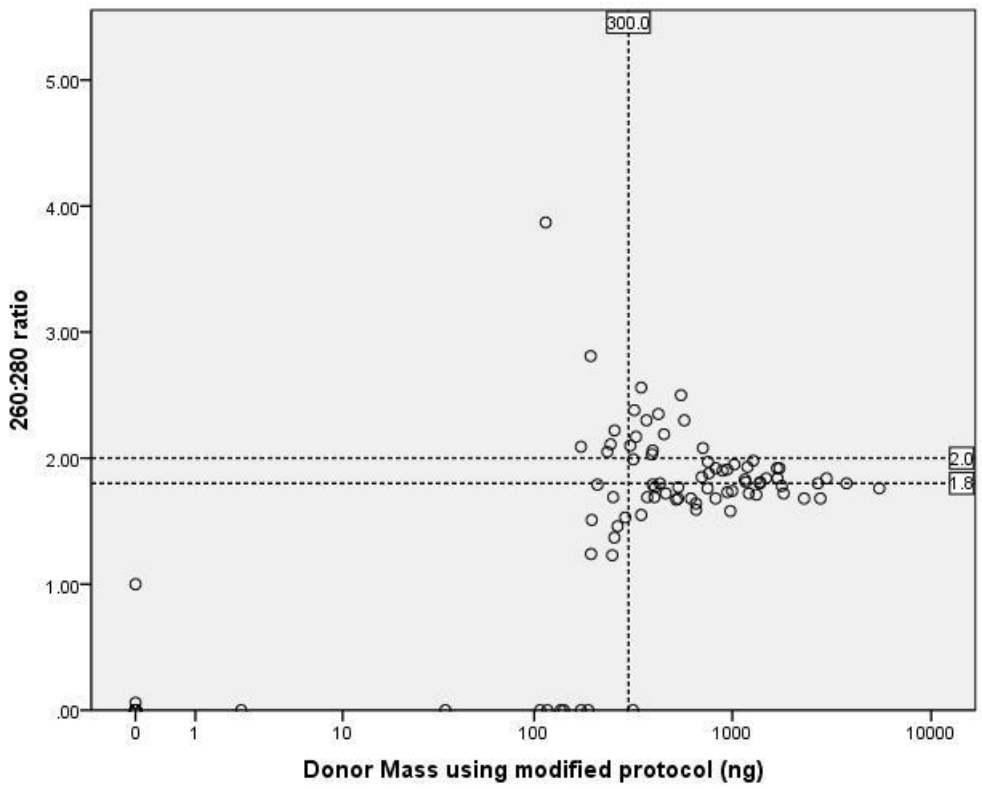
KBioscience genotyping for the *CFH* Y402H SNP. Repeat *CFH* Y402H genotyping with amplified DNA samples (using WGA, page 132) also failed, despite being tested at a variety of conditions (including varying magnesium levels and number of PCR cycles).

Figure 3.16: Comparing DNA extracted from selected FFPE samples between original and modified protocol



Scatter plots showing DNA quantity (x axis, nanograms – log scale) and DNA quality (y axis, 260:280 ratio) for selected samples of DNA extracted from FFPE liver biopsy tissue (n=15) using original protocol (left) and modified protocol (right). DNA quantity was calculated by multiplying concentration with volume of buffer added during DNA extraction. The vertical dashed line represents the minimum amount required for DNA analysis by KBioscience (300ng). The horizontal dashed lines enclose an area representing acceptable DNA quality (260:280 ratio 1.80–2.00), as suggested in the Nanodrop ND100 manual.[236]

Figure 3.17: Results of DNA extraction from all FFPE samples using modified protocol (n=99)



Scatter plot showing DNA quantity (x axis, nanograms – log scale) and DNA quality (y axis, 260:280 ratio) for all samples extracted from formalin-fixed paraffin-embedded (FFPE) liver biopsy tissue (n=84). DNA quantity was calculated by multiplying concentration with volume of buffer added during DNA extraction. The vertical dashed line represents the minimum amount required for DNA analysis by KBioscience (300ng). The horizontal dashed lines enclose an area representing acceptable DNA quality (260:280 ratio 1.80-2.00), as suggested in the Nanodrop ND100 manual.[236] NB: one extreme outlier is excluded from the data presented, with a 260:280 ratio of 12 (this was assumed to be a very poor, highly contaminated sample).

3.4.2 Predicting donor *CFH* Y402H genotype indirectly from recipient plasma CFH Y402 and H402 protein levels

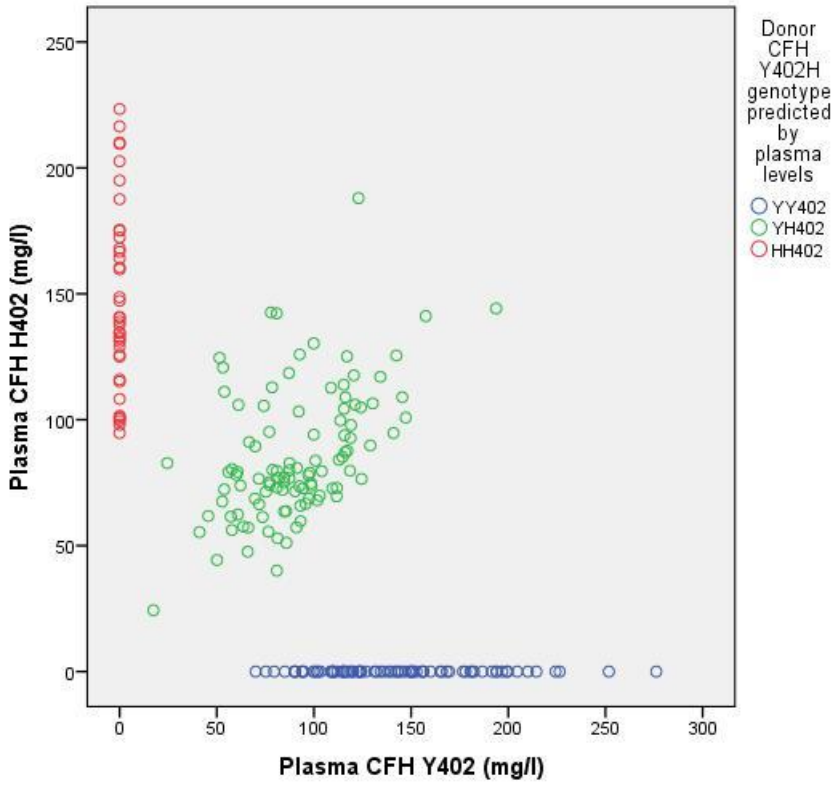
Since donor *CFH* Y402H genotype was only available from 49 donor DNA samples, donor *CFH* Y402H genotype was obtained indirectly by measuring recipient plasma CFH Y402 and H402 protein levels using ELISA (page 132). Plasma CFH Y402 and H402 protein levels with predicted donor liver *CFH* Y402H genotype for all patients are shown in Figure 3.18). A summary of donor *CFH* Y402H genotype (predicted by recipient plasma CFH Y402 and H402 protein levels) is shown in Table 3.6.

Donor *CFH* Y402H genotype was available in 49 recruited LT patients, and these samples allowed verification of whether the use of recipient plasma CFH Y402 and H402 protein levels in predicting donor *CFH* Y402H genotype was appropriate. In fact, a 100 % correlation was observed between actual donor *CFH* Y402H genotype and that predicted by recipient plasma CFH Y402 and H402 protein levels (Figure 3.19).

A subgroup of patients (n=22) was analysed, in whom the liver *CFH* Y402H genotype altered post-LT from one homozygous state to another (i.e. *CFH* YY402 to HH402, or *CFH* HH402 to YY402). In these patients, plasma levels of CFH Y402 or H402 protein corresponded only to the new liver *CFH* Y402H genotype, and not the original *CFH* Y402H of the patient (Figure 3.20).

Donor *CFH* Y402H MAF (C allele) for all patients, as predicted by plasma CFH Y402 or H402 protein levels, was not significantly different compared to the reference population (Rotterdam Study[166]) (40.6% vs 36.2%, chi-square test: p=0.061).

Figure 3.18: Plasma CFH Y402 and H402 protein levels in all patients



Scatter plot showing plasma levels of CFH Y402 and H402 proteins in all recruited patients (n=223). Each circle represents one patient. Patients fall into one of three groups based on whether CFH Y402 or H402 protein is absent / present, and donor liver *CFH* Y402H genotype can thus be predicted (Table 2.19).

1. Blue circles indicate patients with a complete absence of plasma CFH H402 but with presence of plasma CFH Y402 protein. Predicted donor liver genotype is *CFH* YY402.
2. Red circles indicate patients with a complete absence of plasma CFH Y402 but with presence of plasma CFH H402 protein. Predicted donor liver genotype is *CFH* HH402.
3. Green circles indicate patients with both CFH Y402 and H402 proteins present in plasma. Predicted donor liver genotype is *CFH* YH402.

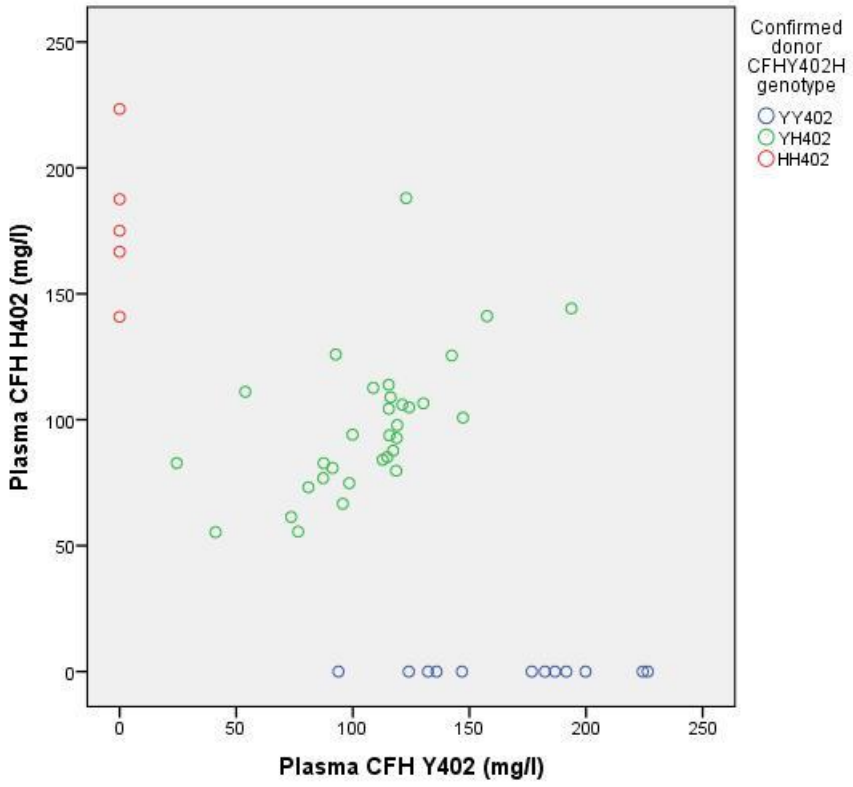
Table 3.6: Results of *CFH* Y402H genotype in LT recipients

***CFH* Y402H status**	**Genotype**	**Frequency**	**Percent**
YY402	TT	80	35.9
YH402	CT	105	47.1
HH402	CC	38	17.0
Total		223	100.0

Table showing distribution of predicted donor *CFH* Y402H genotype based on recipient plasma CFH Y402 and H402 protein levels.

Abbreviations: T = Low risk allele. C = High risk allele. TT = Homozygous for low-risk allele. CT = Heterozygous. CC = Homozygous for high risk allele[4–6].

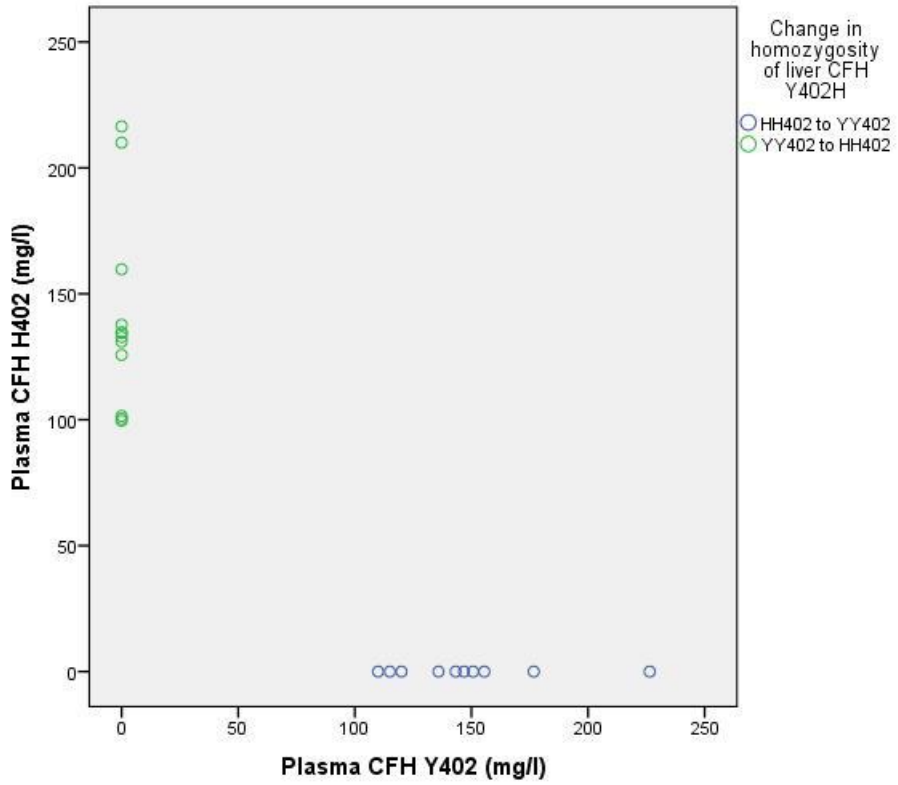
Figure 3.19: Confirmed donor *CFH* Y402H genotype versus plasma CFH Y402 and H402 protein levels



Scatter plot showing plasma levels of CFH Y402 and H402 proteins in patients with confirmed donor tissue *CFH* Y402H genotyping (n=49). Each circle represents one patient. Patients fall into one of three groups based on whether CFH Y402 or H402 protein is absent / present.

1. Blue circles indicate patients with a complete absence of plasma CFH H402 but with presence of plasma CFH Y402 protein. Predicted donor liver genotype is *CFH* YY402 (n=12)
2. Red circles indicate patients with a complete absence of plasma CFH Y402 but with presence of plasma CFH H402 protein. Predicted donor liver genotype is *CFH* HH402 (n=5).
3. Green circles indicate patients with both CFH Y402 and H402 proteins present in plasma. Predicted donor liver genotype is *CFH* YH402 (n=32).

Figure 3.20: Plasma CFH Y402 and H402 protein levels in patients undergoing an alteration in liver *CFH* Y402H homozygosity following LT



Scatter plot showing plasma levels of CFH Y402 and H402 proteins in patients in whom the liver *CFH* Y402H genotype altered post LT from one homozygous state to another (i.e. *CFH* YY402 to HH402, or *CFH* HH402 to YY402) (n=22). Each circle represents one patient. Patients fall into one of two groups based on whether CFH Y402 or H402 protein is absent / present.

1. Blue circles indicate patients with a complete absence of plasma CFH H402 but with presence of plasma CFH Y402 protein (n=12). No plasma H402 protein is seen in *CFH* HH402 patients carrying a transplanted liver with the *CFH* YY402 genotype.
2. Green circles indicate patients with a complete absence of plasma CFH Y402 but with presence of plasma CFH H402 protein (n=10). No plasma Y402 protein is seen in *CFH* YY402 patients carrying a transplanted liver with the *CFH* HH402 genotype.

3.4.3 Summary

Donor tissue samples were successfully obtained from more than half of all patients, the majority in the form of FFPE donor liver biopsy tissue taken from the recipient immediately post-LT. Tissue was also obtained from archives of samples previously retrieved direct from the donor at the time of organ donation, including from spleen and lymph node tissue. *CFH* Y402H was successfully genotyped from DNA extracted from all 49 samples of donor spleen / lymph node tissue, but was unsuccessful from all FFPE tissue despite amplification pre-genotyping. Figure 3.21 summarises the outcome of donor DNA sample acquisition and genotyping.

Use of recipient plasma CFH Y402 and H402 protein levels to determine donor *CFH* Y402H genotype was validated with 100% concordance. *CFH* YY402 patients transplanted with a *CFH* HH402 liver had complete absence of plasma CFH Y402 protein, and likewise *CFH* HH402 patients transplanted with a *CFH* YY402 liver had complete absence of plasma CFH H402 protein. The frequency of donor *CFH* Y402H C allele was no different to that expected from a general population, although did approach statistical significance.

Author contribution
All procedures were carried out by the author, except for the following.

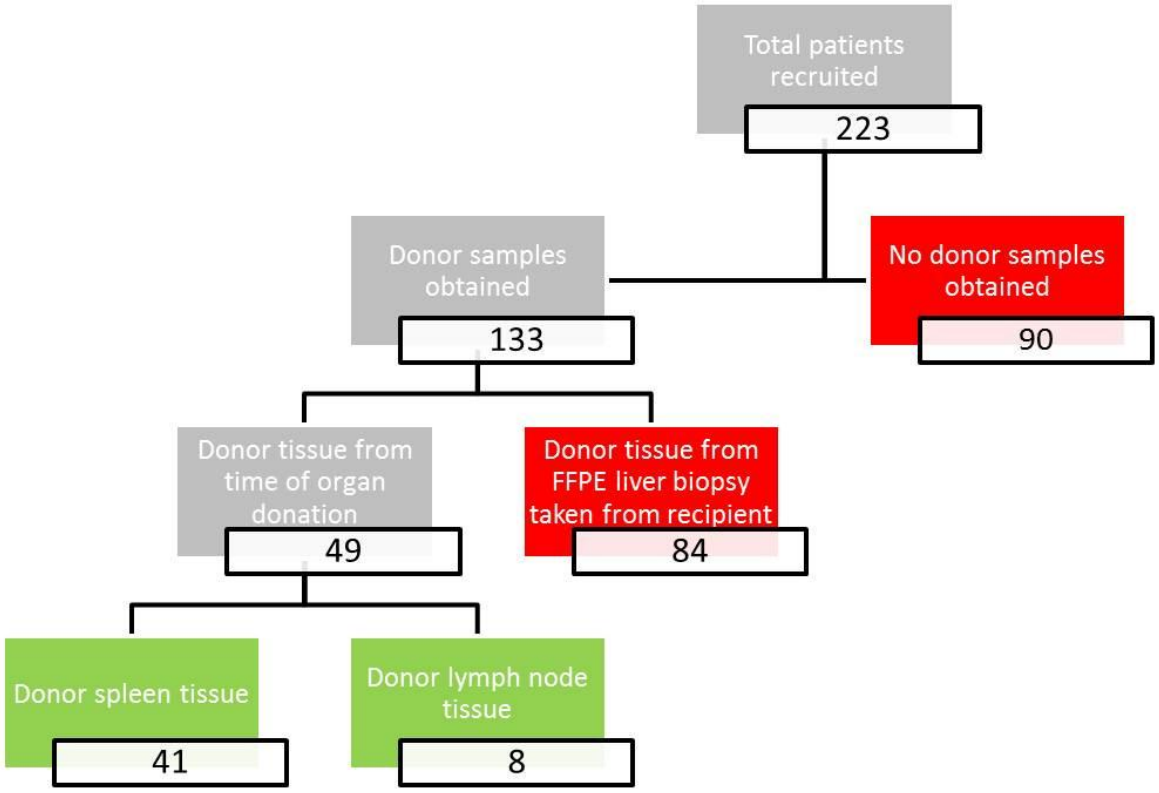
Donor samples were obtained with the kind assistance of the following individuals:

- Addenbrooke's Hospital, Cambridge: Dr Craig Taylor (Head of Department of Tissue Typing), Helen Morgan (Clinical Scientist, Tissue Typing), and Dr Beverley Haynes (Tissue Bank Manager).
- Queen Elizabeth Hospital, Birmingham: Bridget Gunson (Centre for Liver Research Manager), Professor Stefan Hubscher (Professor of Hepatic Pathology), Valerie Adkins (PA to Professor Hubscher).
- Kings College Hospital, London: Lisa Gifford (Clinical Trials Manager, Institute of Liver Studies), Dr Alberto Quaglia (Consultant in Histopathology), Anne Rayner (Biomedical Section Laboratory Manager).
- Samples from recipients recruited at Southampton were obtained from one of the three other sites corresponding to where the patient underwent LT.

Genotyping was outsourced to KBioscience Ltd, Cambridge, UK. Helen Griffiths (Senior Research Technician at University of Southampton) and Xiaoli Chen (PhD student, University of Southampton) provided advice and assistance.

Plasma CFH Y402 and H402 protein assays were carried out at the complement laboratory at Cardiff University, Cardiff, UK. This was kindly arranged by Professor Paul Morgan, and carried out by Dr Svetlana Hakobyan (research associate). I carried out all plasma acquisition, processing and data analysis.

Figure 3.21: Flow diagram of donor DNA analysis



Flow diagram showing breakdown of number and type of donor samples obtained. Green boxes denote samples with successful *CFH* Y402H genotyping by KBioscience Ltd; red boxes denote samples which either failed genotyping or were not available. Donor *CFH* Y402H was available for a total of 49 patients recruited.

3.5 Measurement of plasma complement protein levels in liver transplant patients

Measurement of complement protein levels was carried out as described in section 2.6). Where observed levels exceeded the kit maximum detectable level, the maximum detectable value was used (Table 3.7). The association of plasma complement levels with recipient and donor CFH Y402H genotype, and with AMD status was investigated. Presence of at least one rs1061170 C risk allele was used to define presence of the *CFH* Y402H sequence variation in either recipients or donors (dominant model).[10]

Table 3.7: Upper limits of complement assays

Complement protein	Maximum detectable value
C3a	600 ng/ml
C4a	2 ug/ml
TCC	10 AU /ml
C5a	100 ng/ml

Table showing the maximum detectable values of the complement assay kits. AU = Arbitrary units.

LT patients and recipient rs1061170 C risk allele

Using the Mann-Whitney U test with Bonferroni correction for multiple testing, there was no difference in plasma complement protein levels in LT patients with at least one **recipient** rs1061170 C risk allele (n=146) compared to those with no risk alleles (n=77) (Figure 3.22).

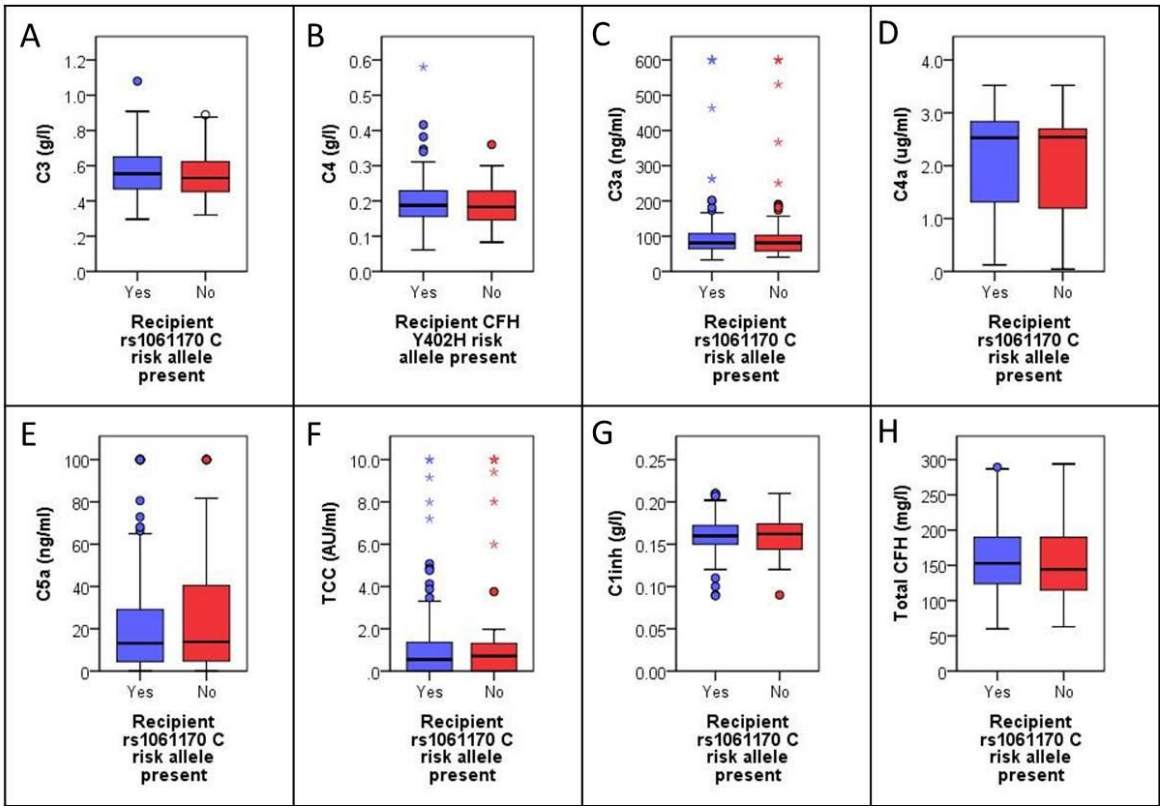
LT patients and donor rs1061170 C risk allele

Using the Mann-Whitney U test with Bonferroni correction for multiple testing, plasma total CFH was elevated in LT patients with at least one **donor** rs1061170 C risk allele (n=143), compared to LT patients with no risk alleles (n=80) (p=0.002 uncorrected, p=0.016 corrected). Levels of all other complement proteins were similar (Figure 3.23).

LT patients and AMD

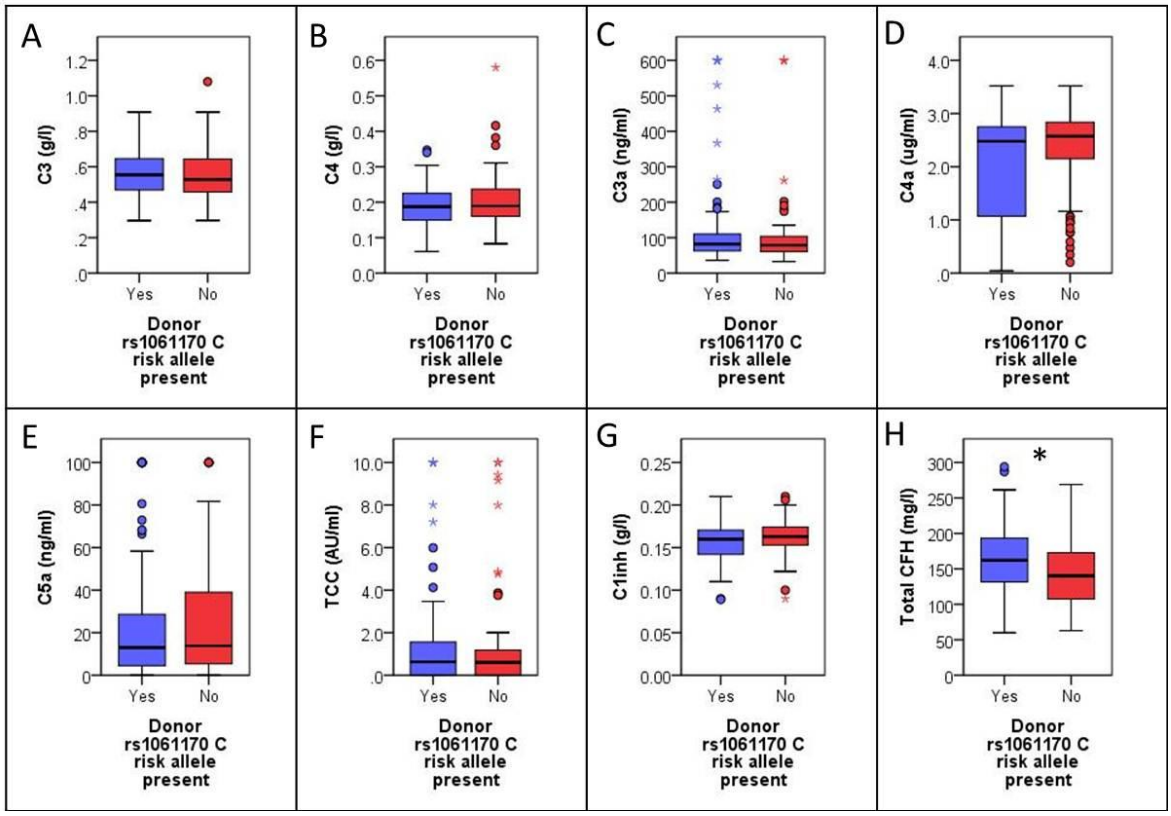
Using the Mann-Whitney U test with Bonferroni correction for multiple testing, there was no difference in plasma complement protein levels between LT patients without AMD (n=79) compared to those with AMD (n=144) (Figure 3.24).

Figure 3.22: Plasma complement levels in LT patients without / with a recipient rs1061170 C risk allele



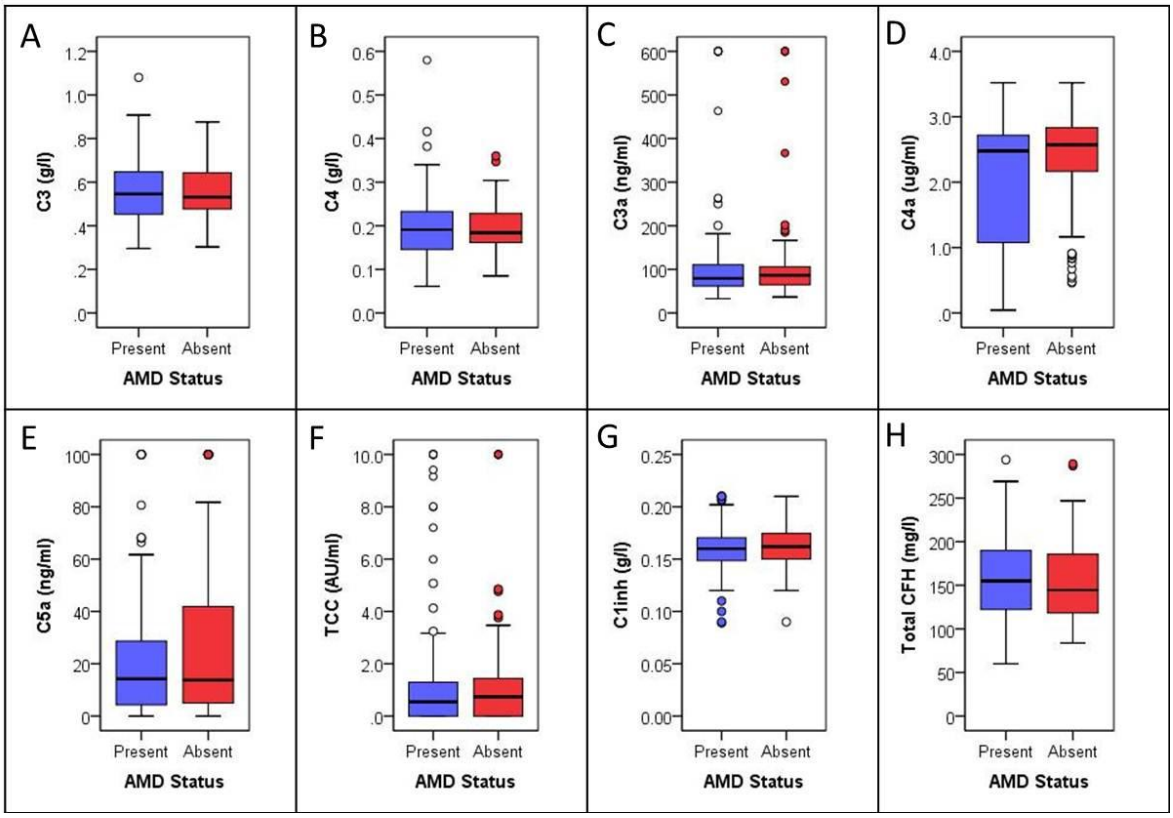
Box and whisker plots showing plasma complement protein levels in LT patients with (n=146) and without (n=77) a **recipient** rs1061170 (*CFH* Y402H) C risk allele. A-B: Complement substrate proteins (C3 and C4). C-F: Complement activation products (C3a, C4a, C5a and TCC). G-H: Complement regulators (C1 inhibitor and total CFH). AU = Arbitrary units. Total n=223 for all analysis (except for C4a: n=222). NB: The box length represents the interquartile range (IQR), and the horizontal line within the box represents the median value. The minimum and maximum values (excluding the outlier and extreme values) are depicted by the whisker ends. Outliers (> (1.5 x IQR) from the upper and lower points of the IQR) are depicted by a circle, and extreme values (> (3xIQR) from upper and lower points of IQR) by an asterisk.

Figure 3.23: Plasma complement levels in LT patients without and with a donor rs1061170 C risk allele



Box and whisker plots showing plasma complement protein levels in LT patients with (n=143) and without (n= 80) a **donor** rs1061170 (*CFH* Y402H) C risk allele. A-B: Complement substrate proteins (C3 and C4). C-F: Complement activation products (C3a, C4a, C5a and TCC). G-H: Complement regulators (C1 inhibitor and total CFH). AU = Arbitrary units. Total n=223 for all analysis (except for C4a: n=222). A black asterisk indicates a significant difference between the 2 groups: total CFH protein levels was significantly greater in LT patients with at least one rs1061170 C risk allele, compared to those with no C alleles (p=0.016 corrected)

Figure 3.24: Plasma complement levels in LT patients without / with AMD.



Box and whisker plots showing plasma complement protein levels in LT patients with (n=144) and without (n=79) AMD. A-B: Complement substrate proteins (C3 and C4). C-F: Complement activation products (C3a, C4a, C5a and TCC). G-H: Complement regulators (C1 inhibitor and total CFH). AU = Arbitrary units. N=223 for all analysis (except for C4a: n=222).

3.5.1 Summary

The presence of a *CFH* Y402H sequence variation in the donor liver was associated with increased plasma CFH levels. There was no association with plasma complement protein levels with recipient *CFH* Y402H genotype and with AMD status in LT patients.

However the analysis carried out in this chapter was univariate only, without taking into account other risk factors for AMD. A multivariate analysis taking into account other AMD risk factors will be described in the next chapter.

Author contribution

All procedures were carried out by the author except the following: Dr Svetlana Hakobyan (Research Associate, Cardiff University) provided guidance and supervision of complement ELISAs. Nephelometric analysis of C3, C4 and C1 inhibitor were carried out by the Clinical Biochemistry department, University Hospital of Wales, Cardiff, UK. All complement analysis was kindly arranged by Professor Paul Morgan (Cardiff University, Cardiff, UK).

3.6 Is AMD associated with recipient or donor *CFH* Y402H genotype?

Having obtained AMD status, as well as recipient + donor *CFH* Y402H genotype status for all recruited patients, the original research hypothesis can be addressed: Is the presence of AMD associated with recipient or donor *CFH* Y402H genotype?

Binary logistic regression analysis was carried out using AMD status (present/absent) as the dependent variable and other covariates added in stepwise blocks using the forced entry method.[266] Genotype was coded using an additive model (high risk homozygotes CC=2, heterozygotes CT=1, low risk homozygotes TT=0). Covariates included in the initial block included recipient and donor *CFH* Y402H genotype and potential confounders known to be significant risk factors for AMD, including age, BMI, and smoking status (pack-years).[2] Other covariates were then added individually to this model.

Recipient and donor *CFH* Y402H genotype for all patients is summarised in Table 3.8. Distribution of *CFH* Y402H genotypes was similar amongst both recipients and donors. Overall, liver *CFH* Y402H genotype changed post-LT in 142 patients (63.7%).

Table 3.8: Recipient versus donor *CFH* Y402H genotype

		Donor *CFH* Y402H genotype			**Total**
		TT	CT	CC	
Recipient *CFH* Y402H genotype	TT	30	35	12	77
	CT	40	45	20	105
	CC	10	25	6	41
Total		80	105	38	223

Table summarising recipient and donor *CFH* Y402H genotype. Donor genotype was based on recipient plasma CFH Y402 and H402 protein levels.

Results of initial binary logistic regression analysis are shown in Table 3.9. Recipient *CFH* Y402H (p=0.036, OR 1.559) and age (p=0.030, OR 1.055) were associated with presence of AMD.

Further covariates (previously collected patient/donor characteristics, page 93) were added individually to this initial model, including duration of current LT, primary cause of LT, concomitant medical conditions, immunosuppressive therapy, other medication, blood pressure, hepatic function, renal function, plasma complement levels, donor age, and donor gender (Table 3.10). The only additional variables associated with AMD was current use of bisphosphonates (p=0.012, OR=2.602, 95% CI 1.234 to 5.483), and low plasma C4a levels (p=0.019, OR=0.681, 95% CI 0.494–0.939).

Table 3.9. Logistic regression analysis (n=223).

VARIABLE	**CODING**	**P VALUE**	**OR**	**95% CI**
Age (years)	(continuous)	0.030	1.055	1.005–1.107
Gender	1=female, 0=male	0.319	1.355	0.746–2.461
Smoking status (pack-years)	(continuous)	0.598	0.995	0.977–1.014
Body mass index (kg/m^2)	(continuous)	0.937	0.997	0.935–1.064
Recipient *CFH* Y402H genotype	2 = CC, 1 = CT, 0 = TT	0.036	1.559	1.030–2.361
Donor *CFH* Y402H genotype	2 = CC, 1 = CT, 0 = TT	0.626	0.905	0.605–1.353

Results from binary logistic regression analysis are shown. AMD status (present/absent) was selected as the dependent variable and other covariates included together using the forced entry method. CFH = Complement factor H. CI = Confidence interval. OR = Odds ratio.

Table 3.10. Addition of individual covariates to initial model

VARIABLES		CODING	P VALUE	OR	95% CI
RECIPIENT	**LT DETAILS**				
	Duration of current LT	Continuous	0.501	0.978	0.917 to 1.043
	PRIMARY CAUSE OF LT				
	Primary biliary cirrhosis (PBC)	Categorical (1=Yes, 0=No)	0.521	1.264	0.618 to 2.588
	Primary sclerosing cholangitis (PSC)	Categorical (1=Yes, 0=No)	0.311	0.648	0.280 to 1.499
	Cholestatic disease (PBC + PSC)	Categorical (1=Yes, 0=No)	0.859	0.946	0.510 to 1.752
	Infection (Hepatitis B and C virus)	Categorical (1=Yes, 0=No)	0.768	1.143	0.469 to 2.786
	Autoimmune causes (PBC, PSC and autoimmune hepatitis)	Categorical (1=Yes, 0=No)	0.605	0.849	0.456 to 1.580
	Inflammatory causes (autoimmune + infectious causes)	Categorical (1=Yes, 0=No)	0.327	0.716	0.367 to 1.396
	CONCOMITANT MEDICAL CONDITIONS				
	Hypertension	Categorical (1=Yes, 0=No)	0.759	1.105	0.583 to 2.098
	Renal impairment	1=Yes, 0=No	0.864	1.053	0.585 to 1.895
	Hypercholesterolemia	Categorical (1=Yes, 0=No)	0.425	0.787	0.437 to 1.418
	Diabetes	Categorical (1=Yes, 0=No)	0.355	0.725	0.367 to 1.434
	Ischemic heart disease / cerebrovascular accident	Categorical (1=Yes, 0=No)	0.218	0.610	0.277 to 1.341
	Colitis	Categorical (1=Yes, 0=No)	0.093	0.495	0.218 to 1.125
	IMMUNOSUPPRESSION				
	Tacrolimus	Categorical (1=Yes, 0=No)	0.352	0.761	0.428 to 1.353
	Steroids	Categorical (1=Yes, 0=No)	0.389	1.296	0.718 to 2.338
	Mycophenolate	Categorical (1=Yes, 0=No)	0.762	1.097	0.602 to 2.000
	Azathioprine	Categorical (1=Yes, 0=No)	0.493	0.800	0.423 to 1.514
	Cyclosporin	Categorical (1=Yes, 0=No)	0.342	0.726	0.375 to 1.406
	Sirolimus	Categorical (1=Yes, 0=No)	0.215	1.900	0.688 to 5.249
	Total number of agents	Ordinal	0.739	0.930	0.607 to 1.426

	OTHER MEDICATION				
	Statin	Categorical (1=Yes, 0=No)	0.514	1.220	0.672 to 2.212
	Bisphosphonate	Categorical (1=Yes, 0=No)	**0.012** *	2.602	1.234 to 5.483
	Beta blockers	Categorical (1=Yes, 0=No)	0.742	1.124	0.560 to 2.255
	Aspirin	Categorical (1=Yes, 0=No)	0.150	0.604	0.303 to 1.201
	Ursodeoxycholic acid	Categorical (1=Yes, 0=No)	0.851	1.075	0.504 to 2.296
	Diuretics (loop or thiazide)	Categorical (1=Yes, 0=No)	0.210	1.821	0.714 to 4.647
	CLINICAL FINDINGS				
	Mean arterial blood pressure (mmHg)	Continuous	0.092	0.978	0.953 to 1.004
	Current hepatocellular dysfunction (AST or ALT >35)	Categorical (1=Yes, 0=No)	0.447	1.307	0.656 to 2.604
	PLASMA COMPLEMENT LEVELS				
	C3 (g/l)	Continuous	0.967	0.955	0.108–8.429
	C4 (g/l)	Continuous	0.876	1.432	0.015–132.771
	C3a (ng/ml)	Continuous	0.630	1.000	0.999–1.002
	C4a (ug/ml)	Continuous	**0.019** *	.681	0.494–0.939
	C5a (ng/ml)	Continuous	0.110	.992	0.982–1.002
	TCC (AU/l)	Continuous	0.849	.990	0.893–1.098
	C1i (g/l)	Continuous	0.515	.011	0.000–8675.657
	CFH (mg/l)	Continuous	0.710	1.001	0.995–1.007
DONOR	**DEMOGRAPHIC INFORMATION**				
	Donor gender	Categorical (1=female, 0=male)	0.406	0.770	0.415 to 1.427
	Donor age	Continuous	0.778	1.003	0.983 to 1.022
	Donor age (55 years and over)	Categorical (1=Yes, 0=No)	0.819	1.090	0.522 to 2.274

The table lists the results of individual addition of further covariates to the initial block regression analysis. The coding used for each covariate in the regression analysis is also shown. ALT = Alanine aminotransferase, AMD = Age-related macular degeneration, AST = Aspartate aminotransferase, AU=Arbitrary Units, CI = Confidence interval, LT = Liver transplant, OR = Odds ratio, PBC = Primary biliary cirrhosis, PSC = Primary sclerosing cholangitis

3.6.1 Summary

AMD in LT patients was associated with recipient rather than donor *CFH* Y402H genotype. Therefore receiving a liver which delivers either risk or protective CFH to plasma for at least five years had no effect that could be detected on the development of AMD. Increasing age as expected was also associated with presence of AMD. The only additional variables associated with AMD were lower plasma C4a levels and current use of bisphosphonates.

Author contribution

All data analysis was carried out by the author, with advice from Dr Jane Gibson (Genetic Epidemiologist, University of Southampton) and Ho Ming Yuen (Medical Statistician, University of Southampton).

CHAPTER 4 Discussion

The discussion begins with a summary of the results, including whether the hypothesis was proved. This is followed by an interpretation of the results, including how the results compare to the literature. The limitations of the research are then described, followed by the conclusion.

4.1 Thesis summary

The initial hypothesis was disproved. AMD status in LT patients was not associated with donor *CFH* Y402H genotype. However AMD status was associated with **recipient** *CFH* Y402H genotype.

The overall prevalence of AMD in 223 LT patients was significantly higher compared to a general population of similar ethnicity and age. This was accounted for by a greater proportion of more severe AMD. There was also a significant increase in frequency of recipient *CFH* Y402H risk (C) allele in LT recipients compared to a general population of similar ethnicity and age. In univariate analysis, plasma CFH protein levels were associated with donor and not recipient *CFH* Y402H genotype. There was no difference in plasma CFH or other complement protein levels between LT patients with or without AMD. However when included in logistic regression, reduced C4a levels and use of bisphosphonates were associated with AMD.

4.2 Interpretation of results and comparison to literature

Recipient and not donor *CFH* Y402H genotype is associated with AMD

The results suggests that receiving a liver which delivers either risk or protective CFH to plasma for at least five years had no detectable effect on the development of AMD. Intraocular *CFH* Y402H genotype remains unchanged, therefore presumably intraocular CFH production does not alter post-LT. Since AMD was associated with recipient (ie. original) *CFH* Y402H genotype, this suggests that local (intraocular), rather than systemic (hepatic) complement activity is more important in AMD pathogenesis. This is consistent with Bomback et al, who reported that systemic intravenous administration of a complement inhibitor (Eculizumab - an anti-C5 antibody, Alexion Pharmaceuticals Ltd) over 12 months for complement-induced glomerulopathy had no discernible effect on retinal drusen; however this was reported in only 2 patients who had retinal drusen at baseline.[267] Several complement inhibitors given both intravenously and locally in the eye by intravitreal injection are in early clinical development for treating both GA and nvAMD, including C3 and C5 and CFD inhibitors.[152;154-156;158-161] These studies are on-going, and time will tell whether local or systemic complement inhibition is more effective in reducing AMD progression in the clinical setting. Early results for ARC1905 (Ophthotech Corp.), an anti-C5 aptamer given by intravitreal injection combined with intravitreal lucentis, shows promise in treating nvAMD.[157]

It should be noted that the sample size chosen was selected on the basis of a 5% chance of a type 1 error. In other words there is a 5% chance that the null hypothesis (that there was no relationship between AMD status and donor *CFH* Y402H genotype) was rejected incorrectly. A possible reason for this may be the assays used for plasma CFH Y402 and H402 protein analysis were not sensitive enough to enable accurate interpretation of donor *CFH* Y402H genotype in all patients. Furthermore, the effects of lifelong exposure to a multitude of individual systemic and environmental factors have not been taken into account due to the cross-sectional nature of the study. These points should be taken into consideration before one can definitively conclude that local intraocular CFH

production and not systemic CFH production is more important in AMD pathogenesis.

No study to date has reported the effect of systemic manipulation of *CFH* (or any other gene) on the development of AMD.

AMD prevalence was increased in LT patients

AMD prevalence in LT patients was increased compared to the Rotterdam Study baseline population.[26] The Rotterdam Study was large scale epidemiological prospective study which recruited almost 5000 patients sampled randomly from a general population, regardless of AMD status. The Rotterdam Study was chosen as the reference population to compare AMD prevalence with due to the similarity in age (≥55 years old) and ethnicity (western European) with the LT patient group. This was also the reason why the Rotterdam grading system was chosen for AMD grading in LT patients, as opposed to other grading systems such as that used in the AREDS study.[31] There are no previous reports of AMD prevalence in LT patients, or in patients with any liver disease.

Immunosuppression and AMD in LT patients

There was no association between type or number of immunosuppressive therapy and AMD. Immunosuppressive therapy is routinely given to all LT patients; consequently this does not directly impact on the findings. Chronic systemic immune suppression has previously been associated with a reduced prevalence of AMD, as observed in rheumatoid arthritis patients.[268] Therefore, systemic immunosuppression would be expected to decrease AMD prevalence, whereas an increase in AMD prevalence was found in LT patients. This suggests immunosuppression had little confounding effect on the overall results.

Recipient *CFH* Y402H sequence variation was more prevalent than expected

The increased frequency of recipient *CFH* Y402H risk allele in LT patients compared to the reference population may be a contributing factor to the increased prevalence of AMD observed in LT patients. There may also be an association between the *CFH* Y402H sequence variation and the requirement of LT. However the aetiology of liver disease requiring LT is very heterogeneous. For example, 23 underlying causes of LT were recorded for the group of patients recruited for this study. Therefore considering LT patients as a whole as one

amalgamated group is too simplistic, and therefore associating *CFH* Y402H to LT patients as a whole should be done with caution.

There are no studies reported in the literature associating the *CFH* Y402H sequence variation with liver disease. Although the *CFH* Y402H sequence variation has been associated with atypical HUS[196;197], the condition itself is not regarded as a liver disease, rather a disease of the kidney resulting from dysfunctional hepatic CFH production.

There is nevertheless considerable evidence associating the complement system as a whole with various forms of liver disease, including viral hepatitis, alcoholic liver disease, liver ischaemia/reperfusion injury, liver fibrosis, injury, and repair (please refer to the review article by Qin et al for more details).[269]

The association of CFH with liver disease in the literature is unclear. CFH may have a role in protection against liver inflammation; Sun et al found that complement activation in mice through intraperitoneal lipopolysaccharide injection displayed reduced liver inflammation and damage when given CR2-CFH (a hybridised more active form of CFH).[270] However histological evidence suggests that CFH may not have an important role in complement regulation within the liver itself. Halme et al showed that blocking the action of CFH protein produced by cultured human hepatocytes had no discernible effect on complement cell lysis activity.[7] CFH production in liver disease appears to vary according to the underlying aetiology. Elevated serum levels of CFH have been found in patients with PBC[271] and liver cirrhosis secondary to chronic HSV.[272] Contrastingly reduced hepatic *CFH* gene expression has been associated with recurrence of hepatocellular carcinoma in Asian patients[273], and in mice exposed to chronic alcohol consumption.[274] Therefore the role of CFH in liver disease is still unclear, and may vary according to the underlying primary disease.

Donor CFH Y402H genotype

Prevalence of donor *CFH* Y402H genotype compared to the reference population (Rotterdam Study) did approach statistical significance. However comparing the donor group to a reference population is problematic since donor ethnicity was not known. This introduces an additional confounder, since prevalence of *CFH*

Y402H genotype is known to vary with ethnicity.[168] The only donor information available was gender and age, due to confidentiality reasons.

Furthermore, prior to organ donation, donor screening is undertaken and donors are excluded if certain co-morbidities are present. Consequently the donor group do not represent a general randomly-selected population. Therefore donors were not used as a control population for comparison of *CFH* Y402H prevalence in recipients.

CFH Y402 and H402 protein levels and change of *CFH* Y402H homozygosity post-LT

The finding that *CFH* YY402 patients transplanted with a *CFH* HH402 liver have absent plasma CFH Y402 protein, and that *CFH* HH402 patients transplanted with a *CFH* YY402 liver have absent plasma CFH H402 protein, confirms previous evidence that the liver is the only producer of CFH protein in the systemic circulation. Schmeling et al used isoelectric focusing to show plasma CFH allotype converted to that of the donor in six out of six LT recipients.[118] The present study verifies this in a larger group, and is novel in using specific CFH Y402 or H402 protein assays and confirmation with actual donor genotype. Furthermore these findings also demonstrate for the first time the absence of extra-hepatic contribution to circulating CFH protein levels.

Systemic CFH levels and donor *CFH* Y402H genotype

An increase in systemic total CFH protein levels was associated with presence of donor (but not recipient) *CFH* Y402H sequence variation. This suggests production of dysfunctional CFH protein by the new liver in LT patients may induce a compensatory increase in hepatic total CFH protein production.

Plasma CFH levels are not associated with *CFH* Y402H genotype in the literature. Scholl et al found an association between systemic complement activation, but not in plasma CFH levels, and a risk *CFH* haplotype which included the Y402H SNP.[145] Both Hecker et al and Silva et al also found no association between *CFH* Y402H genotype and plasma CFH levels in patients of European and Brazilian origin respectively.[144;275]

Systemic CFH levels in LT patients, and AMD

Systemic levels of complement proteins, especially complement activation products, have previously been found to be elevated in AMD.[145;258] LT patients demonstrated no difference in plasma CFH or other complement protein levels according to AMD status. This suggests systemic complement levels do not influence development of AMD in LT patients. Systemic complement levels in LT patients may be influenced by other factors, such as concomitant medical conditions and chronic immunosuppression.

Other findings

Patients were on an average of two immunosuppression agents, and the high prevalence of hypertension, renal impairment and hypercholesterolaemia may reflect the side effect profile of immunosuppression. The observation of low levels of hepatocellular dysfunction suggests LT was generally successful in stabilising hepatic function.

The most common indications of LT in this study population were the autoimmune cholestatic diseases (PBC and PSC). The most common reported indication for LT for all age groups is alcohol-related, followed by Hepatitis C infection, both of which are more common in the younger population.[200] These indications were uncommon in the recruited LT group, and may be a reflection of the older age group of patients recruited in the present study.

Lower plasma C4a levels were associated with increased AMD risk in LT patients. C4a is an activation product formed by cleavage of C4 by C1-antibody complex in the classical pathway. No previous studies could be found investigating how plasma C4a levels vary with AMD. Lower C4a levels suggest decreased classical pathway activation. This could be regarded as consistent with the literature that it is the alternative rather than the classical pathway which is of greater importance in AMD. As described previously, selectively blocking the alternative complement pathway in mice significantly reduced laser-induced CNV, whereas blocking the classical and lectin complement pathways had no effect.[162]

Bisphosphonate use in LT patients was associated with increased AMD risk. The reason for this finding is not clear, and may represent statistical noise. Bisphosphonates are used for the prevention of osteoporosis, but have also been

noted to have anti-angiogenic effects.[276] Honda et al reported that patients with nvAMD given oral bisphosphonates for 6 months showed some improvement in visual acuity and neovascularisation activity.[277] However there are no reports of an association between bisphosphonates and dry AMD.

4.3 Limitations of research

Study design

The cross-sectional design of this study means it is not possible to determine whether AMD was present before LT. However the mean age of patients at the time of LT surgery was 55 years, and AMD typically occurs after this age.[219] It could therefore be assumed that the majority of patients would not have AMD at the time of LT, although this is not possible to demonstrate. Furthermore, patient characteristics such as medications / immunosuppression agents, MABP, BMI and blood tests may vary at different time points, which may affect logistical regression analysis. The inclusion and exclusion criteria applied for this study (Western European descent, age 55 years and over, and LT duration ≥5 years ago) should be taken into account when extrapolating the results of this study to LT patients as a whole.

Difficulty in recruitment

The loss of around 70% of patients who initially expressed an interest was due to several factors. Some patients were initially invited even though they no longer attended the study site hospital. The need to coincide research clinics with the patient's normal outpatient appointment at the study site hospital caused long delays between initial invitation receipt and scheduling of the research clinic appointment, sometimes up to 6 months. This delay caused some patients to lose interest in the research project, and others unfortunately died by the time of the research clinic. Running multicentre research clinics single-handedly meant that it was not possible to recruit patients scheduled to attend their outpatient clinics on the same day simultaneously at multiple sites. An unexpected problem at Kings College Hospital, London was that despite its reputation as the biggest liver unit in Europe, only 145 patients were eligible for recruitment. Reasons for this included a large non-Western European population, high mortality (possibly due to the greater number of complicated cases carried out at Kings), and a large number of patients residing outside the UK. Despite these logistical issues, the number of patients recruited (n=223) was still greater than the desired calculated sample size (n=174) necessary to provide a power of at least 80%.

HRM analysis

HRM analysis could not discriminate alleles with sufficient accuracy, therefore was not used for *CFH* Y402H genotyping for this study. Reasons for this may include problems with insufficient PCR amplification, due to suboptimal primer design. Further modifications to the primers may improve PCR efficiency and therefore may enable successful HRM *CFH* Y402H genotyping.

Failure of donor *CFH* Y402H genotyping of DNA from FFPE liver biopsy samples

KBioscience genotyping failed for all DNA extracted from FFPE samples. One reason for this may be the lack of sufficient tissue within FFPE liver biopsy samples from which to extract DNA, compared to donor spleen / lymph node tissue. It was noted that DNA extracted from several FFPE samples showed very poor 260:280 ratios. Indeed the 260:280 ratio for some samples was zero, suggesting either no DNA was present, significant contaminants were present (including proteins), or DNA was of poor quality.

Many variables could have affected the quality of DNA obtained from FFPE samples, including the quality of fixative used, length of time of fixing, age of the block, and amount of degradation of DNA in paraffin.[278] The process of formalin fixation itself results in highly fragmented DNA, with fragments only a few hundred bases in length. Ferguson et al reported that highly fragmented DNA from FFPE is more likely to fail genotyping.[279]

Whole genome amplification

The DNA polymerase (phi 29 polymerase) found in the Qiagen REPLI-g WGA kit used in this project works best with DNA fragments longer than 2kB. In hindsight it may have been better to use the Qiagen REPLI-g FFPE kit to extract DNA from all FFPE samples. This kit extracts DNA from FFPE tissue, ligates short DNA fragments to produce longer strands of DNA, then allowing WGA to take place more efficiently. Further DNA extraction from FFPE samples using the Qiagen REPLI-g FFPE kit was not possible since all samples had been depleted.

Nevertheless a recent paper by Liang et al suggested that WGA of FFPE tissue using two commercial kits (including one from Qiagen) was associated with a high number of false positives and false negatives, and that variations of <10Mb

could not be reliably detected.[280] Therefore any results of post-WGA DNA genotyping would have to be interpreted with caution.

Complement assays

Samples exceeding the upper limit of the assay ideally should have been retested at a higher dilution, rather than using the upper limit value. This was not done due to cost-implication of requiring further ELISA kits. However since non-parametric statistical analysis was carried out using a rank system (Mann-Whitney U test), the absolute values of complement levels were not taken into account. Therefore using the upper limit value, which would still rank highest regardless of the theoretical value, may introduce less errors in statistical analysis.

Limiting analysis to the *CFH* Y402H variation

The *CFH* Y402H SNP was chosen as the focus of this study since at the time of the study onset, this SNP was the first and most reported sequence variation associated with AMD. A limitation of this study is the focus on a single SNP. Other SNPs associated with the CFH gene, which may be more strongly associated with AMD, were not taken into account during the logistic regression analysis. For example the rs10737680 SNP near the CFH gene was most strongly associated with AMD a in a recent meta-analysis.[140] Other genetic variations within/near the *CFH* gene may be more influential in AMD pathogenesis. Furthermore the *CFH* Y402H SNP may simply be a marker for another more disease-influencing genetic variation, although to date this has not been demonstrated.

The effect of sequence variations in other genes strongly associated with AMD, such as the *ARMS2* gene[13], were also not taken into account in the final logistic regression of this study. AMD is a multifactorial disease with multiple risk factors, and it would not be possible to incorporate every possible variable into an all-encompassing logistic regression analysis. Nevertheless, the original intention of this study was to genotype all recipient and donor DNA for other SNPs in/near other genes associated with AMD (C2, C3, CFB, ARMS2, LIPC, TIMP3) [164], so that a more complete logistic regression could be carried out. However donor DNA was only available for less than 25% of recruited patients, therefore more comprehensive donor genotyping was not possible.

4.4 Future work

Revisiting LT cohort

The LT patients recruited in this study could be revisited in 5, 10 and 15 years' time for repeat fundus photographs, to determine whether AMD status varies over this duration according to recipient / donor *CFH* Y402H genotype.

Prospective study of LT patients and AMD

A prospective study following LT patients from the time of pre-transplantation onwards would provide more definitive evidence as to whether modification of hepatic *CFH* genotype influences development/progression of AMD. A prospective study is presently on the way at Kings College. However recruitment is proving difficult since patients pre-LT have considerable morbidity and understandably are therefore reluctant to take part. There are verbal reports that Professor Gregory Hageman (Moran Eye Centre, Utah, USA) is following a LT cohort prospectively, although to date no publications have emerged.

Comparing intraocular and systemic complement activation

Intraocular fluid could be retrieved from human eyes from patients undergoing routine intraocular surgery / intravitreal injections, and compared to plasma from the same patient. This would enable an assessment on the interplay between intraocular and systemic complement activation.

***CFH* Y402H genotype in liver disease**

This study suggests that *CFH* genotype may influence the severity of hepatic impairment, irrespective of cause, and therefore more likely to require LT. Further investigation into the role of *CFH* genotype in specific liver diseases may therefore be of interest. This could be achieved by genotyping patients attending hepatology centres already diagnosed with particular forms of liver disease.

C4a and AMD

This study found low plasma C4a levels were associated with AMD. Plasma C4a levels could be measured in a pre-existing group of patients with / without AMD to investigate this association further.

Bisphosphonates and AMD

The association of bisphosphonates with AMD may benefit from further investigation by studying whether bisphosphonate use was related to AMD status in a pre-existing group of patients with / without AMD.

4.5 Conclusion

This study suggests replacing abnormal CFH with normal CFH protein in plasma, either directly by systemic protein administration or indirectly through systemic gene therapy, would not be effective in reducing development of AMD. Furthermore, systemic complement activity does not appear to play a part in AMD pathogenesis in LT patients. Together these findings indicate that local intraocular, rather than systemic, complement activity may therefore be more important in AMD pathogenesis. As a result, future complement inhibitory therapy in AMD should concentrate on intraocular delivery systems.

LT recipients have a high prevalence of age related macular degeneration, and consequently should be made aware of the increased risk of AMD through their clinical team. LT recipients should undergo regular annual optician screening, and also should be aware of the symptoms of nvAMD, such as sudden onset of increased distortion, so that urgent advice can be sought.

CHAPTER 5 Appendix

The appendix lists the published papers / abstracts / presentations / prizes arising from this research. Key study documents are also listed.

5.1 AMD and liver transplantation paper published by Ophthalmology
5.2 AMD and the complement system – published review article
5.3 AMD and oxidation – published review article
5.4 Abstract of presentation given at ARVO 2011 + travel award
5.5 Presentation at Royal College of Ophthalmology Conference, 2011
5.6 Ethical approval for project
5.7 Example of patient invitation letter
5.8 Example of patient information sheet
5.9 Example of informed consent form
5.10 Example of proforma

5.1 AMD and liver transplantation paper published by Ophthalmology

ARTICLE IN PRESS

Age-related Macular Degeneration and Modification of Systemic Complement Factor H Production Through Liver Transplantation

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Purpose: To investigate whether modification of liver complement factor H (CFH) production, by alteration of liver CFH Y402H genotype through liver transplantation (LT), influences the development of age-related macular degeneration (AMD).

Design: Multicenter, cross-sectional study.

Participants: We recruited 223 Western European patients ≥55 years old who had undergone LT ≥5 years previously.

Methods: We determined AMD status using a standard grading system. Recipient *CFH* Y402H genotype was obtained from DNA extracted from recipient blood samples. Donor *CFH* Y402H genotype was inferred from recipient plasma CFH Y402H protein allotype, measured using enzyme-linked immunosorbent assays. This approach was verified by genotyping donor tissue from a subgroup of patients. Systemic complement activity was ascertained by measuring levels of plasma complement proteins using an enzyme-linked immunosorbent assay, including substrates (C3, C4), activation products (C3a, C4a, and terminal complement complex), and regulators (total CFH, C1 inhibitor).

Main Outcome Measures: We evaluated AMD status and recipient and donor CFH Y402H genotype.

Results: In LT patients, AMD was associated with recipient *CFH* Y402H genotype ($P = 0.036$; odds ratio [OR], 1.6; 95% confidence interval [CI], 1.0–2.4) but not with donor *CFH* Y402H genotype ($P = 0.626$), after controlling for age, sex, smoking status, and body mass index. Recipient plasma CFH Y402H protein allotype predicted donor *CFH* Y402H genotype with 100% accuracy (n = 49). Plasma complement protein or activation product levels were similar in LT patients with and without AMD. Compared with previously reported prevalence figures (Rotterdam Study), LT patients demonstrated a high prevalence of both AMD (64.6% vs 37.1%; OR, 3.09; $P<0.001$) and the *CFH* Y402H sequence variation (41.9% vs 36.2%; OR, 1.27; $P = 0.014$).

Conclusions: Presence of AMD is not associated with modification of hepatic CFH production. In addition, AMD is not associated with systemic complement activity in LT patients. These findings suggest that local intraocular complement activity is of greater importance in AMD pathogenesis. The high AMD prevalence observed in LT patients may be associated with the increased frequency of the *CFH* Y402H sequence variation.

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Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world.[1] The early stage is usually asymptomatic and typified by the presence of drusen (yellowish deposits) at the macula. The late stage is associated with visual loss and either significant retinal atrophy (geographic atrophy) or growth of new blood vessels (choroidal neovascularization). Although intravitreal anti-vascular endothelial growth factor injections are effective in treating choroidal neovascularization, there is still no satisfactory way of treating geographic atrophy or of preventing the onset and progression of early AMD.

Age-related macular degeneration is a complex, multifactorial disease associated with a range of environmental and genetic risk factors.[2] The complement system in particular seems to play a key role in the pathogenesis of AMD. Sequence variation in several genes coding for complement proteins have been associated with AMD,[3] including the complement factor H (*CFH*) gene.[4-6]

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The association of AMD with the *CFH* gene is intriguing, because the CFH protein is a key regulator of the alternative complement pathway, acting at the level of C3 convertase.[7] Presence of the *CFH* Y402H polymorphism increases the risk of AMD significantly, with an odds ratio (OR) of 2.5 and 6.3 for the heterozygous CT (YH) and homozygous CC (HH) genotypes, respectively.[8] Substitution of tyrosine by histidine in the CFH protein at position 402 affects binding of the CFH protein to multiple ligands, including C-reactive protein, heparin, streptococcal M protein, and glycosaminoglycans.[9] This leads to altered binding to cell surfaces and impaired regulation of alternative pathway C3 convertase. Indeed, in the eye the CFH H402 protein variant has reduced binding in the retina compared with the Y402 variant.[10] Mullins et al[11] demonstrated histologic evidence of increased complement activation in the retina of eyes from *CFH* H402 homozygous human donors compared with *CFH* Y402 homozygotes, suggesting that dysfunctional intraretinal CFH protein increases activation of the alternative complement pathway, which in turn increases the risk of AMD.

The liver is the primary producer of circulating complement proteins, including CFH. The production of hepatic CFH is demonstrated by the abundant secretion of CFH from primary human hepatocyte cultures, and CFH expression in human liver cDNA libraries.[12,13] Liver transplant (LT) patients provide a unique opportunity to test whether development of AMD is modulated by altering hepatic *CFH* genotype and consequently by systemic CFH protein allotype. Such effects have already been demonstrated in atypical hemolytic uremic syndrome (aHUS). Atypical hemolytic uremic syndrome is associated with sequence variations in the *CFH* gene, and patients with severe renal failure from aHUS can benefit from combined renal and liver transplantation. The new liver produces normal CFH protein, thereby reducing the risk of recurrent aHUS.[14] Therefore, our hypothesis was that AMD in LT patients might be associated with donor, and not recipient, *CFH* Y402H genotype.

Methods

Ethics Statement

This study was approved by the National Research Ethics Service Committee South Central—Southampton B, UK (08/H0504/191). The study was conducted according to the tenets of the Declaration of Helsinki. Written, informed consent was obtained from all patients before study recruitment.

Study Design and Patient Recruitment

We used a cross-sectional design to investigate whether donor or recipient *CFH* Y402H genotype was associated with AMD in LT patients.

Patients were recruited between September 2009 and January 2011 from 4 LT centers in the UK (London Kings College Hospital, London; Queen Elizabeth Hospital, Birmingham; Addenbrooke's Hospital, Cambridge; and University Hospital Southampton). Inclusion criteria included patients of Western European origin, aged ≥55 years, with a history of LT ≥5 years ago. Patients were retrospectively identified from preexisting hospital LT databases.

We determined AMD status by dilated fundus examination and digital photography (Topcon TRC 50DX camera, Topcon Corporation, Tokyo, Japan/Nidek NM-200D camera, Nidek Co. Ltd, Gamagori, Japan). Digital photographs were graded according to the Rotterdam grading system by 2 retinal fellows (S.K., L.Z.H.) and any differences were arbitrated by a senior grader (A.J.L.). We defined AMD as the presence of Rotterdam grades 1 through 4 in the worst eye (Table 1).[15] General medical history, smoking history, body mass index, and blood pressure were recorded. Recipient peripheral blood was taken for DNA genotyping and plasma complement component measurement. Donor age and sex information were obtained anonymously from each site. Donor tissue (when available) was obtained for DNA genotyping from samples stored at the time of LT.

Patients discovered to have severe ocular disease were excluded because of the possibility of misdiagnosing AMD. Patients with a history of retransplantation were included only if the current transplant was of duration ≥5 years and the duration of the current transplant exceeded that of the original transplant.

Procedures

Blood for recipient DNA analysis was collected from subjects in 10-ml EDTA collection tubes and immediately stored at −80°C pending analysis. Blood for plasma complement analysis was collected in 10-ml EDTA collection tubes, centrifuged at 2600 revolutions per minute, divided into aliquots, and stored at −80°C pending analysis. Donor tissue (when available) was obtained from frozen archives of donor spleen/lymph node tissue and stored at −80°C pending analysis.

We extracted DNA from both recipient blood and donor tissue using a salting-out method.[16] Donor tissue was homogenized before DNA extraction using phosphate-buffered saline (0.01 mol/L). All DNA was stored at −20°C before analysis. We genotyped DNA for the *CFH* Y402H polymorphism by KBiosciences Ltd using KASPar technology (available at www.kbioscience.co.uk/reagents/KASP/KASP.html; accessed July 17, 2012) and confirmed through replication.

A range of plasma complement protein levels were measured, including complement activation products (C3a, C4a, C5a, and terminal complement complex [TCC]), complement substrates (C3, C4), and complement regulators (C1 inhibitor, total CFH). The C3, C4, and C1 inhibitors were measured by clinically validated and externally quality controlled (United Kingdom National External Quality Assessment Service) rate nephelometric assays (Clinical

Table 1. Distribution of AMD Grading in United Kingdom Liver Transplant Recipients

Grade	Description	Number (n = 223)	%
0	No AMD or drusen <63 μm	79	35.4
1	Drusen ≥63 μm or PI	39	17.5
2	Drusen ≥125 μm or drusen ≥63μm with PI	66	29.6
3	Drusen ≥125 μm with PI	24	10.8
4	CNV/GA	15	6.7
1–4	Total with AMD	144	64.6

AMD = age-related macular degeneration (early AMD = grades 1–3; late AMD = grade 4); CNV = choroidal neovascularization; GA = geographic atrophy; PI = pigmentary irregularities.
Results from AMD grading of all patients using the Rotterdam system[15] are shown.

Biochemistry and Immunology, University Hospital Wales, Cardiff; Dade Behring, Milton Keynes). The C3a, C4a, C5a, and TCC were measured with enzyme-linked immunosorbent assay kits (C3a, C5a; TCC: Hycult Biotech Inc., Uden, The Netherlands; C4a: Becton, Dickinson and Co., Franklin Lakes, NJ). Total CFH and CFH Y402H allotypes were measured using an in-house enzyme-linked immunosorbent assay.[17] When observed levels exceeded the kit's maximum detectable level, the upper limit of the kit range was used.

Statistics

The prevalence of AMD and the frequency of the *CFH* Y402H risk allele were compared with reported general population studies using the chi-square goodness-of-fit test. Binary logistic regression analysis was carried out using AMD status (present/absent) as the dependent variable and other covariates added in stepwise blocks using the forced entry method. Genotype was coded using an additive model (homozygotes = 2; heterozygotes = 1; wild type = 0). Covariates included in the initial block included recipient and donor *CFH* Y402H genotype and potential confounders known to be significant risk factors for AMD, including age, body mass index, and smoking status (pack-years).[2] Other covariates were then added individually to this model. Plasma complement levels in LT patients with or without AMD were compared using the Mann-Whitney *U* test. Plasma complement levels in LT patients as a whole were compared with 30 healthy controls without AMD or a history of AMD (obtained from a preexisting Southampton AMD cohort), also using the Mann-Whitney *U* test. Data analysis was carried out using SPSS version 19 (IBM, Rochester, NY). Statistical significance was defined as $P \leq 0.05$, with Bonferroni correction for multiple testing.

Results

Patient Demographics, AMD Status, and *CFH* Y402H Genotype

We recruited 223 patients. The mean recipient age was 66.9±6.1 years and mean duration after LT was 11.8±4.4 years. There was a marginal preponderance of males (55.2%; n = 123). The mean donor age was 41.4±14.9 years, and 58.3% (n = 130) of the donors were male.

The prevalence of AMD in the LT cohort (Table 1) was higher compared with a general population of similar ethnicity and age (64.6% vs 37.1% in the Rotterdam Study baseline population; $P<0.001$; OR, 3.09; 95% confidence interval, 2.34−4.09).[15] Recipient *CFH* Y402H risk allele frequency was also higher than expected in the general population of similar ethnicity (41.9% vs 36.2% in the Rotterdam Study baseline population; $P = 0.014$; OR, 1.27; 95% confidence interval, 1.05−1.54).[18] Recipient *CFH* Y402H genotype conformed to Hardy-Weinberg equilibrium. Characteristics for variables for the whole group and with/without AMD are shown in Table 2 (available at http://aaojournal.org).

Donor *CFH* Y402H Genotype

Because donor tissue was not available for all patients, donor *CFH* Y402H genotype was inferred from recipient plasma CFH Y402 allotype, measured using novel allotype-specific monoclonal antibodies.[17] This approach was verified by comparing to confirmed *CFH* Y402H genotype obtained from donor spleen or lymph node DNA in 49 patients. This demonstrated a 100% correlation between donor tissue *CFH* Y402H genotype and that predicted by recipient plasma CFH Y402H protein allotype (Fig 1).

Recipient and Not Donor *CFH* Y402 Genotype Was Associated with AMD

Binary logistic regression analysis was performed, investigating the effects of recipient and donor *CFH* Y402H genotype and using covariates known to be significant risk factors for AMD (age, sex, smoking status, body mass index; Table 3). Recipient *CFH* Y402H ($P = 0.036$; OR, 1.559) and age ($P = 0.030$; OR, 1.055) were associated with presence of AMD. Of note, donor *CFH* Y402H genotype (based on recipient plasma *CFH* Y402H allotype) did not predict AMD.

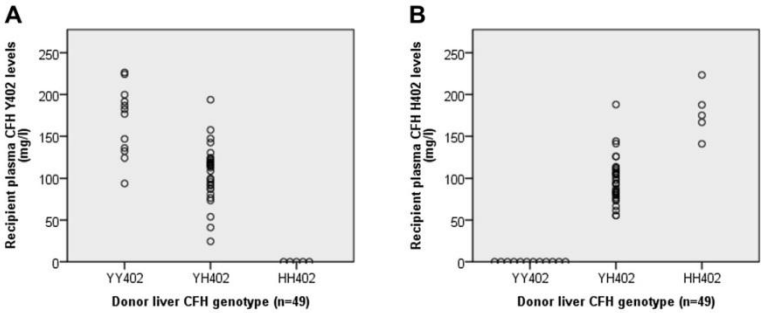

Figure 1. Liver transplant recipient plasma CFH Y402H protein allotype levels and donor *CFH* Y402H genotype. **A**, Recipient plasma CFH Y402 protein levels and confirmed donor *CFH* Y402H genotype. **B**, Recipient plasma CFH H402 protein levels and confirmed donor *CFH* Y402H genotype. Forty-nine patients had confirmed donor tissue *CFH* Y402H genotype. A complete absence of plasma CFH Y402 (normal) protein was found in patients receiving a liver with confirmed *CFH* HH402 genotype (high-risk homozygotes). A complete absence of plasma CFH H402 (abnormal) protein was found in patients receiving a liver with confirmed *CFH* YY402 genotype (low-risk normal). Both CFH Y402 and H402 proteins were found in the plasma of patients receiving a liver with confirmed *CFH* YH402 genotype (heterozygotes). Liver genotype changed from CFH YY402 to HH402, or vice versa in 12 patients—only a single CFH Y402H protein allotype corresponding to the new liver *CFH* Y402H genotype was detected in the plasma of these patients. CFH = complement factor H; HH402 = abnormal (high-risk) *CFH* H402 homozygous genotype; YY402 = normal (low-risk) *CFH* YY402 homozygous genotype; YH402 = *CFH* Y402H heterozygous genotype; Y402 = low-risk CFH protein; H402 = high-risk CFH protein.

Table 3. Logistic Regression Analysis (n = 223)

Variable	Coding	P	OR	95% CI
Age (yrs)	Continuous	0.030	1.055	1.005–1.107
Sex	1 = female, 0 = male	0.319	1.355	0.746–2.461
Smoking status (pack-years)	Continuous	0.598	0.995	0.977–1.014
Body mass index (kg/m^2)	Continuous	0.937	0.997	0.935–1.064
Recipient *CFH* Y402H genotype	2 = C:C, 1 = C:T, 0 = T:T	0.036	1.559	1.030–2.361
Donor *CFH* Y402H genotype	2 = C:C, 1 = C:T, 0 = T:T	0.626	0.905	0.605–1.353

Results from binary logistic regression analysis are shown. Age-related macular degeneration status (present/absent) was selected as the dependent variable and other covariates included together using the forced entry method.
CFH = complement factor H; CI = confidence interval; OR = odds ratio.

Further covariates added individually to this model included duration of current LT, primary cause of LT, concomitant medical conditions, immunosuppressive therapy, other medication, blood pressure, hepatic function, renal function, donor age, and donor sex. The only additional variable associated with AMD was current use of bisphosphonates (P = 0.012; OR, 2.602; 95% confidence interval, 1.234–5.483; Table 4, available at http://aaojournal.org).

Plasma Complement Levels

Plasma complement protein levels (including the substrate proteins C3 and C4, activation products C3a, C4a, C5a, TCC, and regulators C1 inhibitor and CFH) were similar in LT patients with and without AMD (Fig 2). We also compared LT patients with 30 randomly selected healthy controls ≥55 years old without AMD or a history of LT. Plasma total CFH was higher in LT patients compared with controls (P = 0.001); otherwise, levels of all other complement proteins were similar (Fig 3).

Discussion

In LT patients, AMD was associated with recipient rather than donor *CFH* Y402H genotype. Therefore, receiving a liver that delivers either risk or protective CFH to plasma, for ≥5 years, had no effect that we could discern on the development of

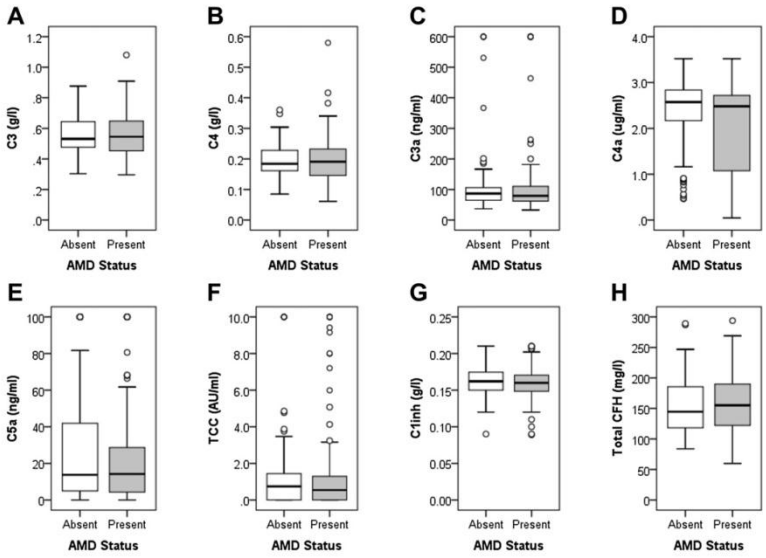

Figure 2. Plasma complement levels in liver transplant (LT) patients without and with age-related macular degeneration (AMD). Box plots showing plasma levels of various complement proteins in LT patients without and with AMD. **A**, **B**, Complement substrate proteins. C3 is involved in the alternate and terminal complement pathway, and C4 is involved in the classical complement pathway. **C**–**F**, Complement activation products. C3a indicates activation of the alternate and terminal complement pathway, C4a indicates activation of the classical complement pathway, and C5a and terminal complement complex (TCC) indicate activation of the terminal complement pathway. **G**, **H**, Complement regulators. C1 inhibitor regulates the classical complement pathway, and complement factor H (CFH) regulates the alternative complement pathway (total plasma CFH measured, i.e., both CFH Y402 and H402 proteins). Using the Mann-Whitney U test with Bonferroni correction for multiple testing, there was no difference between levels of each these plasma complement proteins in LT patients with and without AMD. AU = arbitrary units. N = 223 for all analyses (except for C4a, where n = 222).

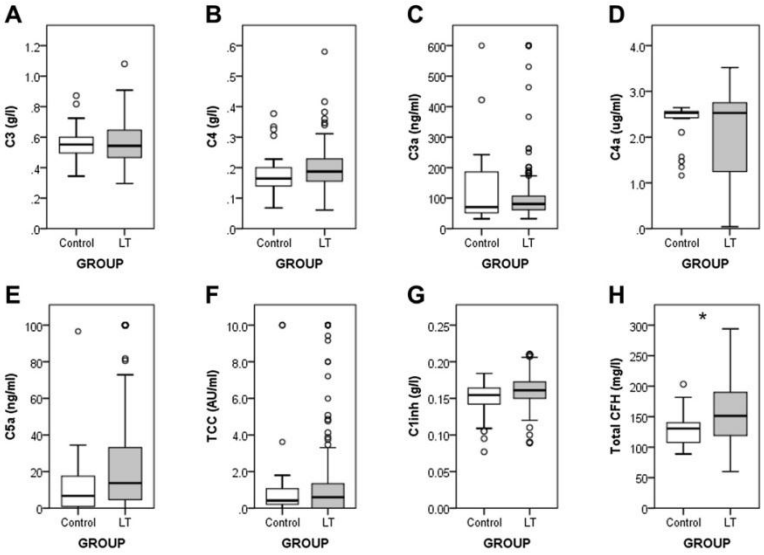

Figure 3. Plasma complement levels in liver transplant (LT) patients compared with healthy controls. Box plots showing plasma levels of various complement proteins in LT patients (n = 223; except for C4a, where n = 222) compared with healthy controls (n = 30). **A**, **B**, Complement substrate proteins. C3 is involved in the alternate and terminal complement pathway, and C4 is involved in the classical complement pathway. **C**–**F**, Complement activation products. C3a indicates activation of the alternate and terminal complement pathway, C4a indicates activation of the classical complement pathway, and C5a and terminal complement complex (TCC) indicate activation of the terminal complement pathway. **G**, **H**, Complement regulators. C1 inhibitor regulates the classical complement pathway, and complement factor H (CFH) regulates the alternative complement pathway (total plasma CFH measured, i.e., both CFH Y402 and H402 proteins). Using the Mann-Whitney *U* test with Bonferroni correction for multiple testing, only plasma total CFH was elevated (*) in LT patients compared with controls ($P = 0.001$ uncorrected). AU = arbitrary units.

AMD. This implies that replacing abnormal CFH with normal CFH protein in plasma either directly or through systemic gene therapy is unlikely to succeed as a treatment for AMD. Interestingly, Bomback et al[19] recently reported that intravenous administration of eculizumab (a monoclonal antibody that binds complement C5) over 12 months had no effect on retinal drusen in 2 patients with C3 glomerulopathy. Their results are consistent with our observations. No study to date has demonstrated whether systemic manipulation of genotype can influence the development of AMD.

Our results suggest that local intraocular complement activity in the eye may be more important in AMD pathogenesis. Lyzogubov et al[20] demonstrated the importance of local production of CFH in the pathogenesis of AMD; selectively silencing retinal CFH expression (with subretinal injections of short-interfering RNA against CFH) worsened choroidal neovascularization in an experimentally induced mouse model without any change in systemic hepatic CFH expression. Indeed, local CFH protein production within the retina is known to occur at relatively high levels. Hageman et el[21] quantified CFH transcripts in the retina using real-time polymerase chain reaction and demonstrated that expression of CFH in the retina approached levels observed in the liver.

The similarity of systemic complement protein and activation product levels in LT patients with and without AMD supports our argument that local rather than systemic complement activity is more important in AMD pathogenesis. Levels of systemic complement proteins, especially complement activation products, have previously been found to be elevated in AMD.[22,23] Systemic complement levels in LT patients may be influenced by other factors, such as concomitant medical conditions and chronic immunosuppression.

The increased prevalence of AMD observed in LT patients may be explained by the observed increased frequency of recipient *CFH* Y402H risk allele. The association with both the *CFH* Y402H risk allele and raised total plasma CFH with LT suggests that CFH has a role to play in influencing the severity of liver disease, necessitating LT. There are no reported associations between the *CFH* Y402H gene sequence change and liver disease. The elevation of total CFH plasma levels in LT patients may reflect an effective regulatory response to increased complement activation, which has been reported previously in liver disease.[24–26]

There was no association between type or amount of immunosuppressive therapy and AMD. Immunosuppressive therapy is routinely given to all LT patients; therefore, this does not directly effect our findings. Furthermore, chronic systemic immunosuppression has previously been associated with a reduced prevalence of AMD, as observed in rheumatoid arthritis patients.[27]

Bisphosphonate use in LT patients was associated with AMD. The reason for this association is unclear. Bisphosphonate use has been reported to have a protective effect in wet AMD,[28] so the association in our study cohort requires further investigation.

The 100% concordance between recipient CFH Y402H protein allotype and donor *CFH* Y402H genotype supports previous studies implicating the liver as the primary producer of circulating CFH protein. Schmeling et al[29] showed that plasma CFH allotype converted to that of the donor in 6 of 6 LT recipients. Our study verifies this in a larger group and is novel in using allotype-specific CFH assays and comparison with donor genotype analysis. The absence of any recipient CFH protein allotype in patients receiving homozygous Y402 or H402 livers in our study also demonstrates for the first time the absence of extrahepatic contribution to circulating CFH protein levels.

Ethnicity was restricted to Western Europe because both minor allele frequency and association of the *CFH* Y402H polymorphism with AMD varies with ethnic background.[30] Patients ≥55 years were selected because AMD prevalence typically increases after this age.[31] The selection of patients a minimum of 5 years after LT was to allow adequate time for any modulation of hepatic *CFH* Y402H genotype to influence AMD development. This duration was based on the minimum follow-up period utilized in the Age-Related Eye Disease Study, which successfully demonstrated a change in outcome (AMD progression) based on a particular intervention (oral supplementation).[32] These exclusions should be considered when extrapolating the results of this study to LT patients as a whole. The cross-sectional design of this study means that it is not possible to determine whether AMD was present before LT. However, the mean age of patients at the time of LT was 55 years; as mentioned, AMD typically occurs after this age.

In conclusion, this study shows that manipulation of systemic CFH is unlikely to alter development of AMD. Local intraocular rather than systemic CFH seems more important in AMD pathogenesis. Liver transplant patients have a high prevalence of AMD and should undergo regular ophthalmic monitoring. It is possible that CFH is associated with increased severity of liver disease, and this should be investigated further.

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References

1. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. Bull World Health Organ 2004;82:844–51.
2. Seddon JM, Reynolds R, Yu Y, et al. Risk models for progression to advanced age-related macular degeneration using demographic, environmental, genetic, and ocular factors. Ophthalmology 2011;118:2203–11.
3. Khandhadia S, Cipriani V, Yates JR, Lotery AJ. Age-related macular degeneration and the complement system. Immunobiology 2012;217:127–46.
4. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. Science 2005;308:385–9.
5. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 2005;308:419–21.
6. Edwards AO, Ritter R III, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. Science 2005;308:421–4.
7. Muller-Eberhard HJ, Schreiber RD. Molecular biology and chemistry of the alternative pathway of complement. Adv Immunol 1980;29:1–53.
8. Thakkinstian A, Han P, McEvoy M, et al. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. Hum Mol Genet 2006;15:2784–90.
9. Ripoche J, Day AJ, Harris TJ, Sim RB. The complete amino acid sequence of human complement factor H. Biochem J 1988;249:593–602.
10. Skerka C, Lauer N, Weinberger AA, et al. Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol Immunol 2007;44:3398–406.
11. Mullins RF, Dewald AD, Streb LM, et al. Elevated membrane attack complex in human choroid with high risk complement factor H genotypes. Exp Eye Res 2011;93:565–7.
12. Halme J, Sachse M, Vogel H, et al. Primary human hepatocytes are protected against complement by multiple regulators. Mol Immunol 2009;46:2284–9.
13. Schulz TF, Schwable W, Stanley KK, Dierich MP. Use of a high-efficiency expression vector to isolate cDNA clones for factor H and map their positions within the molecule. Mol Immunol 1986;23:1243–8.
14. Remuzzi G, Ruggenenti P, Codazzi D, et al. Combined kidney and liver transplantation for familial haemolytic uraemic syndrome. Lancet 2002;359:1671–2.
15. Klaver CC, Assink JJ, van Leeuwen R, et al. Incidence and progression rates of age-related maculopathy: the Rotterdam Study. Invest Ophthalmol Vis Sci 2001;42:2237–41.
16. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.
17. Hakobyan S, Tortajada A, Harris CL, et al. Variant-specific quantification of factor H in plasma identifies null alleles associated with atypical hemolytic uremic syndrome. Kidney Int 2010;78:782–8.
18. Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. JAMA 2006;296:301–9.
19. Bomback AS, Smith RJ, Barile GR, et al. Eculizumab for dense deposit disease and C3 glomerulonephritis. Clin J Am Soc Nephrol 2012;7:748–56.
20. Lyzogubov VV, Tytarenko RG, Jha P, et al. Role of ocular complement factor H in a murine model of choroidal neovascularization. Am J Pathol 2010;177:1870–80.
21. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (*HF1/CFH*) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci U S A 2005;102:7227–32.
22. Scholl HP, Charbel Issa P, Walier M, et al. Systemic complement activation in age-related macular degeneration. PLoS One [serial

online] 2008;3:e2593. Available at: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0002593. Accessed November 8, 2012.
23. Gibson J, Hakobyan S, Cree AJ, et al. Variation in complement component C1 inhibitor in age-related macular degeneration. Immunobiology 2012;217:251–5.
24. Barak V, Selmi C, Schlesinger M, et al. Serum inflammatory cytokines, complement components, and soluble interleukin 2 receptor in primary biliary cirrhosis. J Autoimmun 2009;33:178–82.
25. Kanmura S, Uto H, Sato Y, et al. The complement component C3a fragment is a potential biomarker for hepatitis C virus-related hepatocellular carcinoma. J Gastroenterol 2010;45:459–67.
26. Bykov I, Junnikkala S, Pekna M, et al. Effect of chronic ethanol consumption on the expression of complement components and acute-phase proteins in liver. Clin Immunol 2007;124:213–20.
27. McGeer PL, Sibley J. Sparing of age-related macular degeneration in rheumatoid arthritis. Neurobiol Aging 2005;26:1199–203.
28. Honda S, Nagai T, Kondo N, et al. Therapeutic effect of oral bisphosphonates on choroidal neovascularization in the human eye. J Ophthalmol [serial online] 2010;2010:206837. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2913841/. Accessed July 17, 2012.
29. Schmeling A, Correns A, Staffa G, Geserick G. Changes in the phenotype of polymorphic plasma proteins after liver transplantation: new data and medico-legal consequences. Int J Legal Med 1999;112:364–7.
30. Nonyane BA, Nitsch D, Whittaker JC, et al. An ecological correlation study of late age-related macular degeneration and the complement factor H Y402H polymorphism. Invest Ophthalmol Vis Sci 2010;51:2393–402.
31. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology 1992;99:933–43.
32. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. Arch Ophthalmol 2001;119:1417–36.

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5.2 AMD and the complement system – published review article

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Review

Age-related macular degeneration and the complement system

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ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. It is a complex multifactorial disease, and despite new advances in treatment, many patients still succumb to visual impairment. The complement pathway has been implicated in the pathogenesis of many diseases, and recently variants in several genes encoding complement pathway proteins have been associated with AMD. Complement proteins have been found in histological specimens of eyes with AMD. Altered levels of both intrinsic complement proteins and activated products have been found in the circulation of patients with AMD. Complement activation may be triggered by oxidative stress, resulting from retinal exposure to incoming light; indeed an inter-play between these two pathological processes seems to exist. Finally, complement inhibitors are currently being evaluated in clinical trials. This article reviews the role of the complement system in AMD, and the potential of complement inhibition in preventing the devastating blindness resulting from this disease.

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Abbreviations: AMD, age-related macular degeneration; AREDS, Age-Related Eye Disease Study; BM, Bruch's membrane; CEP, carboxyethylpyrrole; C1, C2, etc., complement C1, complement C2, etc.; CFB, complement factor B; CFD, complement factor D; CFH, complement factor H; CFHR, CFH-related; CFI, complement factor I; CNV, choroidal neovascularisation; CNVM, choroidalneovascular membrane; CP, Chlamydia Pneumoniae; CR1, CR2, complement receptor 1, complement receptor 2; CRP, C-reactive protein; DAF, decay accelerating factor, or CD55; ELISA, enzyme-linked immunosorbent assay; FFA, fundus fluorescein angiogram; GA, geographic atrophy; LD, linkage disequilibrium; MCP, membrane cofactor protein, or CD46; N/A, not available; NV AMD, neovascular (wet) AMD; OCT, optical coherence tomogram; OR, odds ratio; PDT, photodynamic therapy; RPE, retinal pigment epithelium; Rs, reference SNP number (as allocated by the SNP database of the National Centre of Biotechnology Information-NCBI); SCR, short consensual repeat; SNP, single nucleotide polymorphism; TCC, terminal complement complex; VEGF, vascular endothelial growth factor.

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Introduction

Age-related macular degeneration (AMD) is a common disease. It is the leading cause of blindness in Europe, USA and Australia, accounting for up to 50% of all cases (Resnikoff et al. 2004). The prevalence in adults is around 3% (Klein et al. 2010). AMD is a complex multifactorial disease which is difficult to manage. Avoiding smoking is the only proven preventative measure and there has been no effective treatment until the recent advent of intravitreal injections of anti-vascular endothelial growth factor. Even this only benefits a subset of patients – the majority of patients with AMD are still not curable. A multitude of pathological processes are likely to predispose an individual to AMD. Of these, complement activation appears to have a major role. In this article we summarise the current evidence for involvement of the complement system, and the potential of complement inhibition in AMD.

Age-related macular degeneration

AMD is a disease of the elderly, affecting almost two thirds of the population over 80 years old (Friedman et al. 2004; de Jong 2006). The prevalence of visual impairment in those over 65 due to AMD is as high as 3% (Seland et al. 2009). AMD is a degenerative disease of a critical area of the retina known as the macula. This area receives the greatest focus of incoming light, and correspondingly crucial for central vision, fine detail and image resolution. The retina consists of 2 layers, the inner neurosensory retina, and the outer retinal pigment epithelium (RPE) cell layer. The RPE layer is separated from the outer vascular choroid by Bruch's membrane (BM), a modified basement membrane. The choroid consists of an internal network of fenestrated capillaries (choriocapillaris layer) adjacent to BM, and an external large vessel layer. Its role is to supply oxygen and nutrients to and remove waste from the retina, especially at the macula.

Early AMD is often asymptomatic but detectable on examination by the presence of drusen and pigmentary irregularities in the retina. Drusen are clinically apparent as yellowish deposits under the neurosensory or RPE layer. Larger ("soft") drusen are associated with a worse prognosis for AMD (Klein et al. 1992). Pigmentary irregularities are caused by RPE cell hypertrophy, hyperplasia or atrophy. Late (or advanced) AMD is associated with poor central vision, either due to significant RPE/neuroretinal atrophy or new vessel proliferation. Atrophy of the RPE and overlying photoreceptors is described as geographic atrophy (GA). New vessel proliferation, either from the choroid (choroidal neovascularisation, CNV) within the retina (retinal angiomatous proliferation, RAP) is referred to as or neovascular AMD (nvAMD). A key driver of new vessel proliferation is the generation of vascular endothelial growth factor (VEGF) from the RPE cells (Lopez et al. 1996). AMD can also be categorised as wet AMD (nvAMD), or dry AMD (all other subtypes). Presently the dry form is untreatable, although oral antioxidants may have some benefit in limiting progression. CNV is currently treated with anti-VEGF intravitreal injections, given via the sclera directly into the vitreous cavity (Rosenfeld et al. 2006; Brown et al. 2006a). Fig. 1 illustrates the cross-sectional pathological changes occurring within the macula in AMD. Fig. 2 depicts the typical clinical findings seen in AMD. Two investigations used to diagnose CNV (fundus fluorescein angiogram-FFA, and optical coherence tomography-OCT) are shown in Fig. 3.

It is unknown whether GA and nvAMD are differing phenotypes resulting from similar underlying pathological processes, or two distinct conditions. Both GA and nvAMD share a common early phase which is indistinguishable clinically and pathologically. Furthermore, the genetic and environmental factors predisposing to each phenotype appear to be very similar. At some point a cellular event must occur to trigger specific mechanisms, which presumably encourages development of a specific AMD phenotype (i.e. either GA or nvAMD). It is likely there is some genetic influence on this because there is some concordance within familial clusters (Klein et al. 1994), and in patients there is some concordance between eyes (Mann et al. 2011). The definitive triggers are as yet undefined. One possibility may be a difference in VEGF gene expression. Although increased RPE production of VEGF is associated with nvAMD (Lopez et al. 1996), there is some evidence that decreased VEGF expression may precipitate RPE and choroiocapillaris atrophy (Saint-Geniez et al. 2009). GA has been associated with toxic accumulation of AluRNA in RPE cells due to reduced levels of DICER1, a micro-RNA processing

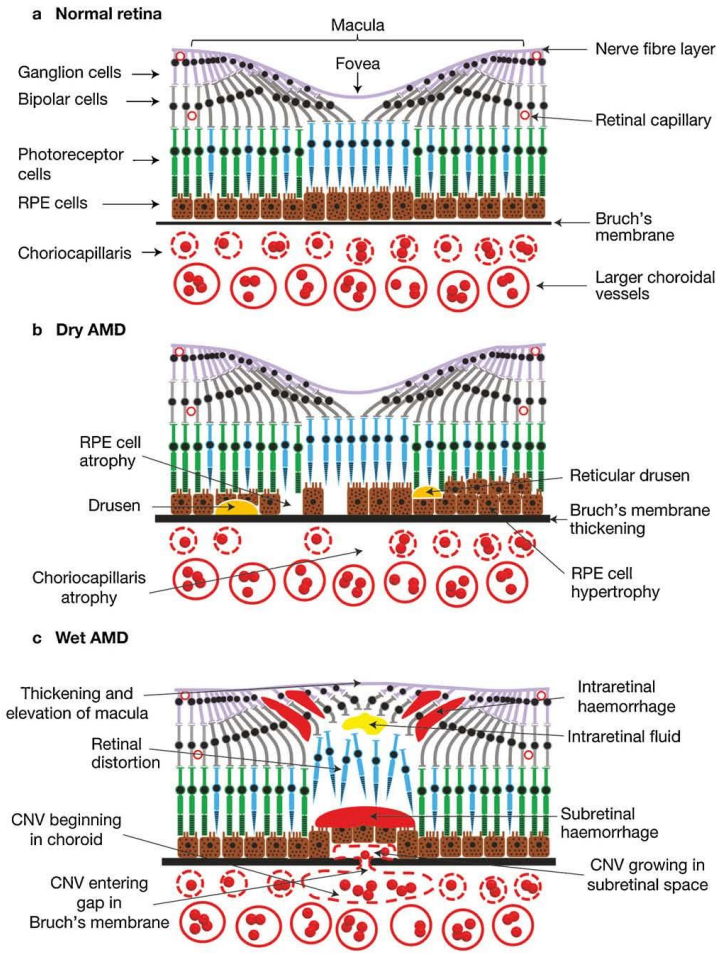

Fig. 1. Cross-sectional pathological changes occurring in macula in age-related macular degeneration (AMD). (a) Normal macula. The outer retinal layer consists of retinal pigment epithelial (RPE) cells. The inner neurosensory layer consists of photoreceptor cells (cone cells for colour vision and fine detail, and rod cells for night vision), a middle bipolar cell layer, and an innermost ganglion cell layer. Light is converted by photoreceptor cells into nerve signals ("photo-transduction"), and is transmitted to the ganglion cells (via the bipolar cells). The long axons of ganglion cells, constituting the nerve fibre layer, conducts nerve signals to the optic nerve and then through the visual pathway to the visual cortex in the brain, where the perception of vision is created. The RPE layer sits on Bruch's membrane (BM). BM iscomposed of collagen and elastin. It is a relative diffusion barrier between the RPE and external choroid layers. The inner choroidal layer, the choriocapillaris, is composed of fenestrated capillaries, and the outer choroid is composed of larger vessels (not shown). The retina derives its blood supply from the choroid, as well as an intra-retinal capillary network. The bipolar and ganglion cells at the macula are displaced laterally. Together with an absence of retinal blood vessels, this ensures minimal interference with the incoming image. The fovea, forming the macular centre, is concave in cross-section and consists only of cone photoreceptors. Furthermore, the RPE cells are larger. (b) Dry AMD. In dry AMD, drusen accumulate between RPE cells and BM. Reticular drusen accumulate between the RPE layer and photoreceptor cells. Pigmentary irregularities, including atrophy and hypertrophy/hyperplasia of RPE cells is seen, along with choriocapillaris atrophy and thickening of BM. Extensive atrophy leads to geographic atrophy (not shown), a form of late/advanced AMD. (c) Wet AMD. Wet AMD, a form of late/advanced AMD, is characterised by choroidal neovascularisation (CNV). When initially forming within the choroid, new vessels break through a gap in BM to grow under/within the retina. The CNV leaks fluid and blood, disrupting the organised architecture of the retinal cells. This results in distortion of central vision, often the first symptom of CNV experienced by the patient. Wet AMD can also occur from intra-retinal neovascularisation (retinal angiomatous proliferation, RAP). Consequent thickening and elevation of the retina can be detected clinically. Eventually the CNV scars, with permanent disruption of the retinal architecture.

enzyme (Kaneko et al. 2011); as yet this has not been reported in nvAMD. It is as yet unclear how important and to what extent such interactions are in the pathogenesis of either nvAMD or GA.

Animal models of AMD, in particular mice, have been developed to help understand the pathogenesis of AMD, as well as providing suitable pre-clinical models for pharmaceutical intervention. The main disadvantages of using mice is that firstly they have

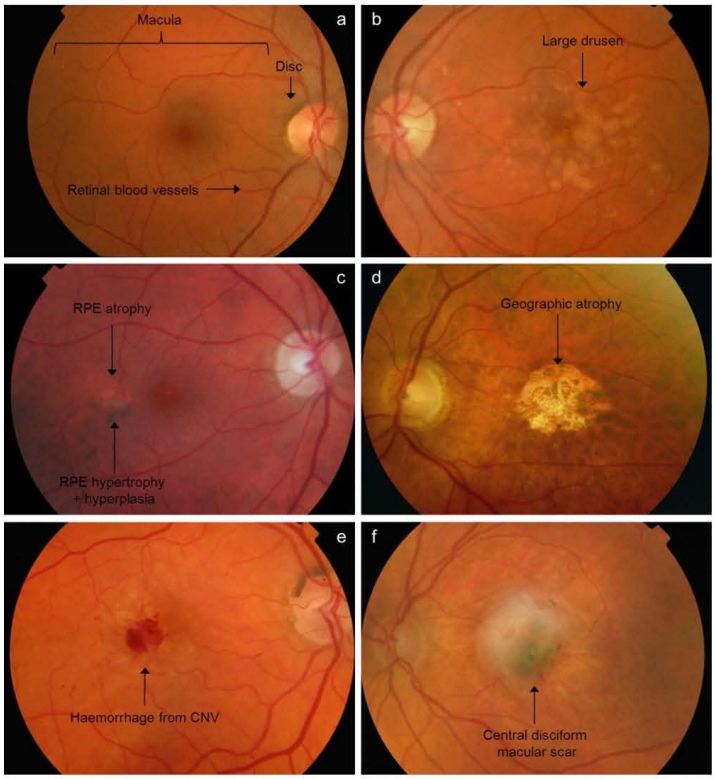

Fig. 2. Fundus photographs depicting various stages of age-related macular degeneration (AMD). (a) Normal eye. This is a photograph of the fundus (back) of the right eye. The optic disc, the beginning of the optic nerve, can be seen – this serves as an anatomical landmark during clinical examination. Radiating out from the optic disc in 4 quadrants are the retinal blood vessels. The macula is the area just temporal to the optic disc. It usually appears darker due to increased pigment, and its centre is marked by an absence of visible blood vessels. (b) Early AMD-drusen. Large drusen can be seen, which appear as multiple yellowish clumps. (c) Early AMD-pigmentary irregularities. An area of hyperpigmentation (due to RPE cell hypertrophy and hyperplasia) and hypopigmentation (RPE atrophy) can be seen just temporal to the centre of the macula. (d) Late AMD-GA. This patient has an extensive area of sharply demarcated RPE atrophy. The underlying choroidal vessels have mostly atrophied as well. Central vision will be affected. (e) Late AMD-CNV. Blood vessels have grown from the choroid (the vascular layer posterior to the retina), to form a choroidal neovascular membrane (CNVM). This has caused a haemorrhage within the retina, causing sudden loss of central vision. This can be treated with intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents. (f) Late AMD-scar. Untreated CNV will eventually result in a permanent macular scar. The central vision will be permanently poor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

no macula, and secondly discrepancies exist in both the innate and adaptive immune system between mice and men (Mestas and Hughes 2004). Despite this, there are broad similarities in underlying physiological and pathological processes compared to humans. For example, certain genes causing retinal degeneration in humans have their counterparts in mice. A further benefit is the relatively short duration required to study pathophysiology in mice, although ideally ageing mouse models should be used to mimic AMD as closely as possible.

Mouse models described in this review include exposure to constant light (causing photo-toxicity resulting in photoreceptor and RPE cell damage) (Lai et al. 1978), application of laser (to induce CNV by disrupting Bruch's membrane) (Tobe et al. 1998), and genetic knock-out models. The latter include the *rd1* mouse, which develops early onset of rapid retinal and photoreceptor degeneration due to a mutation in the β-subunit of the phosphodiesterase gene (β-PDE) gene (Farber 1995), or the *Ccl-2*- or *Ccr-2*-deficient mice, which leads to many cardinal features of AMD due to deficiency in monocyte chemoattractant protein or chemokine receptor 2 protein (Ambati et al. 2003). It should be pointed out that no single mouse model exactly mimics human AMD. For a more extensive description of mouse models please refer to the review by Rakoczy et al. (2006).

The complement pathways

The complement system consists of over 40 proteins and regulators found in the systemic circulation. It plays a key role in host defence against pathogens, the elimination of immune complexes and apoptotic cells, and adaptive immune responses (Walport 2001). Three pathways exist each with a specific trigger – the classical pathway triggered by an antibody–antigen complex, the alternative pathway triggered by binding to a host cell or pathogen surface, and the lectin pathway, triggered by polysaccharides on microbial surfaces.

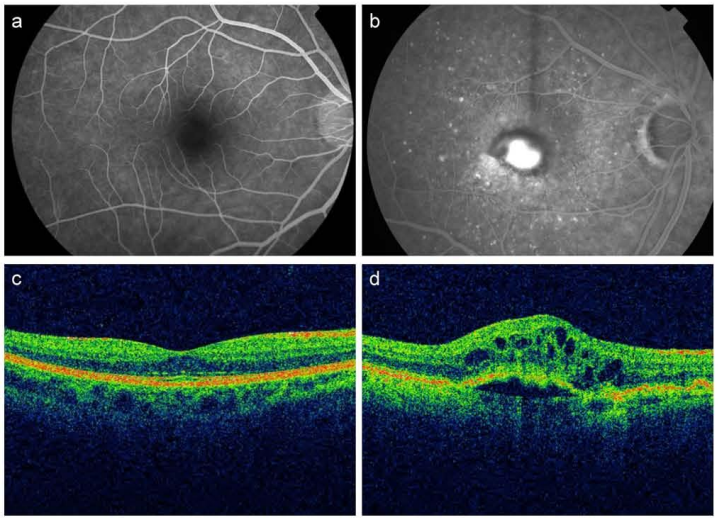

Fig. 3. Investigations used for diagnosing choroidal neovascularisation (CNV) A fundus fluorescein angiogram (FFA) is performed to confirm the presence of CNV. Fluorescein (a fluorescent dye) is given intravenously, usually via the antecubital fossa. Photos are taken as the dye passes through the retinal vessels, using a fundus camera equipped with barrier filters. These exclude reflected light, whilst enabling a clear image of emitted fluorescent light to be captured. Normally the dye does not leak as it passes through the retinal vessels (a). However, if CNV is present, leakage of fluorescein from the permeable walls of the CNV occurs (b). FFA is usually augmented by optical coherence tomography (OCT). This non-invasive test uses low intensity laser to produce high resolution cross-sectional images of the macula (c). Presence of CNV causes retinal thickening due to build-up of fluid, which can also collect in the sub-retinal space (d).

Dysregulation of the complement pathway can lead to autologous damage, and is implicated in the pathogenesis of a wide spectrum of diseases. Growing evidence suggests the complement pathways play a key role in the development of AMD. Drusen, the earliest hallmark of AMD may act as a focus of chronic inflammation (Anderson et al. 2002). Several studies have found evidence of deposition of complement proteins in drusen, including complement components C3a and C5a (Nozaki et al. 2006), C5 and C5b-9 terminal complement complex (TCC) (Mullins et al. 2000; Johnson et al. 2000), as well as fluid-phase complement regulators (complement factor H-CFH, vitronectin and clusterin), and membrane-bound complement inhibitors (complement receptor 1-CR1, also called CD35, and membrane cofactor protein-MCP, also called CD46) (Johnson et al. 2001; Hageman et al. 2005).

Recently discovered variations in genes encoding complement proteins and regulators have been associated with AMD, each appearing to have a largely individual and independent effect on AMD risk. This suggests certain individuals may be genetically predisposed to AMD because of local or systemic aberration of the complement pathway, in particular the alternative pathway. These associations will be described below, in conjunction with histological and systemic evidence for their involvement in AMD. Table 1 depicts a summary of the associations of complement gene sequence variations with AMD (for brevity, only the most significant positive associations in Caucasian populations are shown).

Although this review is concerned with the role of complement, it should be noted that there are other significant genetic associations with AMD, for example the ARMS2 gene on chromosome 10 (Jakobsdottir et al. 2005; Rivera et al. 2005; Fritsche et al. 2008), and genes involved in lipid metabolism (including the Hepatic Lipase LIPC gene) (Chen et al. 2010; Neale et al. 2010; Seddon et al. 2010). Fundamentally, the pathogenesis of AMD is likely to involve the disruption of multiple physiological pathways.

Fig. 4 depicts a simplified summary of the complement pathways, highlighting those components/regulators regarded to have genetic associations with AMD, as well as sites of action of complement inhibitors currently being investigated for AMD (to be discussed later).

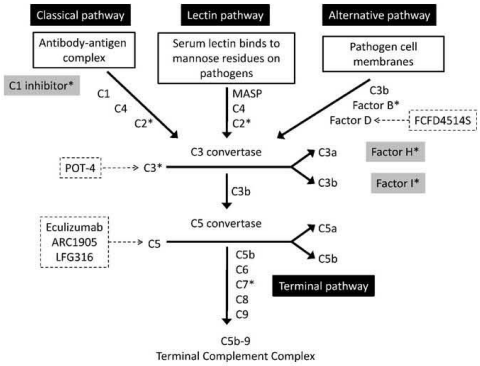

Fig. 4. Simplified diagram of complement pathways - redrawn, based on Zhou et al. (2011). The 3 complement pathways are shown, together with the common terminal pathway. Complement regulators are shown in grey boxes. Complement components and regulators which have genetic associations with AMD are marked with an asterisk (*). Activation products C3a, C5a and C5b-9 have been found in drusen and within surgically removed choroidal neovascular membranes (Mullins et al. 2000; Johnson et al. 2000; Nozaki et al. 2006; Lommatzsch et al. 2007, 2008). They are also elevated systemically in AMD (Sivaprasad et al. 2007; Scholl et al. 2008; Machalinska et al. 2009; Reynolds et al. 2009). Investigational products targeting the complement pathway are depicted in hashed boxes, with a hashed arrow pointing to the site of action. Please refer to text for more information.

Table 1
Complement gene sequence variations associated with age-related macular degeneration.

Category	Complement gene	Gene locus	SNP	Amino acid change	References showing association	Comment
Complement components	C2/CFB	6p21.3	rs9332739 (C2)	E318D	(Gold et al. 2006; Maller et al. 2006), (Spencer et al. 2007; Seddon et al. 2009; Francis et al. 2009)	Unknown whether these SNPs are associated with AMD due to a functional change in either the C2 or CFB genes, or even both.
			rs547154 (C2)	None (SNP in non-coding region)	(Gold et al. 2006; Maller et al. 2006), (Spencer et al. 2007; Jakobsdottir et al. 2008; Richardson et al. 2009; Gibson et al. 2010)	NB: rs9332739 (C2) in LD with rs4151667 (CFB), and rs547154 (C2) in LD with rs641153 (CFB)
			rs4151667 (CFB)	L9H	(Gold et al. 2006; Maller et al. 2006)	
			rs641153 (CFB)	R32Q	(Gold et al. 2006; Maller et al. 2006), (Spencer et al. 2007; Seddon et al. 2009; Richardson et al. 2009; Kopplin et al. 2010)	
	C3	19p13.3–p13.2	rs2230199	R102G	(Yates et al. 2007; Maller et al. 2007; Spencer et al. 2008b; Seddon et al. 2009; Despriet et al. 2009; Park et al. 2009; Francis et al. 2009; Scholl et al. 2009; Chen et al. 2010; McKay et al. 2010; Gibson et al. 2010; Kopplin et al. 2010)	NB. These two SNPs are in LD with each other
			rs1047286	P314L	(Spencer et al. 2008b; Despriet et al. 2009; Park et al. 2009; Gibson et al. 2010)	
	C5	9q34.1	Several SNPs	–	(Baas et al. 2010)	SNPs in the C5 gene only associated with AMD in one population only – not replicated in several other studies or in meta-analysis
	C7	5p13	rs2876849	None (SNP in non-coding region)	(Dinu et al. 2007; Gibson et al. 2010)	
Complement regulators	SERPING1	11q11–q13.1	rs2511989	None (SNP in non-coding region)	(Ennis et al. 2008; Lee et al. 2010)	Evidence for association of SERPING1 gene with AMD inconclusive – see text
			Others	None (SNP in non-coding region)/unknown	(Ennis et al. 2008; Lee et al. 2010)	
	CFH	1q32	rs1061170	Y402H	(Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Despriet et al. 2006; Li et al. 2006; Francis et al. 2007; Seddon et al. 2009; Scholl et al. 2009; Gibson et al. 2010; Kopplin et al. 2010)	First complement gene associated with AMD (Y402H SNP)
			rs800292	V62I	(Hageman et al. 2005; Francis et al. 2007; Gibson et al. 2010; Li et al. 2006)	
			Others	None (SNP in non-coding region or synonymous substitution)	(Hageman et al. 2005; Klein et al. 2005; Francis et al. 2007; Li et al. 2006; Seddon et al. 2009; Chen et al. 2010; Gibson et al. 2010; Kopplin et al. 2010)	
	CFHR1 + 3	1q32	N/A	Large deletion (84 kb)	(Hughes et al. 2006; Hageman et al. 2006; Spencer et al. 2008a; Schmid-Kubista et al. 2009)	
	CFHR2,4,5	1q32	Various	None (SNP in non-coding region or synonymous substitution)	(Zhang et al. 2008; Narendra et al. 2009; Gibson et al. 2010)	
	CFI	4q25	Various	None (SNP in non-coding region or synonymous substitu-tion/unknown	(Fagerness et al. 2009; Ennis et al. 2010; Chen et al. 2010)	SNPs found in CFI and near-by PLA2G12A gene (?function)

CFB: complement factor B, CFH: complement factor H, CFHR: CFH-related, CFI: complement factor I, LD: linkage disequilibrium, Rs: reference SNP number (as allocated by the SNP database of the National Centre of Biotechnology Information-NCBI)m, SNP: single nucleotide polymorphism.

Role of specific complement components in AMD

Complement C1

The complement component 1 (C1) molecule hasthree subunits, C1q, C1r and C1s. C1q binding to an antibody–antigen complex is the trigger for the classical pathway. C1q is found histologically in choroidal neovascular membranes (CNVM) retrieved surgically from patients with wet AMD (Baudouin et al. 1992). Despite this, the classical complement pathway does not presently appear to have a prominent role in the pathogenesis of AMD. For example, the AMD mouse model (the rd1 mouse) carries a recessive mutation in the β-subunit of the phosphodiesterase gene (β-PDE) gene (Farber 1995), leading to early onset of rapid retinal and photoreceptor degeneration. Rd1 C1qα (−/−) knock-out mice are unable to code for the α chain of the C1q subunit, in effect eliminating the classical pathway; these mice exhibit similar degenerative changes to a wild-type rd1 mouse, suggesting the classical pathway is not essential for the development of AMD (Rohrer et al. 2007).

Although there are no reports of an association between complement C1 gene variants and AMD, there is some evidence variants in the SERPING-1 gene, which codes for C1 inhibitor, may be associated with AMD (see below).

Complement C4

Complement component 4 (C4) is cleaved by activated C1 to form C4b and C4a. C4b forms part of the classical C3 convertase (C4b2a). As yet there are no reports of associations with AMD. Plasma C4 levels in AMD patients are similar to controls, with no association with known AMD genotype variations (Scholl et al. 2008).

Complement C2 + complement factor B

Complement component 2 (C2) and complement factor B (CFB) are described together because of similarities in structure, function and genetic characteristics. Structurally almost identical, the C2 and CFB proteins both have serine protease domains and three complement control protein modules (Gold et al. 2006). The C2 and CFB genes are found in close proximity to each other in the major histocompatibility complex (MHC) class lll region on chromosome 6, separated by only 500 base pairs. They both code for precursors of C3 convertase. C2 is cleaved to C2b, which combines with C4b to form the classical pathway C3 convertase. Similarly, CFB is cleaved to Bb, which combines with C3b to form the alternative pathway convertase, C3bBb. Furthermore, single nucleotide polymorphisms (SNPs) spanning the two genes appear to be in strong linkage disequilibrium (LD).

Both these proteins are secreted locally in the eye. Gold et al. localised CFB and C2 proteins to the neurosensory retina, RPE and choroid in donor eyes. CFB protein, unlike C2, is also found in drusen and BM (Gold et al. 2006). The secretion of CFB by RPE cells increases with age, possibly a response to increased demand for complement activation. Chen et al. found that RPE cells in ageing mice demonstrated up-regulation of CFB expression, and this was associated with increased C3 and C3a deposition in RPE cells and BM (Chen et al. 2008). Local inflammatory mediators may self-propagate this secretion. Wang et al. (2009b) found *in vitro* RPE cell CFB production increased indirectly due to macrophage and microglial cell cytokine release.

Variations in both the C2 and CFB genes are associated with AMD, conferring a protective effect. Gold et al. first reported an association between these genes and AMD (Gold et al. 2006). These findings were subsequently replicated in further studies (Maller et al. 2006; Seddon et al. 2009). The non-synonymous SNPs C2 E318D (rs9332739) and CFB L9H (rs4151667) variants are in high LD with each other, as are the C2 (intron 10, rs547154) and CFB R32Q (rs641153) variants (Maller et al. 2006). There is therefore uncertainty as to which of them is the causative variant. The lack of C2 in drusen and the evidence discussed below that the alternative complement pathway is the more important in AMD, points to CFB as the more likely susceptibility gene.

Presumably the protective effect of C2/CFB variations is due to a reduction in formation of either classical or alternative pathway C3 convertase. This was demonstrated by Montes et al., who studied the functional effects of the CFB R32Q variant *in vitro*. They found the 32Q variant had up to 4 times less C3b binding affinity than the non-risk 32R protein, with consequent reduction in C3 convertase formation (Montes et al. 2009). An intronic variation in a near-by gene, SuperkillerViralicidic Activity 2 (SKIV2L) has also been associated with AMD. This gene is considered to have a role in defence against RNA viruses; however its association with complement is uncertain (Chen et al. 2010; Kopplin et al. 2010).

Systemic plasma levels of the activation products Ba and Bb are elevated in AMD, which may be a reflection of increased systemic complement activity (Scholl et al. 2008; Reynolds et al. 2009; Hecker et al. 2010). Francis et al. (2009) looked at the pharmacogenetic effect between C2 and CFB polymorphisms and oral antioxidant supplements on reducing progression of AMD, but found no association.

Complement C3

Complement component 3 (C3) is a key complement protein and its cleavage into C3a and C3b is central to all three complement pathways. Indeed, mice eyes develop many features of AMD when exposed to C3 administered via an adenovirus vector delivered by subretinal injection (Cashman et al. 2011a). C3 and its activation product C3a have been found in drusen, as well as in neuroretina, BM and choroidal stroma (Mullins et al. 2001; Nozaki et al. 2006; Gold et al. 2006). C3 is expressed in RPE cells, neuroretina and choroidal tissue, suggesting local synthesis (Mullins et al. 2001).

C3 may have a particularly important role in the pathogenesis of wet AMD. Deposition of C3 and activation products C3c and C3d occurs in histological specimens of surgically removed CNV tissue (Baudouin et al. 1992; Lommatzsch et al. 2007 2008). Local C3a levels also increase on inducing CNV by laser in an experimental murine model of wet AMD (Nozaki et al. 2006). In fact, eliminating the C3 gene completely, as in C3 gene knock-out mice, protects from CNV development after exposure to laser (Bora et al. 2005). Development of CNV requires stimulation of choroidal endothelial cells by VEGF. Nozaki et al. (2006) found C3a levels increased VEGF secretion from RPE cells *in vitro*, and additionally from the choroid in mice when given intravenously. However, Skeie et al. (2010) found no C3a receptors in human choroid tissue, suggesting C3a may act indirectly on the choroid by recruitment of monocytes via ICAM-1 production.

Several studies demonstrate an elevation in plasma levels of C3 activation products in AMD (Sivaprasad et al. 2007; Scholl et al. 2008; Machalinska et al. 2009; Hecker et al. 2010). Furthermore, plasma C3d levels are associated with CFH, C2 and complement factor B (CFB) gene variations (Scholl et al. 2008; Hecker et al. 2010). However the association between plasma C3 levels and AMD is less convincing, possibly due to the systemic activation of plasma C3 in AMD (Scholl et al. 2008; Reynolds et al. 2009).

Variations in the C3 gene are associated with AMD. Yates et al. (2007) first found an association between the Arg80Gly (rs2230199) non-synonymous variation in the C3 gene and AMD. This has been replicated by later studies (Maller et al. 2007; Spencer et al. 2008b; Despriet et al. 2009; Francis et al. 2009; Seddon et al. 2009; Park et al. 2009a; Chen et al. 2010; McKay et al. 2010). The

estimated population attributable risk for this variant is 22% (Yates et al. 2007). Another C3 variant (rs1047286) is also associated with AMD, but is in strong LD with the Arg80Gly variant (Spencer et al. 2008b; Despriet et al. 2009; Park et al. 2009a). A meta-analysis of 17 studies confirms the association of genetic variants of the C3 gene and AMD (Thakkinstian et al. 2011).

Electrophoretic mobility differentiates the Arg80Gly polymorphism into the non-risk C3S (slow) and variant C3F (fast) proteins (Poznansky et al. 1989). The variant C3F has increased binding to mononuclear phagocytes, although functional variations have not been consistently replicated (Arvilommi 1974; Welch et al. 1990; Bartok and Walport 1995). The C3F protein is implicated not only in AMD but also in several immune-mediated diseases, including systemic vasculitis (Finn et al. 1994) and IgA nephropathy (Finn and Mathieson 1993). The C3F genotype may also be associated with reduced long-term outcome of renal transplantation (Brown et al. 2006b).

Cleavage of C3 to active C3b induces conformational change in the C3 molecule, exposing several binding sites including a thioester-containing domain. This is essential for covalent binding of C3b to target surfaces. The Arg80 amino acid contributes to a positive charge over this domain. Glycine is of neutral charge, and substitution of arginine for glycine at position 80 in the Arg80Gly variant may therefore affect the activity of the thioester-containing domain. This may account for the functional differences in the C3F and C3S proteins (Janssen et al. 2006; Jokiranta et al. 2006; Yates et al. 2007).

Variations in the C3 gene have also been associated with CNV in a Chinese population (Pei et al. 2009). Francis et al. (2009) looked at pharmacogenetic effect of the C3 Arg80Gly variation on reduction of AMD progression by oral antioxidant and zinc supplementation, but found no association.

Complement factor D

Complement factor D (CFD) is required for the cleavage of C3b-bound factor B, which leads to the formation of the alternative pathway C3 convertase, C3bBb (Volanakis and Narayana 1996). CFD can therefore increase alternative complement pathway activation. The function of CFD in the eye was demonstrated by Rohrer et al. They reported that CFD gene (−/−) knock-out mice, exposed to oxidative stress in the form of constant light for 12 h a day for 10 days (a model for AMD) demonstrated a significant reduction in photoreceptor damage. This suggests eliminating CFD may be protective against AMD (Rohrer et al. 2007). However clinical studies are non-confirmatory. Zeng et al. (2010) investigated 6 SNPs in the CFD gene and found no association in Caucasian patients with advanced AMD.

An increase in systemic CFD has been associated with increased prevalence of AMD, although this is not consistent. Scholl et al. reported that plasma levels of CFD were elevated in patients with AMD. They also found that a statistical model based on plasma levels of factor D, in addition to Ba and C3dlevels, was more accurate in predicting AMD than one based on genotype (Scholl et al. 2008). Hecker et al. also found plasma levels of CFD were elevated in patients with AMD and increased with age. Although there was no association with known complement genotype variations, the authors reported the best model for optimal prediction of AMD included factor D, and a SNP from the CFH, CFB and ARMS2 loci (Hecker et al. 2010). Reynolds et al. (2009) however found no association between circulating CFD levels and AMD.

Targeting CFD could offer a mechanism for reducing activation of the alternative complement pathway. Monoclonal antibodies directed against CFD have been developed (van Lookeren Campagne et al. 2010; Loyet et al. 2010), and are undergoing clinical trials (Genentech 2011a,b) (see below).

Complement C5

Complement component 5 (C5) is cleaved by C5 convertase into C5b (which initiates formation of TCC) and C5a (an anaphylatoxin). Systemic plasma levels of C5a, a marker of terminal pathway activation, are elevated in patients with AMD (Scholl et al. 2008; Reynolds et al. 2009). Furthermore, Reynolds et al. found a significant association between C5a levels and ARMS2 and the C3 genotype, suggesting systemic complement activation is under some element of genetic control (Reynolds et al. 2009).

In vitro studies suggest C5 has a role in both early and late AMD. Both C5 and C5a have been found in drusen in human donor eyes with AMD (Mullins et al. 2000; Nozaki et al. 2006). RPE cells overlying drusen show immunoreactivity for C5 and immunoglobulin, suggesting a role for immune complexes in AMD (Johnson et al. 2000). A similar hypothesis was proposed by Ambati et al. They studied an AMD mouse *Ccl-2*-(−/−) or *Ccr-2*-(−/−) knock-out model (as described earlier) and found a macrophage deficiency, which led to inadequate clearance and accumulation of C5 and IgG in the RPE or choroid. Such immune-complex deposition could form a nidus of chronic inflammatory activity that acts as trigger for AMD (Ambati et al. 2003).

C5a may have a more specific role in the pathogenesis of CNV. C5a receptors are expressed by *in vitro* cultured RPE cells; furthermore, RPE cells, demonstrate increased secretion of VEGF when treated with C5a, (Ambati et al. 2003; Nozaki et al. 2006; Cortright et al. 2009). Nozaki et al. confirmed this by showing C5a is generated by experimental inducement of CNV by laser in mice, and genetic ablation of the C5a receptor leads to reduced VEGF production, with consequent reduction in CNV formation (Nozaki et al. 2006). Skeie et al. proposed that C5a may also have an indirect pro-inflammatory role. They reported that stimulation of C5a receptors in human choroid could recruit monocytes by inducing ICAM-1 expression (Skeie et al. 2010).

Despite this evidence, there is no clear association between genetic variants of the C5 gene and AMD. Bass et al. found 8 SNPs in the C5 gene were significantly associated with AMD in the AMRO-NL (Amsterdam/Rotterdam-Netherlands) study population, independent of age, gender and variations in other genes significantly associated with AMD (CFH Y402H and ARMS2A69S). However this could not be replicated in three further populations from the Rotterdam study, Southampton (UK) and New York (USA). Furthermore, a meta-analysis of the pooled data did not support an association (Baas et al. 2010). Yates et al. (2007) also studied SNPs in C5 and found no association with AMD.

The use of C5-targetted pharmaceutical agents in AMD are currently being investigated in clinical trials (Kohl 2006; Apte and Ophthotech Anti-Complement in AMD Study Group 2009; Cousins and Ophthotech Study Group 2010; University of Miami 2011; Novartis Pharmaceuticals 2011; Ophthotech Corporation 2011a,b) (Table 2).

The terminal complement complex: C5b-9

The terminal complement complex (TCC), also called the membrane-attack complex (MAC), is formed from binding of C5b to the plasma complement proteins C6, C7, C8 and C9. TCC has been found histologically in drusen (Mullins et al. 2000) and at the macula, especially in older eyes with AMD (Hageman et al. 2005). TCC forms transmembrane channels, which lead to cell lysis and death. Bora et al. demonstrated that laser-induced CNV in a mouse model contained TCC and inhibition of TCC formation by addition of anti-C6 antibody results in inhibition of CNV formation (Bora et al. 2005).

Table 2
Complement-targetting investigational medicinal products undergoing clinical trials for age-related macular degeneration (AMD).

Investigative product	Pharmaceutical company	Mechanism of Action	Mode of delivery	AMD group targeted	Phase	Status	Clinicaltrials.gov number (+ reference)	Results of clinical trial
POT-4 (AL-78898A)	Potentia Pharmaceuticals/Alcon	C3 inhibitor	Intravitreal injection	NV AMD	1	Ended	NCT00473928 (Potentia Pharmaceuticals 2011b)	No drug-related toxicity in patients treated with up to 450 μg POT-4 (Kaushal et al. 2009)
					2	Suspended	NCT01157065 (Potentia Pharmaceuticals 2011a)	N/A
Eculizumab	University of Miami/Alexion Pharmaceuticals	C5 inhibitor	Intravenous infusion	Dry AMD (drusen and GA)	2	Ongoing	NCT00935883 (University of Miami 2011)	N/A
ARC1905	Ophthotech Corporation	Anti-C5 aptamer	Intravitreal injection	NV AMD	1	Ongoing	NCT00709527 (Ophthotech Corporation 2011a)	58 patients given 3–6 monthly ARC1905 (0.03, 0.3, 1, or 2 mg) with ranibizumab (0.5 mg). No dose-limiting toxicity seen. Mean visual acuity (VA) change was +10.2 letters at week 8. 35% gained ≥ 3 lines of VA. Mean change in macular central thickness (as measured by optical coherence tomography) was -124 μm. (Cousins and Ophthotech Study Group 2010)
				Dry AMD (GA)	1	Ongoing	NCT00950638 (Ophthotech Corporation 2011b)	N/A
LFG316	Novartis Pharmaceuticals	?C5 inhibitor[a]	Intravitreal injection	Advanced AMD (NV AMD or GA)	1		NCT01255462 (Novartis Pharmaceuticals 2011)	N/A
FCFD4514S	Genetech	Anti-CFD	Intravitreal injection	GA	1	Ongoing	NCT00973011 (Genentech 2011b)	N/A
					2	Not yet recruiting	NCT01229215 (Genentech 2011a)	N/A

AMD: age-related macular degeneration, NV AMD: neovascular (wet) AMD, GA: geographic atrophy, N/A: not available.
[a] Details unclear from clinicaltrials.gov website and Medline search.

Bora et al. (2005) reported anti-murine Complement C6 antibodies inhibited TCC formation in mice, and also resulted in inhibition of CNV from laser damage. This suggests targeting C6 or perhaps any of the components making up the TCC may be effective in preventing CNV development, although clinical trials of such products have yet to be conducted. Genetic variations in the C7 gene may be associated with AMD. (Dinu et al. 2007) reported a C7 haplotype was associated with reduced risk of developing wet AMD in patients with at least one CFH gene Y402H risk allele (see below).

Complement pathway regulators

C1 Inhibitor

C1 inhibitor, otherwise known as SERPING1 (serpin peptidase inhibitor, clade G, member 1) (Silverman et al. 2001) forms stable complexes with C1 subunits C1r and C1s (Ziccardi and Cooper 1976; Arlaud et al. 1979), and as a result irreversibly inhibits the classical complement pathway. SERPING1 also inhibits the lectin pathway (Kerr et al. 2008), and has a range of non-specific anti-inflammatory activities (Davis et al. 2007).

Studies reporting associations of the SERPING1 gene with AMD are contradictory. Ennis et al. first reported a protective association between variations in the SERPING1 gene and AMD in a UK population, which was replicated in a US cohort. The most significant was the intronic SNP rs2511989 (Ennis et al. 2008). Lee et al. (2010) reported two SNPS in the SERPING1 gene were associated with wet AMD in a US population, with one SNP conferring risk, the other being protective.

Other groups have been unable to replicate this association. Park et al. (2009b) were unable to find an association between SERPING1 variations in a population derived from the Mayo Clinic, USA and the Age-Related Eye Disease Study (AREDS) group. Carter et al. also could not replicate these findings in a smaller population from the UK (Carter and Churchill 2011). A large meta-analysis of 4881 patients with AMD and 1761 controls from 7 Caucasian populations around the world also demonstrated no association (Allikmets et al. 2009). Lu et al. (2010) found an association between SERPING1 and AMD in a Han Chinese population; however this was not statistically significant after a full Bonferonni correction (Lu et al. 2010). Nakata et al. (2011) could not replicate this association in a Japanese population.

The lack of consistency of an association may be due to a variation in level of LD in different populations, even amongst those defined as Caucasians (Allikmets et al. 2009). The SNPs found in

association with AMD by Ennis et al. are HapMap tags and are intronic; the true causal variant, perhaps yet to be discovered, may be in LD with these (Klaver and Bergen 2008). Kralovicova and Vorechovsky (2009) suggested specific SNPs found in association with AMD close to the coding region may affect mRNA splicing, and therefore may have a functional role. Studying further SNPs in the SERPING1 gene in more detail may shed further light on the relationship of this gene to AMD.

The SERPING1 protein is expressed at the macula. *In vitro* studies of human donor eyes demonstrate that both retina and the RPE/choroid layers from donor eyes express SERPING1, especially at the macula compared to the periphery (van Soest et al. 2007; Ennis et al. 2008; Mullins et al. 2009). However this protein has not been found in drusen, nor has any alteration in localisation and abundance in the SERPING1 protein been found based on the SERPING1 rs2511989 genotype (Ennis et al. 2008; Mullins et al. 2009).

Interestingly, variations in the SERPING1 gene are associated with hereditary angioedema (Gosswein et al. 2008), and this condition has been treated by administration of an infusion of C1 inhibitor concentrate (Agostoni et al. 1980), opening the possibility of a treatment option for AMD. However, the role of SERPING1 in AMD remains uncertain.

Complement factor H

Complement factor H (CFH) was the first complement protein to be implicated in the pathogenesis of AMD, following reports of an association between AMD and a functional variation in the CFH gene in 2005 (see below). As a result much of the literature on the role of complement in AMD is related to CFH.

Complement factor H (CFH) is an important regulator of the alternative pathway. CFH binds C3b, and accelerates the decay of the alternative C3 convertase (C3bBb). It also acts as a cofactor for the inactivation of C3b by complement factor I (CFI) (Pangburn 2000). CFH binds to cell surfaces to regulate amplification of the alternative complement pathway resulting from spontaneous C3b deposition, which occurs on any surface in contact with blood (Pangburn 2000).

Local intraocular CFH and AMD

Many studies have demonstrated that CFH protein is produced locally in the eye, and appears to have an important function in maintaining local homeostasis. *In vitro* studies show that CFH protein is produced by the RPE cells, at levels approaching those in the liver (Hageman et al. 2005). CFH accumulates in drusen, RPE cells, the sub-RPE space, the inter-photoreceptor matrix and in the choroid (Hageman et al. 2005; Chen et al. 2007). Levels are also high in other parts of the eye, including the lens, optic nerve, sclera and ciliary body. Furthermore, CFH expression appears to commence early in embryogenesis, and increases with age (Mandal and Ayyagari 2006). RPE cell CFH production varies according to local environmental factors. *In vitro* studies show an increase in RPE CFH production by interferon-gamma (Kim et al. 2009), and reduction in conditions of oxidative stress (Chen et al. 2007). In addition, CFH secretion is associated with *in vitro* RPE migration and hypertrophy, suggesting the RPE proliferation seen in AMD may be accompanied by a compensatory increase in complement regulatory factors (Kim et al. 2009; Kociok and Joussen 2010).

Studies of CFH gene knock-out animal models demonstrate that CFH has a vital protective role in the eye. Coffey et al. reported that aged 2 year old CFH gene knockout mice exhibited several significant anatomical, histological and functional changes, including increased deposition of auto-fluorescent material, C3 accumulation in the neurosensory retina, BM thinning, alteration in RPE organelle distribution, and rod photoreceptor cell outer segment disruption. This was also associated with significantly attenuated electroretinogram (ERG) responses (Coffey et al. 2007). Lundh von et al. also showed the importance of CFH in retinal perfusion in CFH gene knockout mice. Deposition of C3 and C3b on the endothelium of retinal vessels was seen, which subsequently became atrophic, reducing retinal perfusion, hence increasing oxidative stress (Lundh von et al. 2009). Sub-retinal injection of short interfering RNA (siRNA) directed against CFH can experimentally silence local CFH production. Lyzogubov et al. (2010) showed that this increased TCC deposition and exacerbated laser-induced CNV in mice.

CFH genotype variants and AMD

The CFH gene is located at chromosome position 1q23. Klein et al. (1998) first reported linkage to markers in this region in a large family in which AMD appeared to be segregating as an autosomal dominant Mendelian disorder. Subsequently, a non-synonymous single nucleotide polymorphism (SNP) in the CFH gene (rs1061170) was reported by three groups simultaneously in the journal Science in 2005 (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005). This SNP results in a substitution of tyrosine by histidine at position 402 of the polypeptide (Y402H) (allele substitution: T → C). A meta-analysis by Thakkinstian et al. (2006) suggested that the 402H variant is a contributing factor in over half of all cases of AMD. Patients homozygous for the risk allele (CC) are approximately 6 times, and those heterozygous (TC) 2.5 times more likely to have AMD compared to those with the non-risk genotype (TT).

Hageman et al. (2005) also reported a significant association between a non-synonymous SNP (I62V, rs800292) of the CFH gene, resulting in substitution of isoleucine with a valine residue within the C3b binding site on the CFH protein (Hageman et al. 2005). This has since been replicated (Bergeron-Sawitzke et al. 2009). Other SNPs throughout the CFH gene have subsequently been associated with AMD (Klein et al. 2005; Hageman et al. 2005; Li et al. 2006; Francis et al. 2007; Seddon et al. 2009; Chen et al. 2010; Gibson et al. 2010; Kopplin et al. 2010). CFH genotype has also been associated with AMD progression (Despriet et al. 2006; Schaumberg et al. 2006; Seddon et al. 2007, 2009; Farwick et al. 2010).

Interestingly a CFH mutation is also found in type 2 membranoproliferative glomerulonephritis (Dragon-Durey et al. 2004), which is associated with complement deposition in the glomeruli, together with sub-retinal drusen similar to those found in AMD (Mullins et al. 2001).

Systemic CFH levels, AMD and CFH genotype

A functional alteration in the CFH gene may lead to dysfunctional/deficient levels of systemic CFH protein. This may cause dysregulation of the alternative complement system, with consequent damage to self. Studies of systemic CFH levels in AMD have given inconsistent results. Reynolds et al. reported an inverse relationship with plasma CFH levels and advanced AMD (CNV/GA) (Reynolds et al. 2009). However no association was found in two other studies (Scholl et al. 2008; Hecker et al. 2010), and indeed Hakobyan et al. (2008a) reported elevated plasma CFH levels in AMD. Dhillon et al. (2010) looked at auto antibodies against CFH in plasma, as measured by Enzyme-linked Immunosorbent Assay (ELISA), and found levels to be lower in patients with AMD compared to controls.

Systemic CFH levels are also not consistently associated with CFH genotype in AMD. Hakobyan et al. (2008a) reported that serum CFH levels were higher in patients with AMD carrying the heterozygous Y402H genotype, but not in homozygotes (Hakobyan et al. 2008a). Others have not found an association between CFH genotype and systemic CFH levels (Reynolds et al. 2009; Hecker et al. 2010). CFH Y402H protein isoforms in plasma can also be detected using gel separation with mass spectrometry (Kelly et al. 2009), and can be quantified by ELISA (Hakobyan et al. 2008a). Hakobyan et al. (2008a) measured plasma levels of both Y402 and H402 variants of

the CFH protein using with ELISA, and confirmed this corresponded to underlying Y402H genotype with 100% accuracy.

Variations in the CFH gene appear to be associated with increased systemic complement activation. Scholl et al. (2008) reported a CFH risk haplotype (including the Y402H variant) was associated with elevated plasma levels of complement activation products (Ba and C3d), which was subsequently replicated by another group (Hecker et al. 2010). However no association has been found between CFH genotype and plasma C3-des-Arg levels, an activation product formed on cleavage of C3 (Sivaprasad et al. 2007).

Functional consequences of CFH variants

Y402H. The functional consequences of the non-synonymous CFH Y402H variation have been studied extensively. The CFH polypeptide chain is arranged into 20 similar units, called short consensual repeats (SCR), each containing approximately 60 amino acids (Ripoche et al. 1988). C3 convertase regulating activity is controlled by SCRs 1–4, including C3b binding, decay accelerating and CFI cofactor activity. C3b binding also occurs at SCRs 12–14, and 19–20 (Zipfel et al. 2002). The CFH molecule has multiple binding sites, and is able to bind to numerous ligands, including C-reactive protein (CRP), heparin, sialic acid, and streptococcal M protein (Pangburn 2000). Replacement of tyrosine with a histidine residue at position 402 of the CFH protein causes alteration at SCR 7 (Ripoche et al. 1988) and therefore affects interaction with ligand-binding at this site. This includes binding with CRP, heparin, M protein, and glycosaminoglycans. This may lead to reduced binding to cell surfaces, and therefore impaired regulation of the alternative C3 convertase. Three dimensional computerized mapping suggests that the Y402H amino acid change affects a polyanion binding site on the CFH molecule (Herbert et al. 2006).

CRP is an acute phase protein, which increases substantially as part of the innate immune response to tissue injury and infection. It binds to polysaccharides on pathogens, and damaged cell surfaces. Its presence is thought to provide secondary sites for CFH binding, therefore increasing CFH presence on cell surfaces (Mold et al. 1999). Several studies have confirmed that the CFH H402 variant has reduced affinity for CRP compared toY402 protein (Herbert et al. 2007; Laine et al. 2007; Sjoberg et al. 2007; Skerka et al. 2007; Yu et al. 2007). The CFH–CRP interaction seen in these studies has been questioned by Hakobyan et al. (2008b) who reported that CRP–CFH binding may be the result of denaturing of CRP due to immobilisation on plastic (as used in ELISA), in specific conditions (absence of calcium ions), and that "differential binding to CRP does not explain the association of this polymorphism with pathology in AMD". However Perkins et al. recently reported that native CRP–CFH interaction is present at high levels of CRP in plasma, as is seen during the acute phase response, and indeed the CFH H402 protein does bind weakly to CRP (Perkins et al. 2010). Elevated levels of serum CRP are associated with AMD. A meta-analysis of eleven studies (41,690 patients) carried out by Hong et al. showed that serum levels of CRP above 3 mg/L are associated with double the risk of AMD, compared to CRP levels <1 mg/L (Hong et al. 2011). Furthermore, the homozygous CC Y402H genotype in combination with elevated serum CRP levels is associated with a high odds ratio (OR) of 19.3 for presence of late AMD, and 6.8 for AMD progression (Robman et al. 2010). Despite this, variations in the CRP gene are not associated with AMD (Schaumberg et al. 2006; Kim et al. 2008a). Johnson et al. also found raised CRP levels locally in the RPE and choroid in donor eyes of patients homozygous for H402, compared to Y402 homozygotes. However there was no difference in CFH protein levels between Y402H genotypes. Furthermore a lack of local CRP expression suggests CRP presence in the eye results from deposition through chronic low-grade local inflammation, or from the systemic circulation (Johnson et al. 2006).

The CFH H402 variant also demonstrates alteration in binding to other ligands. Clark et al. showed that the H402 protein binds less well to glycosaminoglycans (heparin sulphate and dermatan sulphate) within BM, compared to the Y402 form. Binding to RPE cells was no different (Clark et al. 2010). Skerka et al. (2007) however found the H402 protein has reduced binding to RPE cells as well as heparin. Furthermore, the H402 variant binds less well to fibromodulin (Sjoberg et al. 2007) and the M6 protein of Streptococcus pyogenes (Yu et al. 2007), which interestingly may decrease susceptibility to group A streptococcal infections (Haapasalo et al. 2008). Zinc may also play a role. Perkins et al. reported that the CFH H402 protein showed increased aggregation with zinc, a CFH activity inhibitor, compared to the H402 protein (Perkins et al. 2010). These alterations may produce functional differences between the Y402H polymorphisms, and may explain the association of this genotype variation and development of AMD.

I62V. The I62V SNP lies within SCR2 of the CFH gene, which is involved in C3b binding. Structural analysis shows that the substitution of isoleucine with a valine residue at this position could have a profound effect on the folding and thermal stability of the CFH molecule (Hocking et al. 2008). This may explain the functional differences seen between the I62V protein polymorphisms. ELISA and surface plasmon resonance studies show the CFH V62 variant protein has decreased capacity to bind C3b, decreased inhibition of C3bB (C3 pro-convertase), and decreased cofactor function in the CFI-mediated inactivation of C3b (Tortajada et al. 2009).

CFHL-1. Skerka et al. reported that the CFH gene produces a second protein, designated CFH-like protein (CFHL-1) as a result of alternative splicing. This protein is also present in the eye. The CFHL-1 402H variant has similar reduction in binding to cellular surfaces as the CFH Y402H variant, and may also lead to localized dysregulation of complement (Skerka et al. 2007).

Association of other risk factors and CFH in AMD

Smoking and CFH. Smoking is a strong risk factor for AMD (Klein et al. 1993; Smith et al. 1996; Vingerling et al. 1996). At risk CFH genotype variants seem to have an additive effect with smoking on AMD, especially with CNV (Sepp et al. 2006; Deangelis et al. 2007; Scott et al. 2007; Wang et al. 2009c). Deanglis et al. found that individuals homozygous for the Y402H risk genotype (CC) and smoking 10 or more pack-years were 144 times more at risk of developing CNV, compared to those heterozygous (CT) or with the non-risk (TT) Y402H genotype, and smoking less than 10 pack-years (Deangelis et al. 2007). Wang et al. looked at a large cohort of 1881 patients from the Blue Mountains Eye Study. They found that current smokers with the at risk CC/CT Y402H genotype were 5 times more likely to have late AMD compared to non-smokers with the non-riskY402H genotype (Wang et al. 2009c). Furthermore, an individual's risk of developing AMD can be predicted by combining genotype data with smoking status (Hughes et al. 2007). Schmidt et al. found that CFH Y402H, ARMS2and smoking status together could account for 61% of the population-attributable risk of AMD (Schmidt et al. 2008).

Fish consumption and CFH. Oily fish consumption is associated with a lower risk of AMD (Augood et al. 2008). A study on a large cohort from the Blue Mountains eye study demonstrated that the incidence of late AMD was lower in patients who ate fish weekly compared to those eating fish less than once a week, but only in those patients with the homozygous (CC) Y402H variation (relative risk 0.15) (Wang et al. 2009c).

Chlamydia Pneumoniae and CFH. Chronic infection with *Chlamydia Pneumoniae* (CP) has been proposed as one possible mechanism which could trigger on-going complement activation in the eye. The association between CP and AMD is, however, tentative (Kalayoglu et al. 2003; Robman et al. 2005; Robman et al. 2007). CFH genotype is also not consistently associated with CP in patients with AMD. Baird et al. reported a substantial increase in OR (11.8) for development of AMD in patients with both the Y402H risk CC allele and upper tertiles of CP antibodies, compared to those with the non-risk T allele and lowest antibody tertile (Baird et al. 2008). However Shen et al. (2009) found no association between CP infection (as evidenced by the presence of the CP gene in blood) and CFH status in patients with AMD.

CFH – interactions with other genes. Although variants in different complement genes appear to have a largely independent effect on risk, several studies have suggested that the CFH gene may interact with other genes known to be associated with AMD, in particular the ARMS2 gene, which suggests a common causative pathway may exist for these genes (Seitsonen et al. 2008b). Patients homozygous for both the CFH Y402 andARMS2 A69S SNPs have a substantially increased risk of AMD (50 times higher), with an even higher risk when smoking and obesity are taken into account (Schaumberg et al. 2007). Tuo et al. (2008) reported that presence of both ARMS2 and CFH risk SNPs have an additive effect on AMD susceptibility (Tuo et al. 2008), but this was not replicated in other studies (Weger et al. 2007; Francis et al. 2008). Dinu et al. (2007) reported patients with either the heterozygous or homozygous CFH Y402H allele and a haplotype of the C7 gene were associated with a reduced risk of developing wet AMD, compared to dry AMD. This suggests that gene interaction may influence AMD phenotype.

CFH polymorphisms and AMD phenotype correlations

CFH polymorphisms appear to be more strongly related to advanced AMD (especially CNV), when compared to early (Despriet et al. 2006) or any AMD (Hageman et al. 2005; Haines et al. 2005). More detailed AMD phenotypic associations have also been reported, as follows.

Early AMD phenotype. CFH genotype may influence early AMD phenotype with regards to drusen and pigmentary irregularities. Thompson et al. (2007) looked at a large population cohort (Beavers Dam Study) and found macular pigmentary irregularities were associated with CFH gene polymorphisms. Tedeschi-Blok et al. (2007) found the presence of the at-risk Y02H C allele (either homozygous or heterozygous) was associated with the presence of bilateral intermediate-to-large drusen, as opposed to unilateral drusen. Magnusson et al. (2006) reported that the CFH Y402H SNP appeared to be associated with the presence of soft drusen, in an Icelandic population. Droz et al. reported individuals homozygous for the Y402H variant were more likely to have both central and peripheral drusen. However they found no association with drusen size, location, or total area, nor with pigmentary changes (Droz et al. 2008). The Inter99 Eye Study looked at the presence of drusen in 1107 subjects, and found that the presence of 20 or more small hard drusen was not associated with Y402H, although there was an association with peripheral drusen (Munch et al. 2010).

CNV characteristics. CNV has been described based on its appearance during FFA as classic (lace-like with early leakage), and occult/minimally classic (late leakage of undetermined origin or stippled hyperfluorescence of the RPE cells). Several groups have looked at whether CFH genotype influences CNV characteristics. Brantley et al. found patients with the at-risk C allele in the Y402H polymorphism were more likely to have predominantly classic CNV (Brantley et al. 2007a). Seitsonen et al. found no correlation between CNV size and CFH Y402H genotype in patients with wet AMD. However they did find a non-significant association in patients with the homozygous at-risk CC allele and incidence of minimally classic CNV (Seitsonen et al. 2008a). Leveziel et al. (2008) found individuals with wet AMD, with the homozygous Y402H genotype were more likely to have minimally classic CNV, although numbers were relatively small in each group.

CFH SNPs and pharmacogenetic correlations

Several studies have investigated pharmacogenetic associations between the CFH Y402H SNP and various treatment modalities.

Oral antioxidants. Oral antioxidant supplements have been shown in the AREDS clinical trial to have some benefit in reducing AMD progression from high risk early AMD (large drusen and pigmentary changes) to CNV/GA (advanced AMD) (Age-Related Eye Disease Study Research Group 2001). Klein et al. looked at whether the CFH Y402H variation influenced the outcome in 876 patients taking the AREDS supplements. In those with the non-risk Y402H genotype (TT), there was a 69% reduction in progression to advanced AMD in patients taking oral antioxidants plus zinc, compared to those taking placebo, decreasing to 11% reduction in homozygotes (CC). This suggested there was an interaction between CFH genotype and oral AREDS supplementation (Klein et al. 2008a).

Photodynamic therapy. Photodynamic therapy (PDT) has been used to treat CNV by local laser activation of a systemically infused photoactive dye (verteporfin) (TAP study group 1999). However, the association of the CFH Y402H variation with PDT outcome is inconsistent. Goverdhan et al. first reported that carrying the at-risk Y402H C allele increased the likelihood of requiring PDT, (OR 2.16), and furthermore, patients carrying the homozygous CC genotype were more likely to have a worse visual outcome following PDT treatment (Goverdhan et al. 2008). Brantley et al. reported, conversely, that the non-risk TT genotype was associated with worse response to PDT compared to the TC and CC genotype (Brantley et al. 2009). Other groups have not found any association (Seitsonen et al. 2007; Feng et al. 2009).

Anti-VEGF injections. Anti-VEGF intravitreal injections presently form the mainstay of treatment of CNV. Brantley et al. reported a pharmacogenetic correlation with regards to treatment with intravitreal bevacizumab in 86 patients with wet AMD. Patients with the homozygous CC Y402H at risk genotype experienced a loss of vision, compared with an improvement for the non-risk TT and heterozygous TC genotype. However there was no correlation with ARMS2A69S polymorphisms the ARMS2 A69S polymorphism and response to treatment (Brantley et al. 2007b). Lee et al. (2009) reported that patients homozygous for the CFH Y402H mutation were 37% more likely to require additional injections (Lee et al. 2009). Investigation of other complement genotypes on anti-VEGF injection outcome may reveal further associations.

Pharmocogenetic associations such as those outlined above may have clinical relevance. Although AMD treatment presently is limited, future management could potentially be guided by predicting treatment outcome based on individual genotype ("personalised medicine").

CFH and other ethnic groups

The CFH Y402H risk C allele occurs at varying frequencies in different ethnic groups. Hageman et al. (2006) looked at a set of 1064 DNA samples from 47 populations (Human Genome Diversity Panel), and found a Y402H C allele frequency of 35% in African + Europeans, 25–30% in North Africans + Middle Easterns, 10–15% in Asians and 0–5% in Native Americans.

Despite this varying frequency, associations of Y402H in the CFH gene with AMD have been seen in non-Caucasian ethnic groups, including Chinese (Lau et al. 2006; Chu et al. 2008; Gao et al. 2010; Liu et al. 2010), Taiwanese Chinese (Lin et al. 2008; Liu et al. 2009), Indian (Kaur et al. 2006), Black (Klein et al. 2008b) and Israeli (Chowers et al. 2008) populations. An association has also been noted in Hispanics in the USA (Klein et al. 2008b), although another study found an association with AMD progression and not AMD prevalence in this population group (Tedeschi-Blok et al. 2007). No association has been identified in Japanese populations (Fuse et al. 2006; Gotoh et al. 2006; Uka et al. 2006), and a marginal association is seen in Koreans (Kim et al. 2008b). Other CFH SNPs appear to be associated with AMD in Chinese (Ng et al. 2008; Liu et al. 2010) and Japanese (Okamoto et al. 2006; Mori et al. 2007) populations.

This evidence suggests that the CFH gene is associated with AMD in most ethnic groups. However further studies are required to augment population allele frequency data, especiallyin African, Middle Eastern, and South American populations (Nonyane et al. 2010).

Potential CFH-based therapeutic agents

Despite CFH being present both locally and in the systemic circulation, supplemental administration of CFH to the eye may be advantageous. Rohrer et al. reported the potential benefit of a novel recombinant form of CFH, CR2-fH. This consists of the N-terminus of mouse CFH (with the C3 convertase regulating activity), bound to complement receptor 2 (CR2). Intravenous administration of CR2-fH was effective in reducing laser-induced CNV in mice, and was seen to co-localise with C3 in RPE and choroid (Rohrer et al. 2009, 2010).

CFH-related genes

Five genes related to CFH (CFHR1–5) are found just downstream of the CFH gene, all containing similar SCR domains to the CFH gene. In particular, SCRs 6–7 and 19–20 appear to be highly conserved. The function of each of these is incompletely understood (Zipfel et al. 2002).

CFHR1 + CFHR3

A large (84 kb) frequently occurring deletion on chromosome 1 encompassing both the CFHR1 and CFHR3 genes is negatively associated with AMD. Hughes et al. reported that a haplotype carrying the deletion was associated with a decreased risk of AMD, independent of Y402H status. They also found the CFHR1 and CFHR3 proteins were absent in the serum of patients homozygous for the deletion (Hughes et al. 2006). This has been successfully replicated in several studies (Hageman et al. 2006; Spencer et al. 2008a; Schmid-Kubista et al. 2009). The deletion is common globally in African, European and Asian populations, suggesting an ancient origin (Hageman et al. 2006).

The reasons why a deletion in the CFHR1 and CFHR3 genes is associated with a protective effect for AMD are unclear. Both CFHR1 and CFHR3 carry the C3 binding domain of CFH at SCRs 18–20, but do not have the C3b inactivating domains at SCRs 1–4 (Zipfel et al. 2002). Hageman et al. proposed that CFHR1 and CFHR3 interfere with CFH binding of C3b. Therefore an absence of CFHR1 and CFHR3 may enhance the interaction between CFH and C3b, therefore increasing regulation of the alternative complement pathway. CFHR1 and CFHR3 are not expressed in the RPE/choroid and neuroretina, suggesting any effect in the eye may be derived from systemic hepatic production (Hageman et al. 2006). However other evidence confuses the issue. *In vitro* studies suggest that CFHR1 inhibits C5 convertase activity, reduces surface deposition of C5b and decreases TCC formation; therefore a deficiency may be pro-inflammatory (Heinen et al. 2009). CFHR1 and CFHR3 deficiency is also associated with increased risk in atypical Haemolytic Uraemic Syndrome (Zipfel et al. 2007). Therefore a deficiency of CFHR1 and CFHR3 appears to result in loss of complement control, but enhanced local regulation by CFH. Fritsche et al. (2010) suggested a critical balance exists between CFHR1, CFHR3 and CFH levels, and a disruption of this balance may precipitate a particular disease phenotype. It should also be noted that Raychaudhuri et al. (2010) have argued that the CFHR1-3 gene deletion is not itself responsible for the protective effect, but is a marker for another causative variant. The relationship of the CFHR1 + 3 deletion with AMD requires further elucidation.

CFHR2, CFHR4, CFHR5

A handful of studies have shown an association between CFHR2, CFHR4 and CFHR5 with AMD. Zhang et al. reported a genome-wide study in the AREDS cohort associating multiple variants in the CFHR2, CFHR4 and CFHR5 genes with CNV. They also found the highest risk was demonstrated by a haplotype spanning the CFH, CFHR2 and CFHR4 genes (Zhang et al. 2008). Narendra et al. reported a possible protective role of a SNP in exon 3 (Asp169Asp). Although this SNP is synonymous, it could confer a protective effects by improving mRNA stability or affecting RNA splicing (Narendra et al. 2009).

Complement factor I

Complement factor I (CFI) is a serine protease, and acts as a regulator of both the classical and alternative pathways, by cleaving and inactivating C4b and C3b respectively. This enzymatic cleavage can only occur in the presence of cofactors, including C4 binding protein (C4BP), CFH, MCP and CR1. It is generally accepted that CFH acts on C3b, and C4BP on C4b, whereas CRI and MCP act on both (Nilsson et al. 2011).

Histological studies demonstrate that CFI may not retain its regulatory function in AMD. Wang et al. showed that a component of drusen, amyloid beta, can bind to CFI and consequently reduce its function, potentially leading to chronic low-grade inflammation (Wang et al. 2008). However proliferating RPE cells, also seen in early AMD, demonstrate increased expression of CFI protein (Kociok and Joussen 2010). RPE hypertrophy may be a response to inflammation, and increased CFI levels may be induced as a compensatory response.

Systemic levels of CFI do not appear to be altered in AMD. Reynolds et al. (2009) found no difference in plasma CFI levels in patients with AMD compared to controls, and no association with CFI gene variations. Despite this, several groups have found an association between variants near the CFI gene and AMD. Fagerness et al. (2009) identified a SNP (rs10033900) close to the CFI gene which was associated with AMD. Other groups have reported similar findings (Chen et al. 2010; Ennis et al. 2010; Neale et al. 2010), and Kondo et al. (2010) replicated this in a Japanese population. However, the lack of association with SNP rs10033900 in a recent UK study leaves uncertainty as to which SNPs/genes are most strongly associated across different populations (Cipriani et al. 2011a). Seddon et al. (2010) reported a possible interaction between SNPs at the CFI locus and variants in the hepatic lipase C (LIPC) gene, also associated with AMD. The functional variants at the CFI locus are still to be determined. It may be relevant that some of the reported associated SNPs are present in the near-by phospholipase A(2) group 12A (PLA2G12A) gene, which has a role in T cell immune responses (Ho et al. 2001).

Other complement regulators

In addition to those discussed above there are several other regulators of the complement cascade, many of which have not been

studied in AMD. In this issue of the journal, Yates et al. report a study of four of these. They investigated SNPs spanning the genes encoding complement factor P (CFP, properdin), CD46 (MCP), CD55 (decay accelerating factor, DAF) and CD59 (protectin) and found no evidence of association with AMD. CFP is a stabilising component of the alternative pathway convertases and can bind to cells and pathogens, promoting convertase assembly and targeted phagocytosis. CD46 and CD55 are membrane bound regulators, CD46 acting as a cofactor for CFI mediated cleavage of C3b and CD55 inhibiting the formation and accelerating the decay of C3 and C5 convertases (Cipriani et al. 2011b).

CD59 is a membrane bound regulatory protein which prevents final assembly of the TCC by binding to complement components 8 and 9 (Meri et al. 1990). CD59 is extensively expressed in the choroid and all layers of the retina (Bora et al. 1993), and has a potent regulatory effect by reducing local TCC formation. An absence of the equivalent gene in mice, as seen in a CD59a gene (−/−) knock-out model, leads to increased TCC deposition, and development of laser-induced CNV earlier (day 3) compared to control mice (day 5) (Bora et al. 2005). Several studies have investigated the role of CD59 as a therapeutic agent in managing CNV. Bora et al. showed that administration of recombinant CD59a via either the intraperitoneal or intravitreal route could inhibit laser-induced CNV, with a corresponding inhibitory effect on angiogenic growth factor expression (Bora et al. 2007). In a later study, the same group showed that intravitreal injection of recombinant CD59 into mice could not only inhibit further growth but also reduce the size of active CNV, due to increased apoptosis and decreased proliferation of endothelial cells within the CNV (Bora et al. 2010). Ramo et al. (2008) used gene therapy to deliver CD59 to the retina using an adenovirus vector, and found a local protective effect. RPE cells taken from mice given sub-retinal injections of recombinant adenovirus-expressing human CD59, subsequently exposed *in vitro* to complement in human serum, were protected from TCC deposition and TCC-associated damage. The same group subsequently used the same technique to deliver the gene vector distal to the site of CNV with similar inhibitory effects. Encouragingly, the benefit was also preserved with less invasive intravitreal delivery (Cashman et al. 2011b). Clinical trials using CD59 delivered either directly or via gene therapy are awaited.

Interaction of complement with other immune pathways in AMD

Other pathways of the immune system may be implicated in AMD, possibly through interaction with complement. For example, there is some evidence that the complement system "cross-talks" with another major system of innate immunity, the toll-like receptor (TLR) system (Zhang et al. 2007). However the association of the TLR gene and AMD is unclear (Cho et al. 2009). Activated complement components act as chemo-attractants to leucocytes, as well as facilitating phagocytosis. Tissue macrophages are likely to contribute to AMD pathogenesis, in particular to nvAMD. However there is some evidence that macrophages may in some circumstances be protective (Skeie and Mullins 2009). Certain human leucocyte antigen (HLA) haplotypes have been associated with AMD (Goverdhan et al. 2005), however the role of complement with HLA in AMD has yet to be determined. Finally, chronic infections may induce chronic complement activation. A particularly studied example is *Chlamydia pneumoniae*, through a possible interaction with the CFH protein, although as mentioned above the association of this micro-organism with AMD is unclear.

Interaction between oxidative stress and complement in AMD

Oxidative stress appears to play a key role in the pathogenesis of AMD (Khandhadia and Lotery 2010). One possible mechanism is through complement dysregulation. Experimental evidence illustrates the possible effects of oxidative stress from a variety of sources. Wang et al. showed RPE cells exposed to benzoapyrene, a toxic component of cigarette smoke inducing oxidative stress, exhibited accumulation of C3, CFH and CFB. Retinal tissue taken from mice exposed to cigarette smoke also exhibited complement deposition surrounding BM, including C3a, C5, C5b-9 and CFH (Wang et al. 2009a). Thurman et al. (2009) showed cultured RPE cells exposed to oxidative stress from hydrogen peroxide exhibited reduced levels of complement inhibitors at the cell surface, associated with increased VEGF production. Rohrer et al. (2007) reported an AMD mouse model, created by exposure to constant light, demonstrated significant up-regulation of complement genes, including C1qα, C1qβ, C1qc, C3, C4, and SERPING1. Hollyfield et al. injected mice systemically with serum carboxyethylpyrrole (CEP). CEP is formed by light-induced oxidation of docosahexaenoic acid (DHA) found in retinal cell membranes. These mice fixed complement in BM, as well as developing sub-RPE drusen and GA-like retinal lesions (Hollyfield et al. 2008). This evidence suggests an interaction exists between two significant pathological processes, oxidative stress and the complement cascade, in the development of AMD.

Clinical trials of complement-based therapies for AMD

A range of complement inhibitors are currently being evaluated in clinical trials for the treatment of both dry and wet AMD (Table 2 – information obtained from www.clinicaltrials.gov). The complement cascade is targeted at several levels, through C3, C5 and CFD inhibition (see Fig. 4). Most are administered by intravitreal injection, as is used for the currently approved treatment of AMD with anti-VEGF agents. Eculizumab is an exception, needing to be given as an intravenous infusion.

POT-4 is a C3 inhibitor being investigated for wet AMD. Although phase 1 studies have reported no drug-toxicity, phase 2 studies have been suspended for unknown reasons. Eculizumab marketed as "Soliris", is a recombinant humanized monoclonal immunoglobulin G antibody against C5 given intravenously, licenced for use in paroxysmal nocturnal haemoglobinuria. However its chronic systemic use is associated with increased risk of *Neisseria meningitides* infection (Parker 2009). This highlights the dangers of immune suppression during chronic complement inhibition. The systemic effects of intravitreal injections are considered less significant (Loyet et al. 2010). ARC1905 is an anti-C5 aptamer. These are non-immunogenic single-stranded oligonucleotides which, due to particular three-dimensional folding characteristics, bind to specific protein targets and inhibit protein–protein interactions (Mayer and Jenne 2004). FCFD4515S is a recombinant humanised monoclonal antibody fragment which binds to the C-terminal portion of CFD, in turn preventing binding to C3bB pro-convertase, and reducing formation of active C3bBb convertase (van Lookeren Campagne et al. 2010).

The ideal complement inhibitor would have beneficial improvement in vision and disease pathogenesis, with minimal systemic effects whilst preserving the more proximal part of the complement pathway to allow local clearance of pathogens. The results of clinical trials are awaited.

Conclusion

There is growing evidence that the complement system plays a vital role in the pathogenesis of AMD. Further research is needed to determine what triggers complement activation and how this leads to AMD. Manipulation of the complement pathway may provide a novel therapeutic option for managing AMD in the future.

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References

Age-Related Eye Disease Study Research Group, 2001. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no 8. Arch. Ophthalmol. 119, 1417–1436 (Erratum appears in Arch Ophthalmol. 2008 Sep;126(9):1251).

Agostoni, A., Bergamaschini, L., Martignoni, G., Cicardi, M., Marasini, B., 1980. Treatment of acute attacks of hereditary angioedema with C1-inhibitor concentrate. Ann. Allergy 44, 299–301.

Allikmets, R., Dean, M., Hageman, G.S., Baird, P.N., Klaver, C.C., Bergen, A.A., Weber, B.H., 2009. The SERPING1 gene and age-related macular degeneration. Lancet 374, 875–876.

Ambati, J., Anand, A., Fernandez, S., Sakurai, E., Lynn, B.C., Kuziel, W.A., Rollins, B.J., Ambati, B.K., 2003. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. Nat. Med. 9, 1390–1397.

Anderson, D.H., Mullins, R.F., Hageman, G.S., Johnson, L.V., 2002. A role for local inflammation in the formation of drusen in the aging eye. Am. J. Ophthalmol. 134, 411–431.

Apte, R.S., 2009. Ophthotech Anti-Complement in AMD Study Group Targeting Complement Factor 5 in Neovascular Age-Related Macular Degeneration (NV-AMD)—results of a Phase 1 Study. In: ARVO Meeting Abstracts 50, p. 5011.

Arlaud, G.J., Reboul, A., Sim, R.B., Colomb, M.G., 1979. Interaction of C1-inhibitor with the C1r and C1s subcomponents in human C1. Biochim. Biophys. Acta 576, 151–162.

Arvilommi, H., 1974. Capacity of complement c3 phenotypes to bind on to mononuclear cells in man. Nature 251, 740–741.

Augood, C., Chakravarthy, U., Young, I., Vioque, J., de Jong, P.T., Bentham, G., Rahu, M., Seland, J., Soubrane, G., Tomazzoli, L., Topouzis, F., Vingerling, J.R., Fletcher, A.E., 2008. Oily fish consumption, dietary docosahexaenoic acid and eicosapentaenoic acid intakes, and associations with neovascular age-related macular degeneration. Am. J. Clin. Nutr. 88, 398–406.

Baas, D.C., Ho, L., Ennis, S., Merriam, J.E., Tanck, M.W., Uitterlinden, A.G., de Jong, P.T., Cree, A.J., Griffiths, H.L., Rivadeneira, F., Hofman, A., van, D.C., Smith, R.T., Barile, G.R., Gorgels, T.G., Vingerling, J.R., Klaver, C.C., Lotery, A.J., Allikmets, R., Bergen, A.A., 2010. The complement component 5 gene and age-related macular degeneration. Ophthalmology 117, 500–511.

Baird, P.N., Robman, L.D., Richardson, A.J., Dimitrov, P.N., Tikellis, G., McCarty, C.A., Guymer, R.H., 2008. Gene–environment interaction in progression of AMD: the CFH gene, smoking and exposure to chronic infection. Hum. Mol. Genet. 17, 1299–1305.

Bartok, I., Walport, M.J., 1995. Comparison of the binding of C3S and C3F to complement receptors types 1, 2, and 3. J. Immunol. 154, 5367–5375.

Baudouin, C., Peyman, G.A., Fredj-Reygrobellet, D., Gordon, W.C., Lapalus, P., Gastaud, P., Bazan, N.G., 1992. Immunohistological study of subretinal membranes in age-related macular degeneration. Jpn. J. Ophthalmol. 36, 443–451.

Bergeron-Sawitzke, J., Gold, B., Olsh, A., Schlotterbeck, S., Lemon, K., Visvanathan, K., Allikmets, R., Dean, M., 2009. Multilocus analysis of age-related macular degeneration. Eur. J. Hum. Genet. 17, 1190–1199.

Bora, N.S., Gobleman, C.L., Atkinson, J.P., Pepose, J.S., Kaplan, H.J., 1993. Differential expression of the complement regulatory proteins in the human eye. Invest. Ophthalmol. Vis. Sci. 34, 3579–3584.

Bora, N.S., Jha, P., Lyzogubov, V.V., Kaliappan, S., Liu, J., Tytarenko, R.G., Fraser, D.A., Morgan, B.P., Bora, P.S., 2010. Recombinant membrane-targeted form of CD59 inhibits the growth of choroidal neovascular complex in mice. J. Biol. Chem. 285, 33826–33833.

Bora, N.S., Kaliappan, S., Jha, P., Xu, Q., Sivasankar, B., Harris, C.L., Morgan, B.P., Bora, P.S., 2007. CD59, a complement regulatory protein, controls choroidal neovascularization in a mouse model of wet-type age-related macular degeneration. J. Immunol. 178, 1783–1790.

Bora, P.S., Sohn, J.H., Cruz, J.M., Jha, P., Nishihori, H., Wang, Y., Kaliappan, S., Kaplan, H.J., Bora, N.S., 2005. Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J. Immunol. 174, 491–497.

Brantley Jr., M.A., Edelstein, S.L., King, J.M., Apte, R.S., Kymes, S.M., Shiels, A., 2007a. Clinical phenotypes associated with the complement factor H Y402H variant in age-related macular degeneration. Am. J. Ophthalmol. 144, 404–408.

Brantley Jr., M.A., Edelstein, S.L., King, J.M., Plotzke, M.R., Apte, R.S., Kymes, S.M., Shiels, A., 2009. Association of complement factor H and LOC387715 genotypes with response of exudative age-related macular degeneration to photodynamic therapy. Eye (Lond.) 23, 626–631.

Brantley Jr., M.A., Fang, A.M., King, J.M., Tewari, A., Kymes, S.M., Shiels, A., 2007b. Association of complement factor H and LOC387715 genotypes with response of exudative age-related macular degeneration to intravitreal bevacizumab. Ophthalmology 114, 2168–2173.

Brown, D.M., Kaiser, P.K., Michels, M., Soubrane, G., Heier, J.S., Kim, R.Y., Sy, J.P., Schneider, S., 2006a. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N. Engl. J. Med. 355, 1432–1444.

Brown, K.M., Kondeatis, E., Vaughan, R.W., Kon, S.P., Farmer, C.K., Taylor, J.D., He, X., Johnston, A., Horsfield, C., Janssen, B.J., Gros, P., Zhou, W., Sacks, S.H., Sheerin, N.S., 2006b. Influence of donor C3 allotype on late renal-transplantation outcome. N. Engl. J. Med. 354, 2014–2023.

Carter, J.G., Churchill, A.J., 2011. Analysis of SERPING1 and its association with age-related macular degeneration. Acta Ophthalmologica 89, e212–e213.

Cashman, S.M., Desai, A., Ramo, K., Kumar-Singh, R., 2011a. Expression of complement component 3 (c3) from an adenovirus leads to pathology in the murine retina. Invest. Ophthalmol. Vis. Sci. 52, 3436–3445.

Cashman, S.M., Ramo, K., Kumar-Singh, R., 2011b. A non membrane-targeted human soluble CD59 attenuates choroidal neovascularization in a model of age related macular degeneration. PLoS One 6, e19078.

Chen, M., Forrester, J.V., Xu, H., 2007. Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. Exp. Eye Res. 84, 635–645.

Chen, M., Muckersie, E., Robertson, M., Forrester, J.V., Xu, H., 2008. Up-regulation of complement factor B in retinal pigment epithelial cells is accompanied by complement activation in the aged retina. Exp. Eye Res. 87, 543–550.

Chen, W., Stambolian, D., Edwards, A.O., Branham, K.E., Othman, M., Jakobsdottir, J., Tosakulwong, N., Pericak-Vance, M.A., Campochiaro, P.A., Klein, M.L., Tan, P.L., Conley, Y.P., Kanda, A., Kopplin, L., Li, Y., Augustaitis, K.J., Karoukis, A.J., Scott, W.K., Agarwal, A., Kovach, J.L., Schwartz, S.G., Postel, E.A., Brooks, M., Baratz, K.H., Brown, W.L., Brucker, A.J., Orlin, A., Brown, G., Ho, A., Regillo, C., Donoso, L., Tian, L., Kaderli, B., Hadley, D., Hagstrom, S.A., Peachey, N.S., Klein, R., Klein, B.E., Gotoh, N., Yamashiro, K., Ferris, I.F., Fagerness, J.A., Reynolds, R., Farrer, L.A., Kim, I.K., Miller, J.W., Corton, M., Carracedo, A., Sanchez-Salorio, M., Pugh, E.W., Doheny, K.F., Brion, M., Deangelis, M.M., Weeks, D.E., Zack, D.J., Chew, E.Y., Heckenlively, J.R., Yoshimura, N., Iyengar, S.K., Francis, P.J., Katsanis, N., Seddon, J.M., Haines, J.L., Gorin, M.B., Abecasis, G.R., Swaroop, A., 2010. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. Proc. Natl. Acad. Sci. U.S.A. 107, 7401–7406.

Cho, Y., Wang, J.J., Chew, E.Y., Ferris III, F.L., Mitchell, P., Chan, C.C., Tuo, J., 2009. Toll-like receptor polymorphisms and age-related macular degeneration: replication in three case-control samples. Invest. Ophthalmol. Vis. Sci. 50, 5614–5618.

Chowers, I., Cohen, Y., Goldenberg-Cohen, N., Vicuna-Kojchen, J., Lichtinger, A., Weinstein, O., Pollack, A., Axer-Siegel, R., Hemo, I., Averbukh, E., Banin, E., Meir, T., Lederman, M., 2008. Association of complement factor H Y402H polymorphism with phenotype of neovascular age related macular degeneration in Israel. Mol. Vis. 14, 1829–1834.

Chu, J., Zhou, C.C., Lu, N., Zhang, X., Dong, F.T., 2008. Genetic variants in three genes and smoking show strong associations with susceptibility to exudative age-related macular degeneration in a Chinese population. Chin. Med. J. (Engl.) 121, 2525–2533.

Cipriani, V., Matharu, B.K., Khan, J.C., Shahid, H., Hayward, C., Wright, A.F., Armbrecht, A.M., Dhillon, B., Harding, S.P., Bishop, P.N., Bunce, C., Clayton, D.G., Moore, A.T., Yates, J.R., 2011a. No evidence of association between complement factor I genetic variant rs10033900 and age-related macular degeneration. Eur. J. Hum. Genet. doi:10.1038/ejhg.2011.118.

Cipriani, V., Matharu, B.K., Khan, J.C., Shahid, H., Stanton, C.M., Hayward, C., Wright, A.F., Bunce, C., Clayton, D.G., Moore, A.T., Yates, J.R.W., 2011b. Genetic variation in complement regulators and susceptibility to age-related macular degeneration. Immunobiology, doi:10.1016/j.imbio.2011.09.002 (This issue).

Clark, S.J., Perveen, R., Hakobyan, S., Morgan, B.P., Sim, R.B., Bishop, P.N., Day, A.J., 2010. Impaired binding of the age-related macular degeneration-associated complement factor H 402H allotype to Bruch's membrane in human retina. J. Biol. Chem. 285, 30192–30202.

Coffey, P.J., Gias, C., McDermott, C.J., Lundh, P., Pickering, M.C., Sethi, C., Bird, A., Fitzke, F.W., Maass, A., Chen, L.L., Holder, G.E., Luthert, P.J., Salt, T.E., Moss, S.E., Greenwood, J., 2007. Complement factor H deficiency in aged mice causes retinal abnormalities and visual dysfunction. Proc. Natl. Acad. Sci. U.S.A. 104, 16651–16656.

Cortright, D.N., Meade, R., Waters, S.M., Chenard, B.L., Krause, J.E., 2009. C5a, but not C3a, increases VEGF secretion in ARPE-19 human retinal pigment epithelial cells. Curr. Eye Res. 34, 57–61.

Cousins, S.W., 2010. Ophthotech Study group targeting complement factor 5 in combination with vascular endothelial growth factor (VEGF) inhibition for

neovascular age related macular degeneration (AMD): results of a phase 1 study. In: ARVO Meeting Abstracts 51, p. 1251.
Davis III, A.E., Cai, S., Liu, D., 2007. C1 inhibitor: biologic activities that are independent of protease inhibition. Immunobiology 212, 313–323.
de Jong, P.T., 2006. Age-related macular degeneration. N. Engl. J. Med. 355, 1474–1485.
Deangelis, M.M., Ji, F., Kim, I.K., Adams, S., Capone Jr., A., Ott, J., Miller, J.W., Dryja, T.P., 2007. Cigarette smoking, CFH, APOE ELOVL4, and risk of neovascular age-related macular degeneration. Arch. Ophthalmol. 125, 49–54.
Despriet, D.D., Klaver, C.C., Witteman, J.C., Bergen, A.A., Kardys, I., de Maat, M.P., Boekhoorn, S.S., Vingerling, J.R., Hofman, A., Oostra, B.A., Uitterlinden, A.G., Stijnen, T., van Duijn, C.M., de Jong, P.T., 2006. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. JAMA 296, 301–309.
Despriet, D.D., van Duijn, C.M., Oostra, B.A., Uitterlinden, A.G., Hofman, A., Wright, A.F., ten Brink, J.B., Bakker, A., de Jong, P.T., Vingerling, J.R., Bergen, A.A., Klaver, C.C., 2009. Complement component C3 and risk of age-related macular degeneration. Ophthalmology 116, 474–480.
Dhillon, B., Wright, A.F., Tufail, A., Pappworth, I., Hayward, C., Moore, I., Strain, L., Kavanagh, D., Barlow, P., Herbert, A.P., Schmidt, C.Q., Armbrecht, A.M., Laude, A., Deary, I.J., Staniforth, S.J., Holmes, L.V., Goodship, T., Marchbank, K.J., 2010. Complement factor H autoantibodies and age-related macular degeneration. Invest. Ophthalmol. Vis. Sci..
Dinu, V., Miller, P.L., Zhao, H., 2007. Evidence for association between multiple complement pathway genes and AMD. Genet. Epidemiol. 31, 224–237.
Dragon-Durey, M.A., Fremeaux-Bacchi, V., Loirat, C., Blouin, J., Niaudet, P., Deschenes, G., Coppo, P., Herman, F.W., Weiss, L., 2004. Heterozygous and homozygous factor h deficiencies associated with hemolytic uremic syndrome or membranoproliferative glomerulonephritis: report and genetic analysis of 16 cases. J. Am. Soc. Nephrol. 15, 787–795.
Droz, I., Mantel, I., Ambresin, A., Faouzi, M., Schorderet, D.F., Munier, F.L., 2008. Genotype-phenotype correlation of age-related macular degeneration: influence of complement factor H polymorphism. Br. J. Ophthalmol. 92, 513–517.
Edwards, A.O., Ritter III, R., Abel, K.J., Manning, A., Panhuysen, C., Farrer, L.A., 2005. Complement factor H polymorphism and age-related macular degeneration. Science 308, 421–424.
Ennis, S., Gibson, J., Cree, A.J., Collins, A., Lotery, A.J., 2010. Support for the involvement of complement factor I in age-related macular degeneration. Eur. J. Hum. Genet. 18, 15–16.
Ennis, S., Jomary, C., Mullins, R., Cree, A., Chen, X., Macleod, A., Jones, S., Collins, A., Stone, E., Lotery, A., 2008. Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. Lancet 372, 1828–1834.
Fagerness, J.A., Maller, J.B., Neale, B.M., Reynolds, R.C., Daly, M.J., Seddon, J.M., 2009. Variation near complement factor I is associated with risk of advanced AMD. Eur. J. Hum. Genet. 17, 100–104.
Farber, D.B., 1995. From mice to men: the cyclic GMP phosphodiesterase gene in vision and disease. The Proctor Lecture. Invest. Ophthalmol. Vis. Sci. 36, 263–275.
Farwick, A., Wellmann, J., Stoll, M., Pauleikhoff, D., Hense, H.W., 2010. Susceptibility genes and progression in age-related maculopathy: a study of single eyes. Invest. Ophthalmol. Vis. Sci. 51, 731–736.
Feng, X., Xiao, J., Longville, B., Tan, A.X., Wu, X.N., Cooper, M.N., McAllister, I.L., Isaacs, T., Palmer, L.J., Constable, I.J., 2009. Complement factor H Y402H and C-reactive protein polymorphism and photodynamic therapy response in age-related macular degeneration. Ophthalmology 116, 1908–1912.
Finn, J.E., Mathieson, P.W., 1993. Molecular analysis of C3 allotypes in patients with nephritic factor. Clin. Exp. Immunol. 91, 410–414.
Finn, J.E., Zhang, L., Agrawal, S., Jayne, D.R., Oliveira, D.B., Mathieson, P.W., 1994. Molecular analysis of C3 allotypes in patients with systemic vasculitis. Nephrol. Dial. Transplant. 9, 1564–1567.
Francis, P.J., Hamon, S.C., Ott, J., Weleber, R.G., Klein, M.L., 2009. Polymorphisms in C2 CFB and C3 are associated with progression to advanced age related macular degeneration associated with visual loss. J. Med. Genet. 46, 300–307.
Francis, P.J., Schultz, D.W., Hamon, S., Ott, J., Weleber, R.G., Klein, M.L., 2007. Haplotypes in the complement factor H (CFH) gene: associations with drusen and advanced age-related macular degeneration. PLoS One 2, e1197.
Francis, P.J., Zhang, H., DeWan, A., Hoh, J., Klein, M.L., 2008. Joint effects of polymorphisms in the HTRA1 LOC387715/ARMS2, and CFH genes on AMD in a Caucasian population. Mol. Vis. 14, 1395–1400.
Friedman, D.S., O'Colmain, B.J., Munoz, B., Tomany, S.C., McCarty, C., de Jong, P.T., Nemesure, B., Mitchell, P., Kempen, J., 2004. Prevalence of age-related macular degeneration in the United States. Arch. Ophthalmol. 122, 564–572.
Fritsche, L.G., Lauer, N., Hartmann, A., Stippa, S., Keilhauer, C.N., Oppermann, M., Pandey, M.K., Kohl, J., Zipfel, P.F., Weber, B.H., Skerka, C., 2010. An imbalance of human complement regulatory proteins CFHR1 CFHR3 and factor H influences risk for age-related macular degeneration (AMD). Hum. Mol. Genet. 19, 4694–4704.
Fritsche, L.G., Loenhardt, T., Janssen, A., Fisher, S.A., Rivera, A., Keilhauer, C.N., Weber, B.H., 2008. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat. Genet. 40, 892–896.
Fuse, N., Miyazawa, A., Mengkegale, M., Yoshida, M., Wakusawa, R., Abe, T., Tamai, M., 2006. Polymorphisms in Complement Factor H and Hemicentin-1 genes in a Japanese population with dry-type age-related macular degeneration. Am. J. Ophthalmol. 142, 1074–1076.
Gao, Y., Li, Y., Xu, L., Li, Y., Zhang, H.T., Jonas, J.B., Sun, B.C., 2010. Complement factor H polymorphism in age-related maculopathy in the Chinese population: the Beijing Eye Study. Retina 30, 443–446.
Genentech., 2011a. A study of safety, tolerability, and evidence of activity of FCFD4514S administered monthly or every other month to patients with geographic atrophy. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda, MD, 2000-[cited 2011 April 01]. Available from: http://clinicaltrials.gov/ct2/show/NCT01229215,. NLM Identifier: NCT01229215.
Genentech., 2011b. A study of the safety, tolerability, pharmacokinetics, and immunogenicity of intravitreal injections of FCFD4514S in patients with geographic atrophy. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda, MD, 2000-[cited 2011 April 01]. Available from: http://clinicaltrials.gov/ct2/show/NCT00973011,. NLM Identifier: NCT00973011.
Gibson, J., Cree, A., Collins, A., Lotery, A., Ennis, S., 2010. Determination of a gene and environment risk model for age-related macular degeneration. Br. J. Ophthalmol. 94, 1382–1387.
Gold, B., Merriam, J.E., Zernant, J., Hancox, L.S., Taiber, A.J., Gehrs, K., Cramer, K., Neel, J., Bergeron, J., Barile, G.R., Smith, R.T., Hageman, G.S., Dean, M., Allikmets, R., 2006. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat. Genet. 38, 458–462.
Gosswein, T., Kocot, A., Emmert, G., Kreuz, W., Martinez-Saguer, I., Aygoren-Pursun, E., Rusicke, E., Bork, K., Oldenburg, J., Muller, C.R., 2008. Mutational spectrum of the C1INH (SERPING1) gene in patients with hereditary angioedema. Cytogenet. Genome Res. 121, 181–188.
Gotoh, N., Yamada, R., Hiratani, H., Renault, V., Kuroiwa, S., Monet, M., Toyoda, S., Chida, S., Mandai, M., Otani, A., Yoshimura, N., Matsuda, F., 2006. No association between complement factor H gene polymorphism and exudative age-related macular degeneration in Japanese. Hum. Genet. 120, 139–143.
Goverdhan, S.V., Hannan, S., Newsom, R.B., Luff, A.J., Griffiths, H., Lotery, A.J., 2008. An analysis of the CFH Y402H genotype in AMD patients and controls from the UK, and response to PDT treatment. Eye (Lond.) 22, 849–854.
Goverdhan, S.V., Howell, M.W., Mullins, R.F., Osmond, C., Hodgkins, P.R., Self, J., Avery, K., Lotery, A.J., 2005. Association of HLA class I and class II polymorphisms with age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 46 (5), 1726–1734.
Haapasalo, K., Jarva, H., Siljander, T., Tewodros, W., Vuopio-Varkila, J., Jokiranta, T.S., 2008. Complement factor H allotype 402H is associated with increased C3b opsonization and phagocytosis of Streptococcus pyogenes. Mol. Microbiol. 70, 583–594.
Hageman, G.S., Anderson, D.H., Johnson, L.V., Hancox, L.S., Taiber, A.J., Hardisty, L.I., Hageman, J.L., Stockman, H.A., Borchardt, J.D., Gehrs, K.M., Smith, R.J., Silvestri, G., Russell, S.R., Klaver, C.C., Barbazetto, I., Chang, S., Yannuzzi, L.A., Barile, G.R., Merriam, J.C., Smith, R.T., Olsh, A.K., Bergeron, J., Zernant, J., Merriam, J.E., Gold, B., Dean, M., Allikmets, R., 2005. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc. Natl. Acad. Sci. U.S.A. 102, 7227–7232.
Hageman, G.S., Hancox, L.S., Taiber, A.J., Gehrs, K.M., Anderson, D.H., Johnson, L.V., Radeke, M.J., Kavanagh, D., Richards, A., Atkinson, J., Meri, S., Bergeron, J., Zernant, J., Merriam, J., Gold, B., Allikmets, R., Dean, M., 2006. Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Ann. Med. 38, 592–604.
Haines, J.L., Hauser, M.A., Schmidt, S., Scott, W.K., Olson, L.M., Gallins, P., Spencer, K.L., Kwan, S.Y., Noureddine, M., Gilbert, J.R., Schnetz-Boutaud, N., Agarwal, A., Postel, E.A., Pericak-Vance, M.A., 2005. Complement factor H variant increases the risk of age-related macular degeneration. Science 308, 419–421.
Hakobyan, S., Harris, C.L., Tortajada, A., Goicochea de, J.E., Garcia-Layana, A., Fernandez-Robredo, P., Rodriguez de, C.S., Morgan, B.P., 2008a. Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: application to assessing risk of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 49, 1983–1990.
Hakobyan, S., Harris, C.L., van den Berg, C.W., Fernandez-Alonso, M.C., de Jorge, E.G., de Cordoba, S.R., Rivas, G., Mangione, P., Pepys, M.B., Morgan, B.P., 2008b. Complement factor H binds to denatured rather than to native pentameric C-reactive protein. J. Biol. Chem. 283, 30451–30460.
Hecker, L.A., Edwards, A.O., Ryu, E., Tosakulwong, N., Baratz, K.H., Brown, W.L., Charbel, I.P., Scholl, H.P., Pollok-Kopp, B., Schmid-Kubista, K.E., Bailey, K.R., Oppermann, M., 2010. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. Hum. Mol. Genet. 19, 209–215.
Heinen, S., Hartmann, A., Lauer, N., Wiehl, U., Dahse, H.M., Schirmer, S., Gropp, K., Enghardt, T., Wallich, R., Halbich, S., Mihlan, M., Schlotzer-Schrehardt, U., Zipfel, P.F., Skerka, C., 2009. Factor H-related protein 1 (CFHR-1) inhibits complement C5 convertase activity and terminal complex formation. Blood 114, 2439–2447.
Herbert, A.P., Deakin, J.A., Schmidt, C.Q., Blaum, B.S., Egan, C., Ferreira, V.P., Pangburn, M.K., Lyon, M., Uhrin, D., Barlow, P.N., 2007. Structure shows that a glycosaminoglycan and protein recognition site in factor H is perturbed by age-related macular degeneration-linked single nucleotide polymorphism. J. Biol. Chem. 282, 18960–18968.
Herbert, A.P., Uhrin, D., Lyon, M., Pangburn, M.K., Barlow, P.N., 2006. Disease-associated sequence variations congregate in a polyanion recognition patch on human factor H revealed in three-dimensional structure. J. Biol. Chem. 281, 16512–16520.

Ho, I.C., Arm, J.P., Bingham III, C.O., Choi, A., Austen, K.F., Glimcher, L.H., 2001. A novel group of phospholipase A2s preferentially expressed in type 2 helper T cells. J. Biol. Chem. 276, 18321–18326.
Hocking, H.G., Herbert, A.P., Kavanagh, D., Soares, D.C., Ferreira, V.P., Pangburn, M.K., Uhrin, D., Barlow, P.N., 2008. Structure of the N-terminal region of complement factor H and conformational implications of disease-linked sequence variations. J. Biol. Chem. 283, 9475–9487.
Hollyfield, J.G., Bonilha, V.L., Rayborn, M.E., Yang, X., Shadrach, K.G., Lu, L., Ufret, R.L., Salomon, R.G., Perez, V.L., 2008. Oxidative damage-induced inflammation initiates age-related macular degeneration. Nat. Med. 14, 194–198.
Hong, T., Tan, A.G., Mitchell, P., Wang, J.J., 2011. A review and meta-analysis of the association between C-reactive protein and age-related macular degeneration. Surv. Ophthalmol. 56, 184–194.
Hughes, A.E., Orr, N., Esfandiary, H., Diaz-Torres, M., Goodship, T., Chakravarthy, U., 2006. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. Nat. Genet. 38, 1173–1177.
Hughes, A.E., Orr, N., Patterson, C., Esfandiary, H., Hogg, R., McConnell, V., Silvestri, G., Chakravarthy, U., 2007. Neovascular age-related macular degeneration risk based on CFH LOC387715/HTRA1, and smoking. PLoS Med. 4, e355.
Jakobsdottir, J., Conley, Y.P., Weeks, D.E., Mah, T.S., Ferrell, R.E., Gorin, M.B., 2005. Susceptibility genes for age-related maculopathy on chromosome 10q26. Am. J. Hum. Genet. 77, 389–407.
Janssen, B.J., Christodoulidou, A., McCarthy, A., Lambris, J.D., Gros, P., 2006. Structure of C3b reveals conformational changes that underlie complement activity. Nature 444, 213–216.
Johnson, L.V., Leitner, W.P., Staples, M.K., Anderson, D.H., 2001. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. Exp. Eye Res. 73, 887–896.
Johnson, L.V., Ozaki, S., Staples, M.K., Erickson, P.A., Anderson, D.H., 2000. A potential role for immune complex pathogenesis in drusen formation. Exp. Eye Res. 70, 441–449.
Johnson, P.T., Betts, K.E., Radeke, M.J., Hageman, G.S., Anderson, D.H., Johnson, L.V., 2006. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. Proc. Natl. Acad. Sci. U.S.A. 103, 17456–17461.
Jokiranta, T.S., Jaakola, V.P., Lehtinen, M.J., Parepalo, M., Meri, S., Goldman, A., 2006. Structure of complement factor H carboxyl-terminus reveals molecular basis of atypical haemolytic uremic syndrome. EMBO J. 25, 1784–1794.
Kalayoglu, M.V., Galvan, C., Mahdi, O.S., Byrne, G.I., Mansour, S., 2003. Serological association between Chlamydia pneumoniae infection and age-related macular degeneration. Arch. Ophthalmol. 121, 478–482.
Kaneko, H., Dridi, S., Tarallo, V., Gelfand, B.D., Fowler, B.J., Cho, W.G., Kleinman, M.E., Ponicsan, S.L., Hauswirth, W.W., Chiodo, V.A., Kariko, K., Yoo, J.W., Lee, D.K., Hadziahmetovic, M., Song, Y., Misra, S., Chaudhuri, G., Buaas, F.W., Braun, R.E., Hinton, D.R., Zhang, Q., Grossniklaus, H.E., Provis, J.M., Madigan, M.C., Milam, A.H., Justice, N.L., Albuquerque, R.J., Blandford, A.D., Bogdanovich, S., Hirano, Y., Witta, J., Fuchs, E., Littman, D.R., Ambati, B.K., Rudin, C.M., Chong, M.M., Provost, P., Kugel, J.F., Goodrich, J.A., Dunaief, J.L., Baffi, J.Z., Ambati, J., 2011. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. Nature 471, 325–330.
Kaur, I., Hussain, A., Hussain, N., Das, T., Pathangay, A., Mathai, A., Hussain, A., Nutheti, R., Nirmalan, P.K., Chakrabarti, S., 2006. Analysis of CFH TLR4, and APOE polymorphism in India suggests the Tyr402His variant of CFH to be a global marker for age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 47, 3729–3735.
Kelly, U., Rickman, C.B., Postel, E.A., Hauser, M.A., Hageman, G.S., Arshavsky, V.Y., Skiba, N.P., 2009. Rapid and sensitive method for detection of Y402, H402 I62, and V62 variants of complement factor H in human plasma samples using mass spectrometry. Invest. Ophthalmol. Vis. Sci. 50, 1540–1545.
Kerr, F.K., Thomas, A.R., Wijeyewickrema, L.C., Whisstock, J.C., Boyd, S.E., Kaiserman, D., Matthews, A.Y., Bird, P.I., Thielens, N.M., Rossi, V., Pike, R.N., 2008. Elucidation of the substrate specificity of the MASP-2 protease of the lectin complement pathway and identification of the enzyme as a major physiological target of the serpin, C1-inhibitor. Mol. Immunol. 45, 670–677.
Khandhadia, S., Lotery, A., 2010. Oxidation and age-related macular degeneration: insights from molecular biology. Expert Rev. Mol. Med. 12, e34.
Kim, I.K., Ji, F., Morrison, M.A., Adams, S., Zhang, Q., Lane, A.M., Capone, A., Dryja, T.P., Ott, J., Miller, J.W., Deangelis, M.M., 2008a. Comprehensive analysis of CRP CFH Y402H and environmental risk factors on risk of neovascular age-related macular degeneration. Mol. Vis. 14, 1487–1495.
Kim, N.R., Kang, J.H., Kwon, O.W., Lee, S.J., Oh, J.H., Chin, H.S., 2008b. Association between complement factor H gene polymorphisms and neovascular age-related macular degeneration in Koreans. Invest. Ophthalmol. Vis. Sci. 49, 2071–2076.
Kim, Y.H., He, S., Kase, S., Kitamura, M., Ryan, S.J., Hinton, D.R., 2009. Regulated secretion of complement factor H by RPE and its role in RPE migration. Graefes Arch. Clin. Exp. Ophthalmol. 247, 651–659.
Klaver, C.C., Bergen, A.A., 2008. The SERPING1 gene and age-related macular degeneration. Lancet 372, 1788–1789.
Klein, M.L., Mauldin, W.M., Stoumbos, V.D., 1994. Heredity and age-related macular degeneration observations in monozygotic twins. Arch. Ophthalmol. 112, 932–937.
Klein, M.L., Schultz, D.W., Edwards, A., Matise, T.C., Rust, K., Berselli, C.B., Trzupek, K., Weleber, R.G., Ott, J., Wirtz, M.K., Acott, T.S., 1998. Age-related macular degeneration. Clinical features in a large family and linkage to chromosome 1q. Arch. Ophthalmol. 116, 1082–1088.
Klein, R., Klein, B.E., Linton, K.L., 1992. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology 99, 933–943.
Klein, R., Klein, B.E., Linton, K.L., DeMets, D.L., 1993. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. Am. J. Epidemiol. 137, 190–200.
Klein, M.L., Francis, P.J., Rosner, B., Reynolds, R., Hamon, S.C., Schultz, D.W., Ott, J., Seddon, J.M., 2008a. CFH and LOC387715/ARMS2 genotypes and treatment with antioxidants and zinc for age-related macular degeneration. Ophthalmology 115, 1019–1025.
Klein, R., Knudtson, M.D., Klein, B.E., Wong, T.Y., Cotch, M.F., Liu, K., Cheng, C.Y., Burke, G.L., Saad, M.F., Jacobs Jr., D.R., Sharrett, A.R., 2008b. Inflammation, complement factor h, and age-related macular degeneration: the Multi-ethnic Study of Atherosclerosis. Ophthalmology 115, 1742–1749.
Klein, R., Cruickshanks, K.J., Nash, S.D., Krantz, E.M., Javier, N.F., Huang, G.H., Pankow, J.S., Klein, B.E., 2010. The prevalence of age-related macular degeneration and associated risk factors. Arch. Ophthalmol. 128, 750–758.
Klein, R.J., Zeiss, C., Chew, E.Y., Tsai, J.Y., Sackler, R.S., Haynes, C., Henning, A.K., SanGiovanni, J.P., Mane, S.M., Mayne, S.T., Bracken, M.B., Ferris, F.L., Ott, J., Barnstable, C., Hoh, J., 2005. Complement factor H polymorphism in age-related macular degeneration. Science 308, 385–389.
Kociok, N., Joussen, A.M., 2010. Enhanced expression of the complement factor H mRNA in proliferating human RPE cells. Graefes Arch. Clin. Exp. Ophthalmol. 248, 1145–1153.
Kohl, J., 2006. Drug evaluation: the C5a receptor antagonist PMX-53. Curr. Opin. Mol. Ther. 8, 529–538.
Kondo, N., Bessho, H., Honda, S., Negi, A., 2010. Additional evidence to support the role of a common variant near the complement factor I gene in susceptibility to age-related macular degeneration. Eur. J. Hum. Genet. 18, 634–635.
Kopplin, L.J., Igo Jr., R.P., Wang, Y., Sivakumaran, T.A., Hagstrom, S.A., Peachey, N.S., Francis, P.J., Klein, M.L., SanGiovanni, J.P., Chew, E.Y., Pauer, G.J., Sturgill, G.M., Joshi, T., Tian, L., Xi, Q., Henning, A.K., Lee, K.E., Klein, R., Klein, B.E., Iyengar, S.K., 2010. Genome-wide association identifies SKIV2L and MYRIP as protective factors for age-related macular degeneration. Genes Immun. 11, 609–621.
Kralovicova, J., Vorechovsky, I., 2009. SERPING1 rs2511988 and age-related macular degeneration. Lancet 373, 461–462.
Lai, Y.L., Jacoby, R.O., Jonas, A.M., 1978. Age-related and light-associated retinal changes in Fischer rats. Invest. Ophthalmol. Vis. Sci. 17, 634–638.
Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Lehtinen, M.J., Lindeman, N., Anderson, D.H., Johnson, P.T., Jarvela, I., Jokiranta, T.S., Hageman, G.S., Immonen, I., Meri, S., 2007. Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. J. Immunol. 178, 3831–3836.
Lau, L.I., Chen, S.J., Cheng, C.Y., Yen, M.Y., Lee, F.L., Lin, M.W., Hsu, W.M., Wei, Y.H., 2006. Association of the Y402H polymorphism in complement factor H gene and neovascular age-related macular degeneration in Chinese patients. Invest. Ophthalmol. Vis. Sci. 47, 3242–3246.
Lee, A.Y., Kulkarni, M., Fang, A.M., Edelstein, S., Osborn, M.P., Brantley, M.A., 2010. The effect of genetic variants in SERPING1 on the risk of neovascular age-related macular degeneration. Br. J. Ophthalmol. 94, 915–917.
Lee, A.Y., Raya, A.K., Kymes, S.M., Shiels, A., Brantley Jr., M.A., 2009. Pharmacogenetics of complement factor H (Y402H) and treatment of exudative age-related macular degeneration with ranibizumab. Br. J. Ophthalmol. 93, 610–613.
Leveziel, N., Zerbib, J., Richard, F., Querques, G., Morineau, G., Fremeaux-Bacchi, V., Coscas, G., Soubrane, G., Benlian, P., Souied, E.H., 2008. Genotype-phenotype correlations for exudative age-related macular degeneration associated with homozygous HTRA1 and CFH genotypes. Invest. Ophthalmol. Vis. Sci. 49, 3090–3094.
Li, M., Atmaca-Sonmez, P., Othman, M., Branham, K.E., Khanna, R., Wade, M.S., Li, Y., Liang, L., Zareparsi, S., Swaroop, A., Abecasis, G.R., 2006. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. Nat. Genet. 38, 1049–1054.
Lin, J.M., Tsai, Y.Y., Wan, L., Lin, H.J., Tsai, Y., Lee, C.C., Tsai, C.H., Tsai, F.J., Tseng, S.H., 2008. Complement factor H variant increases the risk for early age-related macular degeneration. Retina 28, 1416–1420.
Liu, J., Colville, D., Wang, Y.Y., Baird, P.N., Guymer, R.H., Savige, J., 2009. The dot-and-fleck retinopathy of X linked Alport syndrome is independent of complement factor H (CFH) gene polymorphisms. Br. J. Ophthalmol. 93, 379–382.
Liu, X., Zhao, P., Tang, S., Lu, F., Hu, J., Lei, C., Yang, X., Lin, Y., Ma, S., Yang, J., Zhang, D., Shi, Y., Li, T., Chen, Y., Fan, Y., Yang, Z., 2010. Association study of complement factor H, C2 CFB, and C3 and age-related macular degeneration in a Han Chinese population. Retina.
Lommatzsch, A., Hermans, P., Muller, K.D., Bornfeld, N., Bird, A.C., Pauleikhoff, D., 2008. Are low inflammatory reactions involved in exudative age-related macular degeneration? Morphological and immunhistochemical analysis of AMD associated with basal deposits. Graefes Arch. Clin. Exp. Ophthalmol. 246, 803–810.
Lommatzsch, A., Hermans, P., Weber, B., Pauleikhoff, D., 2007. Complement factor H variant Y402H and basal laminar deposits in exudative age-related macular degeneration. Graefes Arch. Clin. Exp. Ophthalmol. 245, 1713–1716.
Lopez, P.F., Sippy, B.D., Lambert, H.M., Thach, A.B., Hinton, D.R., 1996. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest. Ophthalmol. Vis. Sci. 37, 855–868.
Loyet, K.M., Good, J., Sturgeon, L., Davancaze, T., Wong, M., Morimoto, A., van Lookeren Campagne, M., DeForge, L., Haughney, P., Damico, L.A., 2010. Anti-Factor D Fab specifically inhibits the alternative complement pathway: in vitro characterization and in vivo effects following administration to cynomolgus monkeys. In: ARVO Meeting Abstracts 51, p. 2980.

Lu, F., Zhao, P., Fan, Y., Tang, S., Hu, J., Liu, X., Yang, X., Chen, Y., Li, T., Lei, C., Yang, J., Lin, Y., Ma, S., Li, C., Shi, Y., Yang, Z., 2010. An association study of SERPING1 gene and age-related macular degeneration in a Han Chinese population. Mol. Vis. 16, 1–6.

Lundh von, L.P., Kam, J.H., Bainbridge, J., Catchpole, I., Gough, G., Coffey, P., Jeffery, G., 2009. Complement factor h is critical in the maintenance of retinal perfusion. Am. J. Pathol. 175, 412–421.

Lyzogubov, V.V., Tytarenko, R.G., Jha, P., Liu, J., Bora, N.S., Bora, P.S., 2010. Role of ocular complement factor H in a murine model of choroidal neovascularization. Am. J. Pathol. 177, 1870–1880.

Machalinska, A., Dziedziejko, V., Mozolewska-Piotrowska, K., Karczewicz, D., Wiszniewska, B., Machalinski, B., 2009. Elevated plasma levels of C3a complement compound in the exudative form of age-related macular degeneration. Ophthalmic Res. 42, 54–59.

Magnusson, K.P., Duan, S., Sigurdsson, H., Petursson, H., Yang, Z., Zhao, Y., Bernstein, P.S., Ge, J., Jonasson, F., Stefansson, E., Helgadottir, G., Zabriskie, N.A., Jonsson, T., Bjornsson, A., Thorlacius, T., Jonsson, P.V., Thorleifsson, G., Kong, A., Stefansson, H., Zhang, K., Stefansson, K., Gulcher, J.R., 2006. CFH Y402H confers similar risk of soft drusen and both forms of advanced AMD. PLoS Med. 3, e5.

Maller, J., George, S., Purcell, S., Fagerness, J., Altshuler, D., Daly, M.J., Seddon, J.M., 2006. Common variation in three genes, including a noncoding variant in CFH, strongly influences risk of age-related macular degeneration. Nat. Genet. 38, 1055–1059.

Maller, J.B., Fagerness, J.A., Reynolds, R.C., Neale, B.M., Daly, M.J., Seddon, J.M., 2007. Variation in complement factor 3 is associated with risk of age-related macular degeneration. Nat. Genet. 39, 1200–1201.

Mandal, M.N., Ayyagari, R., 2006. Complement factor H: spatial and temporal expression and localization in the eye. Invest. Ophthalmol. Vis. Sci. 47, 4091–4097.

Mann, S.S., Rutishauser-Arnold, Y., Peto, T., Jenkins, S.A., Leung, I., Xing, W., Bird, A.C., Bunce, C., Webster, A.R., 2011. The symmetry of phenotype between eyes of patients with early and late bilateral age-related macular degeneration (AMD). Graefes Arch. Clin. Exp. Ophthalmol. 249, 209–214.

Mayer, G., Jenne, A., 2004. Aptamers in research and drug development. Biodrugs 18, 351–359.

McKay, G.J., Dasari, S., Patterson, C.C., Chakravarthy, U., Silvestri, G., 2010. Complement component 3: an assessment of association with AMD and analysis of gene–gene and gene–environment interactions in a Northern Irish cohort. Mol. Vis. 16, 194–199.

Meri, S., Morgan, B.P., Davies, A., Daniels, R.H., Olavesen, M.G., Waldmann, H., Lachmann, P.J., 1990. Human protectin (CD59), an 18,000–20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology 71, 1–9.

Mestas, J., Hughes, C.C., 2004. Of mice and not men: differences between mouse and human immunology. J. Immunol. 172, 2731–2738.

Mold, C., Gewurz, H., Du Clos, T.W., 1999. Regulation of complement activation by C-reactive protein. Immunopharmacology 42, 23–30.

Montes, T., Tortajada, A., Morgan, B.P., Rodriguez de, C.S., Harris, C.L., 2009. Functional basis of protection against age-related macular degeneration conferred by a common polymorphism in complement factor B. Proc. Natl. Acad. Sci. U.S.A. 106, 4366–4371.

Mori, K., Gehlbach, P.L., Kabasawa, S., Kawasaki, I., Oosaki, M., Iizuka, H., Katayama, S., Awata, T., Yoneya, S., 2007. Coding and noncoding variants in the CFH gene and cigarette smoking influence the risk of age-related macular degeneration in a Japanese population. Invest. Ophthalmol. Vis. Sci. 48, 5315–5319.

Mullins, R.F., Aptsiauri, N., Hageman, G.S., 2001. Structure and composition of drusen associated with glomerulonephritis: implications for the role of complement activation in drusen biogenesis. Eye (Lond.) 15, 390–395.

Mullins, R.F., Faidley, E.A., Daggett, H.T., Jomary, C., Lotery, A.J., Stone, E.M., 2009. Localization of complement 1 inhibitor (C1INH/SERPING1) in human eyes with age-related macular degeneration. Exp. Eye Res. 89, 767–773.

Mullins, R.F., Russell, S.R., Anderson, D.H., Hageman, G.S., 2000. Drusen associated with aging and age-related macular degeneration contain proteins common to extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J. 14, 835–846.

Munch, I.C., Ek, J., Kessel, L., Sander, B., Almind, G.J., Brondum-Nielsen, K., Linneberg, A., Larsen, M., 2010. Small, hard macular drusen and peripheral drusen: associations with AMD genotypes in the Inter99 Eye Study. Invest. Ophthalmol. Vis. Sci. 51, 2317–2321.

Nakata, I., Yamashiro, K., Yamada, R., Gotoh, N., Nakanishi, H., Hayashi, H., Tsujikawa, A., Otani, A., Saito, M., Iida, T., Oishi, A., Matsuo, K., Tajima, K., Matsuda, F., Yoshimura, N., 2011. Association between the SERPING1 gene and age-related macular degeneration and polypoidal choroidal vasculopathy in Japanese. PLoS One 6, e19108.

Narendra, U., Pauer, G.J., Hagstrom, S.A., 2009. Genetic analysis of complement factor H related 5 CFHR5, in patients with age-related macular degeneration. Mol. Vis. 15, 731–736.

Neale, B.M., Fagerness, J., Reynolds, R., Sobrin, L., Parker, M., Raychaudhuri, S., Tan, P.L., Oh, E.C., Merriam, J.E., Souied, E., Bernstein, P.S., Li, B., Frederick, J.M., Zhang, K., Brantley Jr., M.A., Lee, A.Y., Zack, D.J., Campochiaro, B., Campochiaro, P., Ripke, S., Smith, R.T., Barile, G.R., Katsanis, N., Allikmets, R., Daly, M.J., Seddon, J.M., 2010. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). Proc. Natl. Acad. Sci. U.S.A. 107, 7395–7400.

Ng, T.K., Chen, L.J., Liu, D.T., Tam, P.O., Chan, W.M., Liu, K., Hu, Y.J., Chong, K.K., Lau, C.S., Chiang, S.W., Lam, D.S., Pang, C.P., 2008. Multiple gene polymorphisms in the complement factor h gene are associated with exudative age-related macular degeneration in chinese. Invest. Ophthalmol. Vis. Sci. 49, 3312–3317.

Nilsson, S.C., Sim, R.B., Lea, S.M., Fremeaux-Bacchi, V., Blom, A.M., 2011. Complement factor I in health and disease. Mol. Immunol..

Nonyane, B.A., Nitsch, D., Whittaker, J.C., Sofat, R., Smeeth, L., Chakravarthy, U., Fletcher, A.E., 2010. An ecological correlation study of late age-related macular degeneration and the complement factor H Y402H polymorphism. Invest. Ophthalmol. Vis. Sci. 51, 2393–2402.

Novartis Pharmaceuticals, 2011. Intravitreal LFG316 in patients with advanced age-related macular degeneration. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda, MD, 2000-[cited 2011 April 01] Available from: http://clinicaltrials.gov/ct2/show/NCT01255462„ NLM Identifier: NCT01255462.

Nozaki, M., Raisler, B.J., Sakurai, E., Sarma, J.V., Barnum, S.R., Lambris, J.D., Chen, Y., Zhang, K., Ambati, B.K., Baffi, J.Z., Ambati, J., 2006. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc. Natl. Acad. Sci. U.S.A. 103, 2328–2333.

Okamoto, H., Umeda, S., Obazawa, M., Minami, M., Noda, T., Mizota, A., Honda, M., Tanaka, M., Koyama, R., Takagi, I., Sakamoto, Y., Saito, Y., Miyake, Y., Iwata, T., 2006. Complement factor H polymorphisms in Japanese population with age-related macular degeneration. Mol. Vis. 12, 156–158.

Ophthotech Corporation, 2011a. ARC1905 (ANTI-C5 APTAMER) given either in combination therapy with Lucentis® 0.5 mg/eye in subjects with neovascular age-related macular degeneration. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda, MD, 2000-[cited 2011 April 01]. Available from: http://clinicaltrials.gov/ct2/show/NCT00709527„ NLM Identifier: NCT00709527.

Ophthotech Corporation, 2011b. Study of ARC1905 (Anti-C5 Aptamer) in subjects with dry age-related macular degeneration. In: ClinicalTrials.gov [Internet]. National Library of Medicine (US), Bethesda, MD, 2000-[cited 2011 April 01]. Available from: http://clinicaltrials.gov/ct2/show/NCT00950638„ NLM Identifier: NCT00950638.

Pangburn, M.K., 2000. Host recognition and target differentiation by factor H, a regulator of the alternative pathway of complement. Immunopharmacology 49, 149–157.

Park, K.H., Fridley, B.L., Ryu, E., Tosakulwong, N., Edwards, A.O., 2009a. Complement component 3 (C3) haplotypes and risk of advanced age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 50, 3386–3393.

Park, K.H., Ryu, E., Tosakulwong, N., Wu, Y., Edwards, A.O., 2009b. Common variation in the SERPING1 gene is not associated with age-related macular degeneration in two independent groups of subjects. Mol. Vis. 15, 200–207.

Parker, C., 2009. Eculizumab for paroxysmal nocturnal haemoglobinuria. Lancet 373, 759–767.

Pei, X.T., Li, X.X., Bao, Y.Z., Yu, W.Z., Yan, Z., Qi, H.J., Qian, T., Xiao, H.X., 2009. Association of c3 gene polymorphisms with neovascular age-related macular degeneration in a chinese population. Curr. Eye Res. 34, 615–622.

Perkins, S.J., Nan, R., Okemefuna, A.I., Li, K., Khan, S., Miller, A., 2010. Multiple interactions of complement Factor H with its ligands in solution: a progress report. Adv. Exp. Med. Biol. 703, 25–47.

Poznansky, M.C., Clissold, P.M., Lachmann, P.J., 1989. The difference between human C3F and C3S results from a single amino acid change from an asparagine to an aspartate residue at position 1216 on the alpha-chain of the complement component C3. J. Immunol. 143, 1254–1258.

Rakoczy, E.P., Yu, M.J., Nusinowitz, S., Chang, B., Heckenlively, J.R., 2006. Mouse models of age-related macular degeneration. Exp. Eye Res. 82, 741–752.

Ramo, K., Cashman, S.M., Kumar-Singh, R., 2008. Evaluation of adenovirus-delivered human CD59 as a potential therapy for AMD in a model of human membrane attack complex formation on murine RPE. Invest. Ophthalmol. Vis. Sci. 49, 4126–4136.

Raychaudhuri, S., Ripke, S., Li, M., Neale, B.M., Fagerness, J., Reynolds, R., Sobrin, L., Swaroop, A., Abecasis, G., Seddon, J.M., Daly, M.J., 2010. Associations of CFHR1-CFHR3 deletion and a CFH SNP to age-related macular degeneration are not independent. Nat. Genet. 42, 553–555.

Resnikoff, S., Pascolini, D., Etya'ale, D., Kocur, I., Pararajasegaram, R., Pokharel, G.P., Mariotti, S.P., 2004. Global data on visual impairment in the year 2002. Bull. World Health Organ. 82, 844–851.

Reynolds, R., Hartnett, M.E., Atkinson, J.P., Giclas, P.C., Rosner, B., Seddon, J.M., 2009. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. Invest. Ophthalmol. Vis. Sci. 50, 5818–5827.

Ripoche, J., Day, A.J., Harris, T.J., Sim, R.B., 1988. The complete amino acid sequence of human complement factor H. Biochem. J. 249, 593–602.

Rivera, A., Fisher, S.A., Fritsche, L.G., Keilhauer, C.N., Lichtner, P., Meitinger, T., Weber, B.H., 2005. Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Hum. Mol. Genet. 14, 3227–3236.

Robman, L., Baird, P.N., Dimitrov, P.N., Richardson, A.J., Guymer, R.H., 2010. C-reactive protein levels and complement factor H polymorphism interaction in age-related macular degeneration and its progression. Ophthalmology.

Robman, L., Mahdi, O., McCarty, C., Dimitrov, P., Tikellis, G., McNeil, J., Byrne, G., Taylor, H., Guymer, R., 2005. Exposure to Chlamydia pneumoniae infection and progression of age-related macular degeneration. Am. J. Epidemiol. 161, 1013–1019.

Robman, L., Mahdi, O.S., Wang, J.J., Burlutsky, G., Mitchell, P., Byrne, G., Guymer, R., Taylor, H., 2007. Exposure to Chlamydia pneumoniae infection and age-related

macular degeneration: the Blue Mountains Eye Study. Invest. Ophthalmol. Vis. Sci. 48, 4007–4011.
Rohrer, B., Guo, Y., Kunchithapautham, K., Gilkeson, G.S., 2007. Eliminating complement factor D reduces photoreceptor susceptibility to light-induced damage. Invest. Ophthalmol. Vis. Sci. 48, 5282–5289.
Rohrer, B., Long, Q., Coughlin, B., Renner, B., Huang, Y., Kunchithapautham, K., Ferreira, V.P., Pangburn, M.K., Gilkeson, G.S., Thurman, J.M., Tomlinson, S., Holers, V.M., 2010. A targeted inhibitor of the complement alternative pathway reduces RPE injury and angiogenesis in models of age-related macular degeneration. Adv. Exp. Med. Biol. 703, 137–149.
Rohrer, B., Long, Q., Coughlin, B., Wilson, R.B., Huang, Y., Qiao, F., Tang, P.H., Kunchithapautham, K., Gilkeson, G.S., Tomlinson, S., 2009. A targeted inhibitor of the alternative complement pathway reduces angiogenesis in a mouse model of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 50, 3056–3064.
Rosenfeld, P.J., Brown, D.M., Heier, J.S., Boyer, D.S., Kaiser, P.K., Chung, C.Y., Kim, R.Y., 2006. Ranibizumab for neovascular age-related macular degeneration. N. Engl. J. Med. 355, 1419–1431.
Saint-Geniez, M., Kurihara, T., Sekiyama, E., Maldonado, A.E., D'Amore, P.A., 2009. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. Proc. Natl. Acad. Sci. U.S.A. 106, 18751–18756.
Schaumberg, D.A., Christen, W.G., Kozlowski, P., Miller, D.T., Ridker, P.M., Zee, R.Y., 2006. A prospective assessment of the Y402H variant in complement factor H, genetic variants in C-reactive protein, and risk of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 47, 2336–2340.
Schaumberg, D.A., Hankinson, S.E., Guo, Q., Rimm, E., Hunter, D.J., 2007. A prospective study of 2 major age-related macular degeneration susceptibility alleles and interactions with modifiable risk factors. Arch. Ophthalmol. 125, 55–62.
Schmid-Kubista, K.E., Tosakulwong, N., Wu, Y., Ryu, E., Hecker, L.A., Baratz, K.H., Brown, W.L., Edwards, A.O., 2009. Contribution of copy number variation in the regulation of complement activation locus to development of age-related macular degeneration. Invest. Ophthalmol. Vis. Sci. 50, 5070–5079.
Schmidt, C.Q., Herbert, A.P., Hocking, H.G., Uhrin, D., Barlow, P.N., 2008. Translational mini-review series on complement factor H: structural and functional correlations for factor H. Clin. Exp. Immunol. 151, 14–24.
Scholl, H.P., Charbel, I.P., Walier, M., Janzer, S., Pollok-Kopp, B., Borncke, F., Fritsche, L.G., Chong, N.V., Fimmers, R., Wienker, T., Holz, F.G., Weber, B.H., Oppermann, M., 2008. Systemic complement activation in age-related macular degeneration. PLoS One 3, e2593.
Scott, W.K., Schmidt, S., Hauser, M.A., Gallins, P., Schnetz-Boutaud, N., Spencer, K.L., Gilbert, J.R., Agarwal, A., Postel, E.A., Haines, J.L., Pericak-Vance, M.A., 2007. Independent effects of complement factor H Y402H polymorphism and cigarette smoking on risk of age-related macular degeneration. Ophthalmology 114, 1151–1156.
Seddon, J.M., Francis, P.J., George, S., Schultz, D.W., Rosner, B., Klein, M.L., 2007. Association of CFH Y402H and LOC387715 A69S with progression of age-related macular degeneration. JAMA 297, 1793–1800.
Seddon, J.M., Reynolds, R., Maller, J., Fagerness, J.A., Daly, M.J., Rosner, B., 2009. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. Invest. Ophthalmol. Vis. Sci. 50, 2044–2053.
Seddon, J.M., Reynolds, R., Rosner, B., 2010. Associations of smoking, body mass index, dietary lutein, and the LIPC gene variant rs10468017 with advanced age-related macular degeneration. Mol. Vis. 16, 2412–2424.
Seitsonen, S., Jarvela, I., Meri, S., Tommila, P., Ranta, P., Immonen, I., 2008a. Complement factor H Y402H polymorphism and characteristics of exudative age-related macular degeneration lesions. Acta Ophthalmologica 86, 390–394.
Seitsonen, S.P., Jarvela, I.E., Meri, S., Tommila, P.V., Ranta, P.H., Immonen, I.J., 2007. The effect of complement factor H Y402H polymorphism on the outcome of photodynamic therapy in age-related macular degeneration. Eur. J. Ophthalmol. 17, 943–949.
Seitsonen, S.P., Onkamo, P., Peng, G., Xiong, M., Tommila, P.V., Ranta, P.H., Holopainen, J.M., Moilanen, J.A., Palosaari, T., Kaarniranta, K., Meri, S., Immonen, I.R., Jarvela, I.E., 2008b. Multifactor effects and evidence of potential interaction between complement factor H Y402H and LOC387715 A69S in age-related macular degeneration. PLoS One 3, e3833.
Seland, J.H., Vingerling, J.R., Augood, C.A., Bentham, G., Chakravarthy, U., Dejong, P.T., Rahu, M., Soubrane, G., Tomazzoli, L., Topouzis, F., Fletcher, A.E., 2009. Visual impairment and quality of life in the older European population, the EUREYE study. Acta Ophthalmologica.
Sepp, T., Khan, J.C., Thurlby, D.A., Shahid, H., Clayton, D.G., Moore, A.T., Bird, A.C., Yates, J.R., 2006. Complement factor H variant Y402H is a major risk determinant for geographic atrophy and choroidal neovascularization in smokers and nonsmokers. Invest. Ophthalmol. Vis. Sci. 47, 536–540.
Shen, D., Tuo, J., Patel, M., Herzlich, A.A., Ding, X., Chew, E.Y., Chan, C.C., 2009. Chlamydia pneumoniae infection, complement factor H variants and age-related macular degeneration. Br. J. Ophthalmol. 93, 405–408.
Silverman, G.A., Bird, P.I., Carrell, R.W., Church, F.C., Coughlin, P.B., Gettins, P.G., Irving, J.A., Lomas, D.A., Luke, C.J., Moyer, R.W., Pemberton, P.A., Remold-O'Donnell, E., Salvesen, G.S., Travis, J., Whisstock, J.C., 2001. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J. Biol. Chem. 276, 33293–33296.
Sivaprasad, S., Adewoyin, T., Bailey, T.A., Dandekar, S.S., Jenkins, S., Webster, A.R., Chong, N.V., 2007. Estimation of systemic complement C3 activity in age-related macular degeneration. Arch. Ophthalmol. 125, 515–519.
Sjoberg, A.P., Trouw, L.A., Clark, S.J., Sjolander, J., Heinegard, D., Sim, R.B., Day, A.J., Blom, A.M., 2007. The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. J. Biol. Chem. 282, 10894–10900.
Skeie, J.M., Fingert, J.H., Russell, S.R., Stone, E.M., Mullins, R.F., 2010. Complement component C5a activates ICAM-1 expression on human choroidal endothelial cells. Invest. Ophthalmol. Vis. Sci. 51, 5336–5342.
Skeie, J.M., Mullins, R.F., 2009. Macrophages in neovascular age-related macular degeneration: friends or foes? Eye (Lond.) 23, 747–755.
Skerka, C., Lauer, N., Weinberger, A.A., Keilhauer, C.N., Suhnel, J., Smith, R., Schlotzer-Schrehardt, U., Fritsche, L., Heinen, S., Hartmann, A., Weber, B.H., Zipfel, P.F., 2007. Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol. Immunol. 44, 3398–3406.
Smith, W., Mitchell, P., Leeder, S.R., 1996. Smoking and age-related maculopathy. The Blue Mountains Eye Study. Arch. Ophthalmol. 114, 1518–1523.
Spencer, K.L., Hauser, M.A., Olson, L.M., Schmidt, S., Scott, W.K., Gallins, P., Agarwal, A., Postel, E.A., Pericak-Vance, M.A., Haines, J.L., 2008a. Deletion of CFHR3 and CFHR1 genes in age-related macular degeneration. Hum. Mol. Genet. 17, 971–977.
Spencer, K.L., Olson, L.M., Anderson, B.M., Schnetz-Boutaud, N., Scott, W.K., Gallins, P., Agarwal, A., Postel, E.A., Pericak-Vance, M.A., Haines, J.L., 2008b. C3 R102G polymorphism increases risk of age-related macular degeneration. Hum. Mol. Genet. 17, 1821–1824.
TAP study group, 1999. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials—TAP report Treatment of age-related macular degeneration with photodynamic therapy (TAP) Study Group. Arch. Ophthalmol. 117, 1329–1345.
Tedeschi-Blok, N., Buckley, J., Varma, R., Triche, T.J., Hinton, D.R., 2007. Population-based study of early age-related macular degeneration: role of the complement factor H Y402H polymorphism in bilateral but not unilateral disease. Ophthalmology 114, 99–103.
Thakkinstian, A., Han, P., McEvoy, M., Smith, W., Hoh, J., Magnusson, K., Zhang, K., Attia, J., 2006. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. Hum. Mol. Genet. 15, 2784–2790.
Thakkinstian, A., McKay, G.J., McEvoy, M., Chakravarthy, U., Chakrabarti, S., Silvestri, G., Kaur, I., Li, X., Attia, J., 2011. Systematic review and meta-analysis of the association between complement component 3 and age-related macular degeneration: a huge review and meta-analysis. Am. J. Epidemiol. 173, 1365–1379.
Thompson, C.L., Jun, G., Klein, B.E., Klein, R., Capriotti, J., Lee, K.E., Iyengar, S.K., 2007. Genetics of pigment changes and geographic atrophy. Invest. Ophthalmol. Vis. Sci. 48, 3005–3013.
Thurman, J.M., Renner, B., Kunchithapautham, K., Ferreira, V.P., Pangburn, M.K., Ablonczy, Z., Tomlinson, S., Holers, V.M., Rohrer, B., 2009. Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. J. Biol. Chem. 284, 16939–16947.
Tobe, T., Ortega, S., Luna, J.D., Ozaki, H., Okamoto, N., Derevjanik, N.L., Vinores, S.A., Basilico, C., Campochiaro, P.A., 1998. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. Am. J. Pathol. 153, 1641–1646.
Tortajada, A., Montes, T., Martinez-Barricarte, R., Morgan, B.P., Harris, C.L., de Cordoba, S.R., 2009. The disease-protective complement factor H allotypic variant Ile62 shows increased binding affinity for C3b and enhanced cofactor activity. Hum. Mol. Genet. 18, 3452–3461.
Tuo, J., Ross, R.J., Reed, G.F., Yan, Q., Wang, J.J., Bojanowski, C.M., Chew, E.Y., Feng, X., Olsen, T.W., Ferris III, F.L., Mitchell, P., Chan, C.C., 2008. The HtrA1 promoter polymorphism, smoking, and age-related macular degeneration in multiple case-control samples. Ophthalmology 115, 1891–1898.
Uka, J., Tamura, H., Kobayashi, T., Yamane, K., Kawakami, H., Minamoto, A., Mishima, H.K., 2006. No association of complement factor H gene polymorphism and age-related macular degeneration in the Japanese population. Retina 26, 985–987.
University of Miami, 2011. Complement inhibition with eculizumab for the treatment of non-exudative macular degeneration (AMD) (complete). In: ClinicalTrials.gov [Internet]. Bethesda, MD, National Library of Medicine (US), 2000-[cited 2011 April 01]. Available from: http://clinicaltrials.gov/ct2/show/NCT00935883., NLM Identifier: NCT00935883.
van Lookeren Campagne, M., Katschke Jr., K.J., Wu, P., Wiesmann, C., Kelley, R.F., Loyet, K., DeForge, L., Hass, P.E., Haughney, P., Damico, L.A., 2010. Targeting the alternative pathway of complement in AMD with a factor D selective antibody. In: ARVO Meeting Abstracts 51, p. 4511.
van Soest, S.S., de Wit, G.M., Essing, A.H., ten Brink, J.B., Kamphuis, W., de Jong, P.T., Bergen, A.A., 2007. Comparison of human retinal pigment epithelium gene expression in macula and periphery highlights potential topographic differences in Bruch's membrane. Mol. Vis. 13, 1608–1617.
Vingerling, J.R., Hofman, A., Grobbee, D.E., de Jong, P.T., 1996. Age-related macular degeneration and smoking. The Rotterdam Study. Arch. Ophthalmol. 114, 1193–1196.
Volanakis, J.E., Narayana, S.V., 1996. Complement factor D, a novel serine protease. Protein Sci. 5, 553–564.
Walport, M.J., 2001. Complement. First of two parts. N. Engl. J. Med. 344, 1058–1066.
Wang, A.L., Lukas, T.J., Yuan, M., Du, N., Handa, J.T., Neufeld, A.H., 2009a. Changes in retinal pigment epithelium related to cigarette smoke: possible relevance to smoking as a risk factor for age-related macular degeneration. PLoS One 4, e5304.

Wang, J., Ohno-Matsui, K., Yoshida, T., Kojima, A., Shimada, N., Nakahama, K., Safranova, O., Iwata, N., Saido, T.C., Mochizuki, M., Morita, I., 2008. Altered function of factor I caused by amyloid beta: implication for pathogenesis of age-related macular degeneration from Drusen. J. Immunol. 181, 712–720.

Wang, J., Ohno-Matsui, K., Yoshida, T., Shimada, N., Ichinose, S., Sato, T., Mochizuki, M., Morita, I., 2009b. Amyloid-beta up-regulates complement factor B in retinal pigment epithelial cells through cytokines released from recruited macrophages/microglia: another mechanism of complement activation in age-related macular degeneration. J. Cell. Physiol. 220, 119–128.

Wang, J.J., Rochtchina, E., Smith, W., Klein, R., Klein, B.E., Joshi, T., Sivakumaran, T.A., Iyengar, S., Mitchell, P., 2009c. Combined effects of complement factor H genotypes, fish consumption, and inflammatory markers on long-term risk for age-related macular degeneration in a cohort. Am. J. Epidemiol. 169, 633–641.

Weger, M., Renner, W., Steinbrugger, I., Kofer, K., Wedrich, A., Groselj-Strele, A., El-Shabrawi, Y., Schmut, O., Haas, A., 2007. Association of the HTRA1-625G>A promoter gene polymorphism with exudative age-related macular degeneration in a Central European population. Mol. Vis. 13, 1274–1279.

Welch, T.R., Beischel, L., Kleesattel, A., 1990. Functional consequences of the genetic polymorphism of the third component of complement. J. Pediatr. 116, S92–S97.

Yates, J.R., Sepp, T., Matharu, B.K., Khan, J.C., Thurlby, D.A., Shahid, H., Clayton, D.G., Hayward, C., Morgan, J., Wright, A.F., Armbrecht, A.M., Dhillon, B., Deary, I.J., Redmond, E., Bird, A.C., Moore, A.T., 2007. Complement C3 variant and the risk of age-related macular degeneration. N. Engl. J. Med. 357, 553–561.

Yu, J., Wiita, P., Kawaguchi, R., Honda, J., Jorgensen, A., Zhang, K., Fischetti, V.A., Sun, H., 2007. Biochemical analysis of a common human polymorphism associated with age-related macular degeneration. Biochemistry 46, 8451–8461.

Zeng, J., Chen, Y., Tong, Z., Zhou, X., Zhao, C., Wang, K., Hughes, G., Kasuga, D., Bedell, M., Lee, C., Ferreyra, H., Kozak, I., Haw, W., Guan, J., Shaw, R., Stevenson, W., Weishaar, P.D., Nelson, M.H., Tang, L., Zhang, K., 2010. Lack of association of CFD polymorphisms with advanced age-related macular degeneration. Mol. Vis. 16, 2273–2278.

Zhang, H., Morrison, M.A., DeWan, A., Adams, S., Andreoli, M., Huynh, N., Regan, M., Brown, A., Miller, J.W., Kim, I.K., Hoh, J., Deangelis, M.M., 2008. The NEI/NCBI dbGAP database: genotypes and haplotypes that may specifically predispose to risk of neovascular age-related macular degeneration. BMC Med. Genet. 9, 51.

Zhang, X., Kimura, Y., Fang, C., Zhou, L., Sfyroera, G., Lambris, J.D., Wetsel, R.A., Miwa, T., Song, W.C., 2007. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. Blood 110, 228–236.

Zhou, W., Marsh, J.E., Sacks, S.H., 2011. Intrarenal synthesis of complement., http://www.nature.com/ki/journal/v59/n4/fig_tab/4492147f1.html.

Ziccardi, R.J., Cooper, N.R., 1976. Activation of C1r by proteolytic cleavage. J. Immunol. 116, 504–509.

Zipfel, P.F., Edey, M., Heinen, S., Jozsi, M., Richter, H., Misselwitz, J., Hoppe, B., Routledge, D., Strain, L., Hughes, A.E., Goodship, J.A., Licht, C., Goodship, T.H., Skerka, C., 2007. Deletion of complement factor H-related genes CFHR1 and CFHR3 is associated with atypical hemolytic uremic syndrome. PLoS Genet. 3, e41.

Zipfel, P.F., Skerka, C., Hellwage, J., Jokiranta, S.T., Meri, S., Brade, V., Kraiczy, P., Noris, M., Remuzzi, G., 2002. Factor H family proteins: on complement, microbes and human diseases. Biochem. Soc. Trans. 30, 971–978.

5.3 AMD and oxidation – published review article

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Oxidation and age-related macular degeneration: insights from molecular biology

Sam Khandhadia[1] and Andrew Lotery[1,2,*]

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world. It is a multifactorial disease, and current therapy predominantly limits damage only when it has already occurred. The macula is a source of high metabolic activity, and is therefore exposed to correspondingly high levels of reactive oxygen species (ROS). With age, the balance between production of ROS and local antioxidant levels is shifted, and damage ensues. Systemic ROS and antioxidant levels in AMD reflect these local processes. Genetic studies investigating mutations in antioxidant genes in AMD are inconclusive and further studies are indicated, especially to determine the role of mitochondria. Oral antioxidant supplements could be beneficial, and diet modification may help. Future treatments might either increase antioxidant capacity or reduce the production of ROS, using methods such as genetic manipulation. This article reviews the role of oxidative stress in AMD and the potential therapies that might have a role in preventing the blindness resulting from this disease.

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world, causing 50% of blindness in Europe, the USA and Australia (Ref. 1). It is a disease that is prevalent with increasing age, and affects up to 64% of the population over the age of 80 years (Refs 2, 3). Most patients with AMD have the 'dry' form of AMD, which causes gradual deterioration of central vision as a result of atrophy of the retina and retinal pigment epithelium (RPE), and is at present untreatable. However, a significant fraction of patients develop 'wet' AMD, which causes sudden visual loss due to neovascularisation. This results in structural damage of the macula and consequent loss of central vision. Wet AMD can be treated with intraocular injections of monoclonal antibody fragments that inhibit vascular endothelial growth factor (VEGF), which can lead to an improvement in vision in up to 30% of patients (Refs 4, 5). Occasionally, laser treatment or surgery may help. The dry

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form is more prevalent, although the relative proportion of each form of AMD is variable and depends on how the disease is classified and which population is studied. However, regardless of subtype, vision improves in few patients; there is no restorative treatment for dry AMD, or any form of adequate prevention for either form of AMD.

Oxidative damage is one of the key disease-inducing processes in the human body. Although AMD is recognised as a multifactorial disease, oxidative stress may have a pivotal role in its development. In this article, we provide a brief description of AMD and the oxidative processes in the body, followed by evidence of oxidative damage in relation to the pathogenesis of AMD, and how these may be translated into clinical practice.

Anatomical changes to the retina in AMD

The structure of the normal retina is illustrated in Figure 1a. With progressive age, the retina develops characteristic anatomical features. Localised deposits form under the retina, usually between the RPE and Bruch's membrane. These are called 'drusen' and usually appear either as small, discrete yellow dots, called 'hard drusen', or larger more confluent and less discrete yellow spots, called 'soft drusen'. Drusen can also collect more internally to the RPE and within the photoreceptor cell layer. These deposits are called 'reticular drusen' and appear as an interlacing network of small yellow round or oval spots (Ref. 6). Both soft and reticular drusen are associated with increased progression of AMD (Refs 7, 8). Histologically, these are composed of a multitude of proteins (e.g. complement proteins, immunoglobulins, acute-phase proteins, amyloid-β) and lipids (e.g. phospholipids, cholesterol, apolipoproteins) (Ref. 9).

AMD is also associated with alterations in the architecture of the retina and choroid. The cells of the RPE, retina and choroid can all diminish in size and number, eventually becoming atrophic. RPE cells can also become hypertrophic, which is possibly a reactive change. Bruch's membrane becomes thicker and more impermeable. The photoreceptor cells at the macula are inadequately supported because of these changes and become atrophic, possibly leading to long-term impairment of central vision. In addition, as a result of neovascularisation (see below), photoreceptor cells can lose their normal orientation and become disorganised. This results in visual symptoms such as distortion and metamorphopsia (change in image size).

Neovascularisation, a late sign of AMD, originates either in the choroid [choroidal neovascularisation (CNV)] or within the retina [retinal angiomatous proliferation (RAP)]. In CNV, endothelial cells begin to proliferate within the choroid, and a defect in Bruch's membrane allows these blood vessels to pass into the sub-RPE or subretinal space. In RAP, these endothelial vessels proliferate within the retina itself. In both cases, these vessels are fragile and tend to leak or bleed easily. Oedema tends to form either in the subretinal space or within the retinal tissue (intraretinal fluid). Haemorrhage that occurs in the sub-RPE or subretinal space can break through the retina to sit in the potential space between the retina and vitreous (subhyaloid space), or can break through into the vitreous. New vessels tend to proliferate for several months, eventually undergoing fibrosis, which can potentially lead to irreversible vision loss. Figure 1b and c depict the typical changes, as seen in cross-section, in dry and wet AMD.

Clinical features of AMD

Early AMD can be asymptomatic, despite the presence of drusen and changes in the RPE. Patients with dry AMD tend to exhibit a gradual deterioration in the ability to read small print, which sometimes progresses to distortion and central scotomas (blind spots). These changes are very gradual and can develop and progress over many years. Drusen may increase in size and number and this is accompanied by hypo- or hyperpigmentation that results from changes in the RPE. As the choriocapillaris layer becomes atrophic, the larger choroidal vessels become visible through areas of RPE atrophy. Eventually, the neurosensory retina becomes atrophic. Large areas of RPE and retinal atrophy are termed 'geographic atrophy' (GA) (Refs 7, 8). Widespread, extensive GA of the neurosensory retina and RPE at the macula, associated with severe central visual impairment, represents end-stage dry AMD.

In wet AMD, the development of neovascularisation typically presents suddenly, with a dramatic worsening of central vision,

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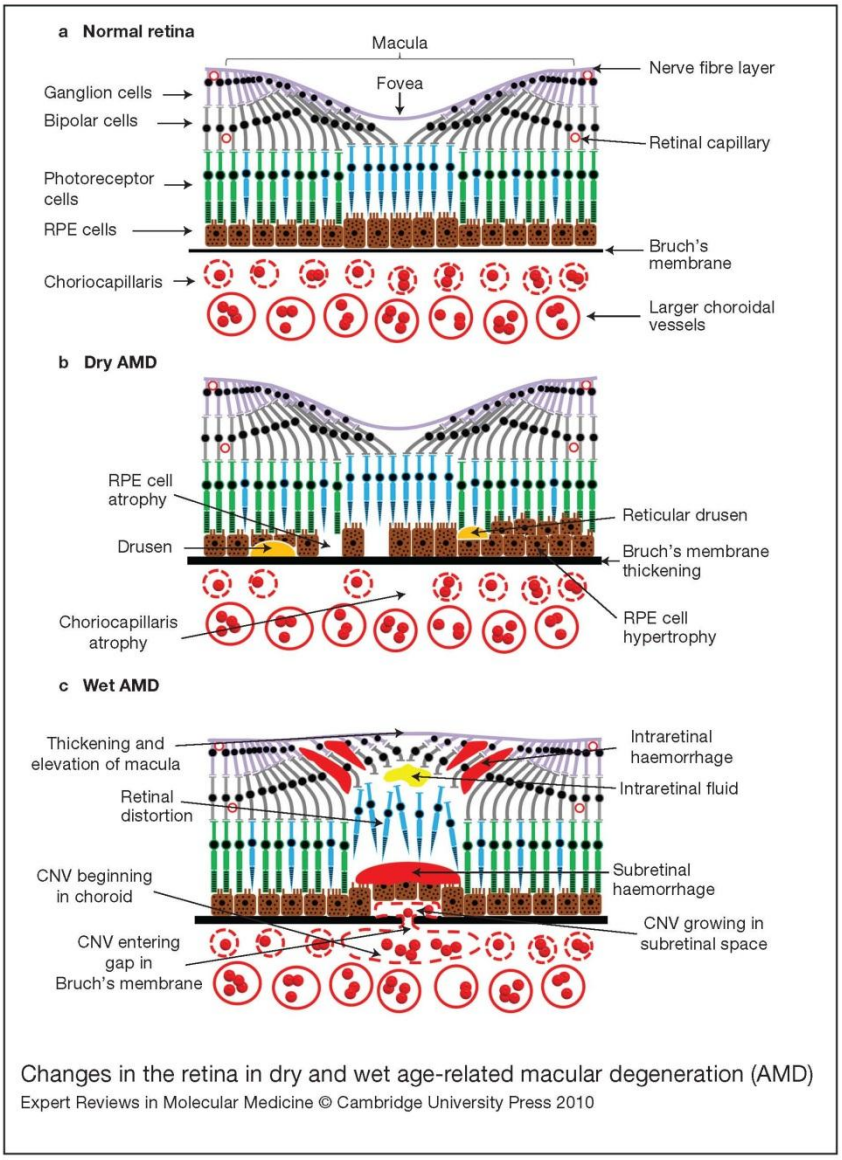

Changes in the retina in dry and wet age-related macular degeneration (AMD)

Figure 1. Changes in the retina in dry and wet age-related macular degeneration (AMD). (*See next page for legend.*)

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Figure 1. Changes in the retina in dry and wet age-related macular degeneration (AMD). (*See previous page for figure.*) (a) Structure of the normal retina. The outermost layer of the retina consists of a monolayer of retinal pigment epithelial (RPE) cells, containing granules (melanosomes) with melanin. Microvilli project from the inner surface of RPE cells, which interdigitate with the outer segments of photoreceptors (rods, green; cones, blue). Multiple photoreceptors usually communicate with each bipolar cell (not shown), which in turn synapse with ganglion cells. The long axons of ganglion cells constitute the nerve fibre layer, which conducts nerve impulses to the optic nerve and then onto the visual cortex in the brain. A network of capillaries is found in the bipolar cell layer, and further capillaries are found within the ganglion cell and nerve fibre layers. Numerous other supporting cells are found in the retina, including amacrine, horizontal and Muller cells (not shown). The outer layer of the retina, the RPE layer, sits on Bruch's membrane. This is composed of collagen and elastin, and acts as a barrier between the RPE cells and the external choroid. The inner layer of the choroid is called the choriocapillaris and is composed of fenestrated capillaries. Larger vessels constitute the outer choriodal layer (not shown). The macula demonstrates characteristic anatomical features compared with the rest of the retina. The bipolar and ganglion cells are displaced to allow maximal light exposure to the photoreceptors, and there are no blood vessels within the retinal layers. The centre of the macula, the fovea, is concave in cross-section and consists of cone photoreceptors only. Furthermore, the RPE cells are taller. (b) Specific anatomical changes are seen in dry AMD. Drusen (shown in yellow) accumulates between RPE cells and Bruch's membrane. Reticular drusen (also shown in yellow) is a specific form of drusen that accumulates between the RPE layer and photoreceptors. Both atrophy and hypertrophy of RPE cells occur, along with choriocapillaris atrophy and thickening of Bruch's membrane. (c) Wet AMD is characterised by the presence of choroidal neovascular membrane (CNV). This forms within the choroid and enters the sub-RPE space through a break in Bruch's membrane. The CNV then proliferates, which can lead to further growth between the retina and RPE layers, leakage of fluid (shown in yellow) and haemorrhage (shown in red). This disruption of retinal layers can cause image distortion. These changes lead to thickening and elevation of the retina, which can be detected clinically.

distortion and development of a central scotoma. Clinically, fluid, hard exudates and haemorrhage may be present, which stem from the abnormal neovascularisation. Eventually, the abnormal blood vessels will either regress or fibrose, leaving a scar. End-stage wet AMD signifies the establishment of fibrous tissue at the macula, known as a 'disciform' scar (because of its circular or 'disc-like' shape). The majority of severe visual loss occurring in AMD is associated with the wet form (neovascularisation). The overall prevalence of GA or CNV is about 3% among all patients with AMD (Ref. 10). Figure 2 depicts the various forms of AMD, as observed clinically.

Oxidation and the body

A full description of the oxidative processes in the body and their relation to ageing is beyond the scope of this article; however, readers are directed to a review by Muller for further information (Ref. 11). Reactive oxygen species (ROS) are highly reactive atoms, ions or molecules that contain oxygen. These are either free radicals or peroxides. Free radicals have an unpaired electron in the outer shell. Oxygen-containing free radicals include hydroxyl ($OH^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), superoxide anion ($O_2^{-\bullet}$) and singlet oxygen ($^1O_2$). Peroxides, by definition, contain a single oxygen–oxygen bond and include hydrogen peroxide (H_2O_2). ROS within the body are formed as normal byproducts of the body's normal metabolic processes, including glycolysis and the Krebs cycle. The superoxide anion is produced by all cells in the mitochondria during the electron transport stage of cellular respiration, and is converted to H_2O_2 by the enzyme superoxide dismutase (SOD) (Ref. 11). ROS are also formed by the immune system as a defence against invading pathogens, and by environmental factors such as smoking, pollution and radiation. The majority of ROS in the body are produced by mitochondria through the electron transport chain, which converts almost 4% of all utilised oxygen into ROS (Ref. 12).

The two main targets of oxidative damage are mitochondria and lipids. Damage from ROS results in point mutations and deletions to mitochondrial DNA (mtDNA) (Ref. 13). Oxidative damage also leads to earlier senescence (loss of ability of cells to divide) (Refs 14, 15), and this may be related to damage and shortening of telomeric DNA (Refs 16, 17).

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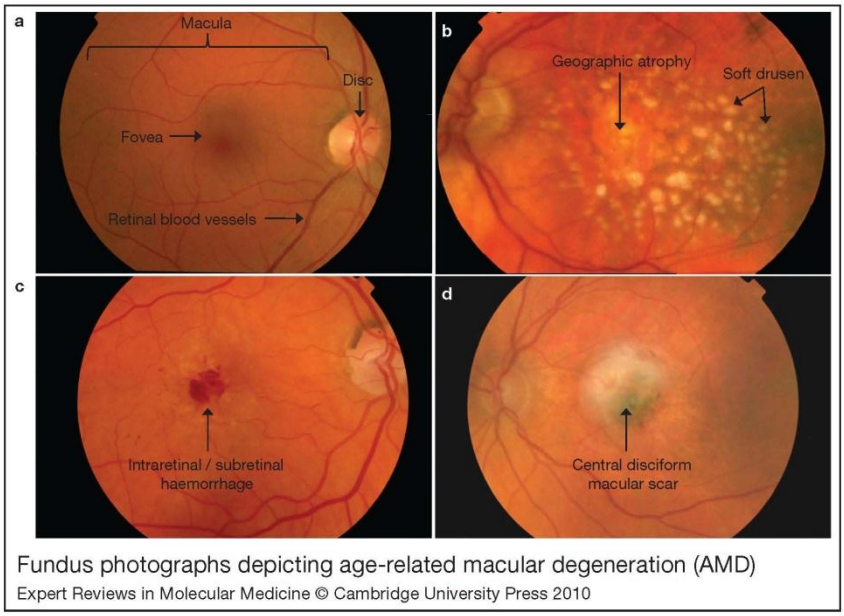

Fundus photographs depicting age-related macular degeneration (AMD)

Figure 2. Fundus photographs depicting age-related macular degeneration (AMD). (a) Fundus of a normal right eye. The optic disc is a pink–yellow area that represents the beginning of the optic nerve. It serves as an anatomical landmark during clinical examination. Radiating out from the optic disc in four quadrants are the retinal blood vessels. Veins are darker and slightly thicker than arteries. Note the 'arcades' created by the vessels on the left of the disc as they arc round the macula. The macula, encompassed by the arcades, lies temporal to the optic disc. The fovea is the centre of the macula and appears darker because of increased xanthophyll pigment levels and taller retinal pigment epithelial (RPE) cells. (b) Severe dry AMD. There is extensive soft drusen, which is visible as yellowish clumps. This patient has geographic atrophy as a result of extensive RPE cell and choriocapillaris atrophy. The sharply demarcated pale area with visible large choroidal vessels is characteristic. As a result this patient may have significantly reduced central vision. (c) Acute wet AMD. This patient developed sudden deterioration of central vision, as a result of subretinal or intraretinal haemorrhage from the choroidal neovascular membrane (CNV). (d) End-stage wet AMD — macular scar. Untreated wet AMD will eventually result in a permanent disciform (disc-shaped) macular scar. Central vision will inevitably be poor.

Furthermore, mtDNA is highly susceptible to oxidative damage, as it is preferentially damaged over nuclear DNA in the presence of ROS (Ref. 18). This could be because mtDNA is closer to the site of creation of ROS, is not protected by histones, and is more prone to errors in replication owing to a less-effective repair system than that for nuclear DNA (Ref. 19). Mitochondrial damage also leads to the release of lethal enzymes, such as caspase and cytochrome *c*, which can lead to apoptosis (Refs 20, 21). ROS damage lipids by oxidation in a process called lipid peroxidation. Polyunsaturated fatty acids (PUFAs) form a significant part of cell membranes. They are most vulnerable because they carry highly reactive hydrogen atoms (Ref. 22). These oxidised lipids, in turn, are toxic and can cause further damage by combining with other molecules (especially proteins), leading to modification of function.

Uncontrolled ROS will cause severe damage to cells. The body has therefore evolved a protective system of antioxidants, which neutralise the

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damaging effects of ROS. This system includes a wide range of enzymes that are measurable in the circulation, such as SOD, glutathione peroxidase (GPX), catalase, metallothionein, peroxiredoxin and thioredoxin, as well as non-enzymatic compounds, such as vitamin C, vitamin E and the carotenoids (Ref. 11). Normally, these antioxidants help to eliminate free ROS to prevent cellular damage. Despite this, some inevitable cellular damage occurs owing to the highly reactive state of ROS. This is usually kept in check by the body's own repair and regeneration processes. However, if ROS levels exceed antioxidation capacity, as a result of either excess ROS production or reduced antioxidant capability, then oxidative damage may ensue. Unfortunately, this is what tends to happen with increasing age, because these steps are key drivers of the ageing process. Furthermore, the body's repair and regeneration capabilities also decrease with age (Ref. 11). This can lead to progressive dysfunction due to abnormal production of proteins and lipids that are essential for cellular activity (Ref. 23). In particular, mtDNA damage increases with age as a result of accumulation of mutations (Ref. 13). Figure 3 summarises the main oxidative processes that occur in the body.

The retina and oxidative stress

The retina is exposed to a constant source of oxidative stress from incoming light, leading to photo-oxidation, which in turn generates ROS within the retina. Male albino rats raised for 12 months in dark conditions had a reduced age-related susceptibility to damage from intense photo-oxidative stress, in the form of intense light, compared with rats raised in dim cyclic light conditions (Ref. 24). Intense light also increases H_2O_2 levels in the outer retina of albino mice (Ref. 25). Blue wavelength [ultraviolet-B (UV-B)] light in particular is considered the more harmful constituent of visible light. In-vitro-cultured RPE cells exposed to blue light develop increased levels of intracellular ROS and stress-related proteins (including osteopontin, heat shock protein 27 and cathepsin), show increased formation of advanced glycation end products and have greater mitochondrial damage (demonstrated by structural changes and reduced membrane potential). These changes are accompanied by a compensatory increase in antioxidant levels (Refs 26, 27). Furthermore, high levels of easily oxidised polyunsaturated fats are present in the retina, as measured by gas chromatography in vitro (Ref. 25). Such lipid peroxidation products are in turn toxic to the retina (see below). Even the phagocytic process of the RPE itself generates ROS through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. In vitro studies of cultured human RPE cells incubated with probe-labelled bovine outer segments demonstrate increased production of intracellular H_2O_2 accompanying increased phagocytosis (Ref. 28).

Oxidative damage induces RPE apoptosis through loss of mitochondrial membrane potential, cytochrome *c* production and caspase 3 activation, as seen in vitro in human cultured RPE cells exposed to *t*-butylhydroperoxide (tBHP) (Ref. 29). Human cultured RPE cells also exhibit premature senescence when exposed in vitro to oxidative stress from tBHP or H_2O_2, as recognised by increased senescence-associated β-galactosidase activity, altered gene expression and consequent alteration in cellular function (Refs 30, 31). Because of their intimate relationship with RPE cells, photoreceptor cells are susceptible to secondary damage once RPE cells are affected, but can also be directly damaged by oxidative stress. Cultured photoreceptor cells exposed to sodium nitroprusside in vitro demonstrate increased activation of protease enzymes such as caplains and caspases, with consequent apoptosis (Ref. 32). ROS damage also increases the expression of connective tissue growth factor, plasminogen activator inhibitor-1, collagen type IV and fibronectin in vitro in cultured human RPE cells. This can lead to accumulation of the extracellular matrix in the RPE layer and might also be a factor in the thickening of Bruch's membrane seen in AMD (Ref. 33). Oxidative stress also appears to impair retinal vascular endothelial function. Normally, retinal vessels undergo vasoconstriction in the presence of high oxygen levels; a randomised placebo-controlled clinical trial on 21 healthy volunteers demonstrated a reduction in this response during oxidative stress upon administration of intravenous lipopolysaccharide (Ref. 34). The choriocapillaris could also be a source of ROS. Protoporphrin IX, a photosensitive precursor of haemoglobin normally found in erythrocytes and plasma, produces ROS

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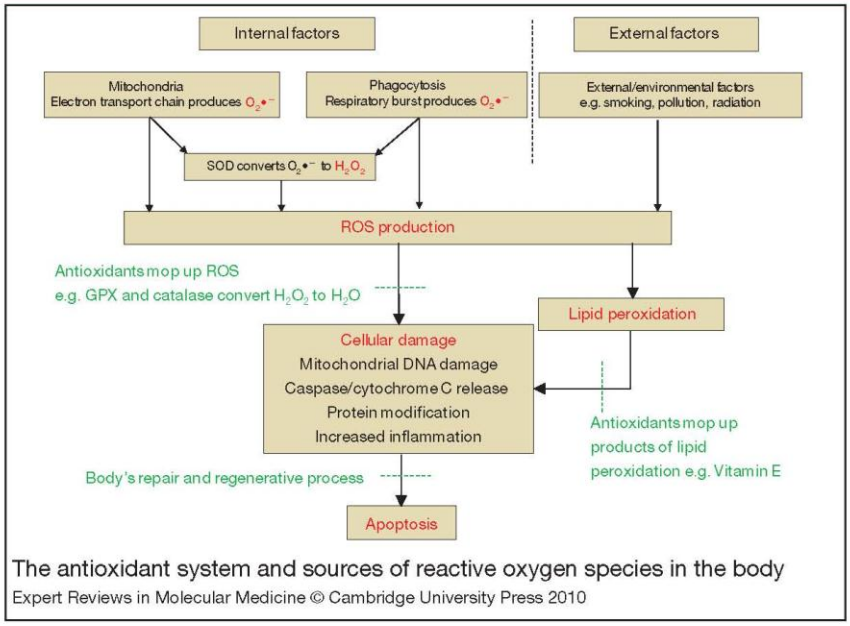

Figure 3. The antioxidant system and sources of reactive oxygen species in the body. Mitochondria are the main source of reactive oxygen species (ROS) in the body. The highly reactive superoxide anion ($O_2^{\bullet-}$) is generated within the mitochondrial inner membrane via the electron transport chain, an essential component of cellular respiration. Its function is to produce energy, after-glycolysis and the Krebs cycle. ROS are also produced by phagocytosis via the NADPH system. This process, called the 'respiratory burst', leads to $O_2^{\bullet-}$ production, required for digestion of internalised particles and pathogens. This is an important component of the immune system, enabling neutrophils and macrophages to clear invading microorganisms. These ROS in turn generate further toxic products by oxidisation of polyunsaturated fatty acids, especially in cellular membranes. ROS can exert a range of toxic effects, including protein modifications, mitochondrial DNA damage and release of the apoptosis-associated enzymes caspase and cytochrome c (ROS and their effects shown in red). Increased inflammation as a result of cellular damage may worsen the situation. Antioxidants (shown in green) help to prevent cellular damage. Most $O_2^{\bullet-}$ is converted, or 'dismutated' to H_2O_2 by superoxide dismutase (SOD). H_2O_2 itself is also toxic, but this in turn is usually converted to H_2O by glutathione peroxidase (GPX) and catalase. A range of other antioxidants (e.g. thioredoxin, metallothionein, peroxiredoxin, vitamin C, vitamin E and carotenoids) also help to mop up ROS. Any ROS that are not eliminated could still produce some cellular damage. However the body's repair and regenerative facilities come into play to maintain normal function (also in green).

when experimentally exposed to light, as detected by electron spin resonance spectrometry (Ref. 35).

A disorder of the immune system is considered to be the major underlying pathological feature of AMD. Several interactions exist between components of the immune system and the oxidative process within the normal retina. Mononuclear phagocytes increase intracellular ROS levels, activate caspase, decrease mitochondrial membrane potential and induce apoptosis in mouse cultured RPE cells. These changes are more exaggerated in *Sod2*-knockout mice RPE cells and after mononuclear phagocyte stimulation with interferon gamma (Ref. 36). The oxidative process may activate the

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complement cascade, which can cause tissue damage, as demonstrated by the following in vitro experiments. Cultured human and mouse RPE cells normally express high levels of complement factor H (CFH), a regulator of the alternate complement cascade. Prolonged phagocytosis of the outer segments reduces intracellular CFH protein production by RPE cells (Ref. 37). Cultured RPE cells exposed to oxidative stress induced by H_2O_2 demonstrate increased surface deposition of C3 and reduced surface expression of the complement inhibitors DAF, CD55 and CD59 (Ref. 38). Mice immunised in vivo with carboxyethylpyrrole (CEP), a product of lipid peroxidation, fix complement C3 in Bruch's membrane, and develop features of AMD (Ref. 39). SERPINA3K, a serine protease inhibitor, is an endogenous regulator of inflammation. It has been shown to significantly decrease ROS production and increase antioxidant levels in vitro in cultured retinal cells exposed to hypoxia (Ref. 40).

VEGF is upregulated in AMD (Ref. 41), and as mentioned previously, anti-VEGF intravitreal injections are now the mainstay of treatment of wet AMD (Refs 4, 5). Cultured RPE cells exposed in vitro to oxidative stress from tBHP demonstrate upregulation of VEGF (Ref. 42). Anti-VEGF treatment could exert some therapeutic benefit by reducing oxidative stress. Bevacizumab, one of the anti-VEGF treatments available clinically, reduces ROS production, lipid peroxidation and apoptosis in choroid and retinal tissue when injected in the eyes of rabbits in vivo (Ref. 43).

Protective mechanisms in the normal retina

Because the retina is highly susceptible to oxidative damage, it has developed a range of protective mechanisms. These include carotenoids, antioxidant enzymes and other substances. The term 'carotenoid' encompasses a range of diet-derived substances, which indude carotenes (α-carotene, β-carotene and lycopene) and xanthophylls (zeaxanthin, lutein and β-cryptoxanthin) (Ref. 44). Lutein and zeaxanthin are found in the retina and might be protective through reduction of lipid peroxidation (Refs 45, 46). Pretreatment of cultured rat photoreceptors with lutein and zeaxanthin protects from oxidative damage due to paraquat and H_2O_2 exposure (Ref. 47). Zeaxanthin also protects cultured rat retinal ganglion cells from oxidative damage induced by in vitro H_2O_2 exposure or serum deprivation (Ref. 48). Furthermore, proteomic studies utilising high-performance liquid chromatography (HPLC) and mass spectrometry demonstrate the presence of several carotenoid oxidation products in extracts of human donor retina, demonstrating their ability to mop up ROS (Refs 49, 50).

The retina and RPE may have higher levels of antioxidant activity compared with other ocular tissues. In vitro measurement of antioxidant enzyme activity in human eyes by direct spectrophotometry shows that the retina has high levels of the copper and zinc-containing isoform of SOD (CuZn-SOD), but relatively low levels of the other SOD isoforms (Ref. 51). However, there seems to be no correlation between RPE SOD levels and the presence of AMD or ageing (Ref. 52). Human donor RPE cells have six times more catalase activity than other ocular tissues (Ref. 52).

Antioxidant levels increase as a protective mechanism during conditions of oxidative stress (Refs 53, 54, 55). In vitro exposure of cultured RPE cells to H_2O_2 demonstrates increased expression of the gene coding for the antioxidant enzyme methionine sulfoxide reductase within the cytosol and mitochondria. This enzyme is also found in sub-RPE drusen from the macula of donors with AMD (Ref. 53). Another antioxidant, haem oxygenase-1 (HO-1), is barely detectable normally, but mRNA levels for this enzyme increase dramatically in vitro in rat retinas exposed to intense visible light. However, there does not appear to be an increase in HO-2 levels in similar conditions, suggesting that the two isoforms have different functions (Ref. 54). Methamphetamine, a psychostimulant that induces oxidative stress, increases SOD levels in the retina as well as in plasma when given to rats in vivo (Ref. 55). Other substances with antioxidant properties found in the retina indude melanin and melantonin. In rat eyes, melanin appears to protect retinal and choroidal cells from the phototoxic effects of light (Ref. 56). Melatonin (*N*-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland and retina, might also have antioxidant effects because cultured RPE cells are protected from oxidative damage by the prolonged addition of melatonin (Ref. 57).

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Changes in oxidative stress in the retina with age

Advancing age increases the susceptibility of the retina to oxidative stress. With age, RPE cells accumulate granules containing a fluorescent pigment called lipofuscin. This is composed of undigested end products from the phagocytic process of photoreceptor cell outer segment discs (Ref. 58). Autophagy also results in accumulation of lipofuscin granules in photoreceptor inner segments (Ref. 59). When purified from extragranular debris, lipofuscin granules contain toxic substances, including retinoids (products of the visual cycle), and modified protein and lipid products (Ref. 60). In vitro, RPE cells loaded with lipofuscin exhibit a significant reduction in phagocytic capacity, therefore compounding the problem (Ref. 61). When exposed to light in vitro, lipofuscin can form ROS, which in turn can damage the RPE. In vitro illumination of lipofuscin with visible light results in extragranular lipid peroxidation, enzyme inactivation and protein oxidation, which are reduced upon the addition of antioxidants (Ref. 62). Using liquid chromatography, mass spectrometry and immuno-analysis, more than 180 proteins in lipofuscin have been identified, many of which are modified, possibly as a result of oxidative influence (Refs 63, 64). Lipofuscin can also reduce intracellular antioxidant capacity; isolated lipofuscin granules fed to human cultured RPE cells in vitro can reduce the activity of the antioxidant enzymes SOD and catalase by up to 60% (Ref. 65).

One key component of lipofuscin is *N*-retinylidene-*N*-retinylethanolamine (A2E), a byproduct of the retinoid visual cycle. Oxidation of A2E can lead to harmful ROS, which may damage DNA. A2E self-generates singlet oxygen, which in turn reacts with A2E to form A2E epoxide. This damages DNA in cultured RPE cells, as detected by the presence of 'comet tails' on single-cell gel electrophoresis, indicating DNA strand breaks (Ref. 66). Cell death can follow. Human cultured RPE cells loaded with A2E exposed in vitro to photo-oxidative stress in the form of blue light show increased apoptosis compared with RPE cells devoid of A2E (Ref. 67). A rise in cytoplasmic caspase, which is associated with cell apoptosis, appears to accompany this process (Ref. 68). There is also evidence that the retinoids within lipofuscin can activate the complement cascade. Oxidative products of A2E in lipofuscin can activate complement in vitro, which in turn can damage local tissue if left unchecked (Ref. 69). Protection against the harmful effects of lipofuscin may be one of the roles of melanin. The presence of calf melanin in cultured RPE cells in vitro is protective against photo-oxidation from blue light (Ref. 70). Furthermore, pigmented rabbit and bovine cultured RPE cells fed photoreceptor outer segments over 4 weeks accumulate significantly less lipofuscin compared with nonpigmented RPE cells. This suggests that melanin may also reduce lipofuscin build-up in RPE cells (Ref. 61).

In vitro studies suggest that the antioxidant capacity of the RPE and retina decreases with age: the rat retina shows decreased SOD, GPX and catalase activity at 4 months compared with 1 month (Ref. 71). Catalase levels appear to diminish in RPE cells of donor eyes with increasing age (although in this study, SOD levels appear unchanged) (Ref. 52). Furthermore, cumulative light exposure of RPE cells with age may reduce the effectiveness of local antioxidants. Porcine RPE cells exposed to high-intensity light demonstrate degradation of melanosomes, reduced melanin levels, with increased ROS production (Ref. 72). Cultured RPE cells fed melanosomes in vitro from older human donors, exposed to blue light, sustain greater cellular damage with prolonged exposure to blue light than RPE cells fed melanosomes from younger human donors (Ref. 73).

The macula and oxidation

There are several reasons why the macula may be particularly susceptible to oxidative damage. The optics of the eye are designed to focus incoming light rays on the macula, therefore maximising photo-oxidative stress, especially from UV-B light. The highest metabolic activity within the retina occurs at the macula. This can be demonstrated indirectly in several ways. The multifocal electroretinogram is an objective, noninvasive method of recording retinal function at several areas throughout the retina simultaneously, after exposure to a light stimulus. This shows greatest response at the macula (Ref. 74). Blood flow within the choroid is greatest in the macular region (Ref. 75). Furthermore, lipofuscin levels are also relatively high at the macula (with a dip at the fovea due

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to its anatomical structure – see Fig. 1a), suggesting high levels of phagocytosis (Ref. 76). Finally, in keeping with increased oxidative stress at the macula, concentrations of the antioxidants lutein and zeaxanthin are also highest in the macula, and specifically in the outer segments of photoreceptor cells, as measured by HPLC in vitro in the adult human retina (Refs 77, 78).

Oxidation and AMD

Certain factors that increase oxidative stress may also be associated with an increased risk of AMD. Cigarette smoke is highly damaging to RPE cells in vitro. Cultured human RPE cells exposed to cigarette smoke extract or components of cigarette smoke (including hydroquinone, acrolein and cadmium) exhibit ROS production and reduction in antioxidant capacity, leading to impairment of mitochondrial function and eventually apoptosis of RPE cells. These detrimental processes can be reduced experimentally with the addition of antioxidants (Refs 79, 80, 81). This in vitro effect may explain the strong association of smoking with an increased risk of AMD seen epidemiologically, as demonstrated by three large population-based cross-sectional studies (Refs 82, 83, 84).

The Beaver Dam Study (1993) in the USA examined 4771 patients aged between 43 and 89 years, and found that the relative odds for the presence of wet AMD in smokers was 3.29 and 2.50 (in males and females, respectively) compared with nonsmokers. However, there was no relationship between the presence of GA and smoking (Ref. 82). The Blue Mountain Eye Study (1996) in Australia looked at 3654 patients aged 49 years and over. This study found an odds ratio (OR) of 3.20 for the presence of wet AMD in smokers and 4.54 for GA, compared with nonsmokers (Ref. 83). Yet another large study, the Rotterdam Study (1996), examined 6174 patients from the Netherlands aged 55 years and over, and found, in subjects younger than 85 years, an OR of 6.6 for the presence of wet AMD in smokers compared with non-smokers. However, there was no association with GA. There was also a dose-dependent relationship, with a significant increased risk (relative risk 6.5) of wet AMD in those smoking in excess of 10 pack years (Ref. 84).

The association between increased sunlight exposure and AMD has been studied extensively; however, both retrospective and prospective epidemiological studies have been inconclusive. The following studies have all used a questionnaire-based system for ascertaining sunlight exposure, which may introduce subject variability. A cohort of 2764 patients from the Beaver Dam Study, aged 43–86 at baseline were followed up prospectively over 10 years. An association of increased incidence of early AMD (including drusen and pigmentary changes) and increased exposure to the summer sun was found. However, no association was found with sun exposure and progression of AMD, or with the incidence of more advanced AMD (Ref. 85). A retrospective study of 769 watermen over 50 years old working on Chesapeake Bay, Maryland in the USA found no relationship between light exposure (either UV-A or UV-B) and presence of AMD (Ref. 86). The researchers subsequently reported a subgroup analysis on the same population, and found that patients with advanced AMD (extensive GA or wet AMD, $n = 8$) had significantly higher blue and visible light exposure in the previous 20 years compared with age-matched controls (Wilcoxon sign rank test, $P = 0.027$). However, the small numbers of patients with advanced AMD should be noted; furthermore, there was no association when considering lifetime sunlight exposure (Ref. 87). The Beaver Dam Study found, in 4771 subjects aged 43–84 years, that the presence of 'late maculopathy' (defined as GA or wet AMD, $n = 77$) was significantly associated with the amount of leisure time spent outdoors in summer (OR 2.19). However, there was no association between estimated UV exposure and AMD (Ref. 88). Furthermore, in Newcastle, Australia, 409 patients with AMD actually had significantly less sun exposure than 286 controls ($P>0.0001$) (Ref. 89). More recently, 4753 patients in the European Eye Study aged 65 years and over showed no association between adult lifetime blue light exposure and early or wet AMD. However, a subgroup analysis showed that patients with wet AMD and low blood antioxidant levels had increased blue light exposure, suggesting that antioxidant supplements could be beneficial (Ref. 90).

Drusen is the earliest manifestation of AMD and may also contribute to oxidative damage. For example, amyloid-β, which is found in drusen, increases ROS formation and reduces antioxidant capacity in RPE cells (Ref. 91).

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However, drusen formation itself may be infuenced by the oxidative process. Liquid chromatography and mass spectrometry analysis of drusen from donor human retinas demonstrates oxidative protein modifications, including CEP protein adducts (see below), as well as crosslinking of tissue metalloproteinase inhibitor-3 and vitronectin (Ref. 92).

As a result of oxidative damage at the macula, local alterations in cellular function can occur. To offset this, compensatory local antioxidant activity within the retina appears to increase in AMD. SOD levels measured in vitro are higher in histological specimens in the cytoplasm and lysosomes of RPE cells in eyes with AMD compared with levels in disease-free eyes. However, this particular experiment demonstrated no change in GPX levels (Ref. 93). Furthermore, antioxidant activity seems to increase with AMD severity (Ref. 94). This suggests that there is a localised compensatory response to increased ROS activity with progressive AMD. Nevertheless, ROS activity may exceed antioxidant capacity within the macula, with subsequent progression of AMD.

Systemic markers of oxidation in AMD

It is possible that development and progression of AMD is influenced by a systemic imbalance between ROS and the antioxidant system. In vitro, RPE cells are more likely to be damaged in the presence of a more oxidative extracellular redox environment (Ref. 29). In vivo, plasma or serum levels of both ROS and antioxidants in patients with AMD have been investigated, with varying results.

Systemic ROS levels and AMD

There are no consistent associations of systemic ROS levels in AMD. Recently, significantly higher levels of serum protein carbonyl (a marker of protein oxidation), 8-hydroxy-29-deoxyguanosine (a marker of oxidative damage to DNA) and total serum oxidation state were found in 47 patients with wet AMD compared with 25 healthy controls (Ref. 95). Nitric oxide (NO) is the most abundant free radical in the body; however, levels of circulating NO are not consistent in AMD. A relatively small study (n = 66) found elevated plasma levels of NO with AMD compared with controls (Ref. 96). However, in a smaller study (n = 30), the opposite was seen, implying possible damage to endothelial cells and reduced endothelial production of NO as a result (Ref. 97). Another study found no difference between AMD subgroups (Ref. 98). Homocysteine is an amino acid that rapidly oxidises to form ROS, but again with inconsistent associations with AMD. In a large cohort of over 2000 patients from the Blue Mountain Study population, higher systemic homocysteine levels were found in patients with AMD than in controls (Ref. 99). Numerous other smaller studies have confirmed this association (Refs 100, 101, 102, 103, 104, 105). Other studies show increased homocysteine levels in wet AMD compared with dry AMD (Refs 105, 106). However, in the third National Health and Nutrition Examination Survey study of more than 3000 patients, no association was found with serum homocysteine (Ref. 107). This was also the case in another study drawn from the Blue Mountain population (with 630 patients) (Ref. 108).

Systemic antioxidant levels and AMD

In contrast to the above, there is possibly greater evidence suggesting an association between systemic levels of certain antioxidants and AMD. Of the individual antioxidants, carotenoids have been studied the most extensively, perhaps because of the ease with which dietary supplementation is possible. The Eye Disease Case-Control Study Group looked at the serum levels of carotenoids in a large number of patients (n = 1036), stratifying levels into low, medium and high groups. They found that patients with medium and high levels of carotenoids had up to a 50% reduced risk of wet AMD (Ref. 109). In 722 Japanese patients, reduced total carotene and total carotenoid serum levels, as well as individual levels of α-tocopherol and β-cryptoxanthin levels, were associated with late AMD. However, there was no association with early AMD (Ref. 110). A smaller study on 334 subjects from the Beaver Dam population found similar levels of individual carotenoids in both patients and controls; only very low levels of lycopene were associated with AMD (Ref. 111). Further smaller studies have reported variable results. A French study (n = 55) did not find any difference in the total serum carotenoid level, or in the other carotenoids between AMD patients and controls (again with the exception of reduced serum lycopene levels in the AMD group) (Ref. 112). In

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a study of Italian patients (n=94), lower levels of circulating vitamin C, vitamin E, β-cryptoxanthin and total carotenoids were found in patients with late AMD (GA or wet AMD) than in those with early AMD. However, there was no difference in total antioxidant capacity and individual carotenoid levels between the two groups. Furthermore, there was no difference in all the measured values between patients with AMD and controls (except again, for lower lycopene levels in the AMD group)(Ref. 113). Another study found increased serum total antioxidant capacity in patients with AMD compared with controls (Ref. 95).

There is less consistency of evidence depiciting the association between systemic levels of antioxidant enzymes and AMD. Several studies have found that systemic antioxidant enzyme levels, including SOD GPX (Refs 96, 114), catalase (Refs 96, 115), glutathione reductase (Ref. 116) and paroxonase (Refs 102, 117), are lower in AMD patients compared with controls. Lower antioxidant levels have also been reported in patients with late AMD compared with early AMD (Ref. 96). Some studies have, however, report no difference in systemic antioxidant levels and AMD (Refs 116, 118, 119). This may be associated with the Furthermore, several studies actually report an increase in systemic antioxidant levels in AMD. A higher level of plasma GPX was associated with a ninefold increase in prevalence of advanced AMD (Ref. 119).

Other antioxidants include copper (found in RPE cells) and melatonin. The serum levels of ceruloplasmin (a globulin that binds copper, found in RPE cells) are higher in patients with AMD (Ref. 120). Daytime serum levels of melatonin are significantly increased in patients with AMD than in controls (Ref. 121). This may be associated with the higher systemic levels of products of lipid peroxidation found in AMD (discussed below). Overall, the evidence suggests that lower systemic antioxidant levels, particularly total carotenoid levels, as well as lycopene individually, are associated with AMD, especially the late form. However the hypothesis that a systemic imbalance of ROS and antioxidant levels is a predisposing factor in AMD, is far from confirmed.

Lipid peroxidation and AMD

The retina is abundant in fatty acids (FAs), including palmitic, stearic, oleic, arachidonic and docosahexaenoic acids (Ref. 26). Under conditions of oxidative stress, PUFAs are particularly susceptible to oxidation (peroxidation). The oxidation of phospholipids in RPE and photoreceptor cells from donor eyes increases with age and in AMD (Ref. 122). These oxidative products can induce apoptosis of RPE cells (Ref. 123). One omega-3 long-chain PUFA particularly susceptible to oxidative damage is docosahexaenoate (DHA), which is found at high concentrations in photoreceptor cells, where it comprises 80% of the total amount of PUFAs in the outer segment discs (Ref. 124). Interestingly, the concentration of FAs is lower at the macula than in the peripheral retina, perhaps because of increased lipid peroxidation in the macular area (Ref. 26).

CEP is produced by the oxidation of DHA-containing phospholipids, which can subsequently form bonds (adducts) with proteins, leading to functional modification (Ref. 125). CEP-adducted proteins tend to accumulate in the outer retina, in rod outer segments and in the RPE, as shown in vitro in mouse and human donor retina (Ref. 126). Several studies suggest that CEP-adducted proteins have a key role in the pathogenesis of AMD. Mice immunised with CEP-adducted albumin accumulate complement C3 in Bruch's membrane, develop drusenoid changes under the RPE and also develop RPE atrophic-like changes (Ref. 39). CEP-adducted proteins are elevated in Bruch's membrane and drusen in donor eyes with AMD (Ref. 92). New vessel growth can be stimulated by CEP adducts that exacerbate laser-induced CNV (Ref. 127). Plasma anti-CEP antibodies are elevated in patients with AMD (Ref. 126). Proteomic analysis also demonstrates increased plasma levels of CEP adducts and CEP autoantibodies in AMD, especially in association with the *ARMS2* and *HTRA1* risk alleles (Ref. 128).

CEP is likely to be only one of several products of lipid peroxidation associated with AMD (Ref. 125). For example, levels of oxidised LDL as well as susceptibility of LDL to oxidation are both increased in the plasma of AMD patients (Refs 129, 130). 4-Hydroxy-2-nonenal (HNE) is a highly toxic product of lipid peroxidation. However, levels of HNE-adducted proteins found at the macula in donor eyes with AMD are similar to levels in the periphery. Furthermore, HNE levels do not increase with

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AMD severity. This suggests that the retina has sufficient antioxidation capacity to remove this product (Ref. 131). Malondialdehyde (MDA), a product of lipid peroxidation, is used as a marker of oxidative stress in studies of oxidation. Several studies have shown that circulating MDA levels are increased in AMD, compared with controls (Refs 95, 96, 97, 115, 117).

Genetic studies of oxidation and AMD

Antioxidant enzyme genes

Current studies in the literature examining the association between genes encoding antioxidant enzymes and AMD in human populations are not confirmatory; however, antioxidant gene knockout mice do develop retinal features reminiscent of AMD. The most studied antioxidant genes are those encoding SOD proteins. The SOD1 protein is the most abundant and is located in the cytoplasm (associated with copper or zinc, gene position 21q22.1); SOD2 is found in the mitochondria (associated with manganese, gene position 6q25.3); SOD3 is found in the extracellular space (gene position 4p15.3–p15.1) (Refs 132, 133). An association with a polymorphism in the *SOD2* gene with AMD was found in a Japanese population (Ref. 134). However, two other groups found no association in Japanese populations (Refs 135, 136), and futhermore, no association was observed in a Northern Irish population (Ref. 137). Interestingly, it has also been suggested that a SOD gene polymorphism could actually be protective in AMD (Ref. 135). Other antioxidant genes have been studied in human populations, again with variable outcomes. One report suggests that there is no association between polymorphisms in the GPX or catalase gene and AMD (Ref. 137). However, polymorphisms in the paroxonase gene (which protects LDL from oxidation) have been associated with AMD (Ref. 129).

Despite the above results, animal models of AMD have been created by manipulation of antioxidant genes. *Sod1*-knockout mice are more likely to develop CNV in the presence of VEGF (Ref. 138). *Sod1*-knockout mice also demonstrate necrotic swelling in the inner and outer nuclear layer of the retina, as well as mitochondrial degeneration associated with increased drusen, thickened Bruch's membrane and CNV (Refs 139, 140). RPE cells from *Sod2* heterozygous knockout mice have lower levels of SOD2 correlating with disruption of mitochondrial transmembrane potential, release of cytochrome *c* and resulting apoptosis (Ref. 141). However, overexpression of SOD in transgenic mice can be detrimental, suggesting that a careful balance of SOD levels in the body is usually essential (Ref. 142). The lack of translation from animal studies to human populations suggests that AMD pathogenesis in humans is likely to be a far more complex, multifactorial disease.

ARMS2

Because mitochondria have a key role in oxidative damage (as described above), it is possible that genes affecting mitochondrial function alter the susceptibility to oxidative damage. The *ARMS2/LOC387715* gene located on chromosome 10q6 has been associated with AMD and might have a role in mitochondrial function (Ref. 143). A single-nucleotide polymorphism (SNP) (G→T in exon 1, with A69S amino acid change) at this locus has been associated with AMD (Ref. 144). The same group later reported an association of several further SNPs with AMD as well as a deletion–insertion (del443ins54) in the *ARMS2* gene (Refs 145). The gene encodes a 12 kDa protein; however, its function is not known. *ARMS2/LOC387715* mRNA has been detected in vitro in the human retina, localising to the mitochondrial outer membrane in mammalian cells and in particular the mitochondria-rich ellipsoid region of photoreceptors (Refs 145, 223). However, more recently, ARMS2 protein was found in the cytosol in cultured RPE cells (Ref. 146). As mentioned previously, patients with AMD are more likely to have elevated systemic markers of lipid peroxidation (plasma CEP adduct or CEP antibody levels) if also carrying the G→T SNP (Ref. 128). Interestingly, the same SNP almost doubled the risk of AMD in patients who smoked (OR, 2.7 versus 1.2) in heterozygotes (GT) and almost quadrupled the risk (OR, 8.2 versus 2.1) in homozygotes (TT), compared with nonsmokers, when taking into account CFH status (Ref. 147). It may be that SNPs in the *ARMS2* gene increase susceptibility to oxidative damage, possibly in association with smoking. However, this is speculative and further studies are indicated.

Mitochondrial genes

Despite the integral role of mitochondria in oxidative damage, there are few studies

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documenting the association of genes within the mitochondria and AMD. Varying outcomes are reported for different mitochondrial haplogroups. Haplogroup H appears to be protective and is associated with reduced AMD prevalence. Haplogroup J is associated with a higher prevalence of large soft drusen, and haplogroup U with higher prevalence of retinal pigment abnormalities (Ref. 148). Patients with variations in the mitochondrial T2 haplogroup and complex I genes are up to 2.5 times more likely to have advanced AMD (Ref. 149). More studies would be useful to investigate the incidence of genetic mutations in mtDNA and AMD.

Complement genes

As mentioned above, there may be an interaction between the immune system and the oxidative process. The immune system has a key role in the pathogenesis of AMD (Refs 9, 150). Several genes encoding components of the complement cascade have been associated with AMD. Three landmark publications in 2005 documented the association of the Y402H sequence variation in the complement factor H (*CFH*) gene and susceptibility to AMD (this gene encodes a regulator of the alternate complement pathway) (Refs 151, 152, 153). Additional strong genetic associations with AMD have also been found with mutations in other complement-related genes, including complement C3 (Ref. 154), complement factor B (*CFB*) (Ref. 155), complement factor I (Refs 156, 157) and a deletion in *CFHR1* and *CFHR3* (Refs 158, 159). Recently, an association with AMD and the *SERPING1* gene was reported (this encodes a C1 inhibitor, which is a regulator of the classic complement pathway) (Ref. 160). Whether these genetic variants increase the risk of AMD by increasing oxidative stress as well as through inflammatory processes remains to be seen.

Clinical implications

Treatment aimed at reducing oxidative damage can therefore be approached in two different ways, either by reducing the source of oxidative damage or by increasing protection against oxidative damage, using antioxidants. The following measures can reduce the risk of AMD in patients by lowering oxidative stress.

Reducing the source of oxidative damage

As described above, the association between sunlight exposure and AMD is not confirmatory. Appropriately filtering sunglasses can reduce A2E and ROS production in RPE and photoreceptor cells in vitro (Ref. 161). However, there is no evidence that sunglasses can reduce the risk of developing AMD (Ref. 85). Smoking, by contrast, is strongly associated with AMD. Stopping smoking is associated with a reduced risk of developing AMD, and individuals who have not smoked for more than 20 years have a risk of AMD that is equivalent to that of nonsmokers (Ref. 162).

Antioxidant supplementation

In vitro, lipofuscin content in cultured RPE cells is significantly reduced by the addition of antioxidants such as α-tocopherol, lycopene, zeaxanthin and lutein (Refs 61, 163). Oral supplementation of antioxidants is a relatively simple intervention, and such products are widely available. However, there does not seem to be sufficient evidence to suggest that oral antioxidants can prevent the onset of AMD. A meta-analysis of three prospective interventional randomised controlled trials and nine prospective questionnaire-based studies looking at incidence of AMD over 5–18 years was recently performed. The authors concluded that 'vitamin A, vitamin C, vitamin E, zinc, lutein, zeaxanthin, α carotene, β carotene, β cryptoxanthin, and lycopene have little or no effect in the primary prevention of early AMD' (Ref. 164).

Alternatively, there may be a place for oral antioxidants in reducing the progression of pre-existing mild AMD. The Age Related Eye Disease Study (AREDS) was a 6 year study observing the effects of high-dose antioxidants, including vitamins C and E and β-carotene, along with zinc, in over 3000 subjects. This demonstrated an odds-risk reduction of one-third in the highest risk group of developing progression to severe AMD (as observed on fundus images). The highest risk group was defined as those with extensive intermediate-size drusen (greatest linear diameter 63–124 μm), at least one large druse (>124 μm diameter), noncentral GA in one or both eyes, or advanced AMD or vision loss because of AMD in one eye (Ref. 165). Another study found that the AREDS formulation appeared to protect

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patients from retinal vascular endothelial dysfunction produced by oxidative stress (Ref. 34). In an ongoing study, a group from Vanderbilt University, USA is investigating whether the original AREDS vitamins change the plasma redox state in 140 patients over 5 years (Ref. 166). Use of β-carotene, however, is contraindicated in smokers because of an association with lung cancer (Ref. 167); AREDS 'smokers' formulations' are available as alternatives. Nevertheless, the benefits of taking such a formulation are likely to be outweighed by the harm resulting from continued smoking.

Potential future treatments

There is a wide spectrum of compounds under investigation that may provide antioxidant benefit. A brief description of the clinical and non-clinical studies of potential therapeutic agents in the literature follows.

Dietary antioxidants

The AREDS2 study is an ongoing 5-year prospective study based in the USA, ending in 2013, that is designed to look at the effects of oral supplementation of xanthophylls (lutein and zeaxanthin) and omega-3 FAs (DHA and eicosapentaenoic acid – EPA) on the risk of progression of pre-existing early AMD to late AMD. A total of 550 patients will be randomised into one of three groups: one taking 10 mg lutein and 2 mg zeaxanthin, another taking 350 mg DHA and 650 mg EPA, and the third taking a combination of both (Ref. 168).

Other oral supplements may be beneficial in providing antioxidant protection. The antioxidant enzyme SOD is available in oral form, called 'Glisodin' (derived from SOD found in melons). A French team has completed a 2 year study (no results are available at the time of writing) investigating whether this supplement can reduce the progression of AMD in 46 patients who have unilateral wet AMD in the fellow eye (Ref. 169). Epigallocatechin gallate, a component of green tea, appears to protect the retina against oxidative damage when injected into rats' eyes (Ref. 170). The National Eye Institute, USA has completed a study examining the effects of oral lutein plus green tea extract, on macular pigment density (a reflection of lutein and zeaxanthin concentrations), as measured by heterochromatic flicker photometry, in 40 patients over 4 months (no results are available at the time of writing) (Ref. 171). However, this combination does not appear to alter oxidative stress systemically in adequately nourished older adults (Ref. 172).

Several other potential supplements appear to produce antioxidant benefits in vitro, but have yet to be studied in well-structured clinical trials. Bilberry extract appears to reduce the formation of reactive products from the oxidation of A2E in cultured RPE cells (Ref. 66). Curcumin, a naturally occurring compound found in the spice turmeric, appears to have an antioxidant effect through several mechanisms, including inhibition of the nuclear factor kappa B (NF-κB) pathway (see below) (Ref. 173), upregulation of antioxidant gene expression (Ref. 174) and downregulation of gene expression of inflammatory mediators (Ref. 175). Cells of rats given a curcumin-supplemented diet for 2 weeks appear to sustain less photo-oxidative damage from light exposure. Furthermore, cultured RPE cells undergo lower rates of apoptosis when pretreated with curcumin (Ref. 176). Lipoic acid, an essential cofactor for mitochondrial enzymes, is found in most foods and appears to reduce the oxidative damage to RPE cells in vitro (Ref. 80). This suggests that lipoic acid supplements may be beneficial, although no longitudinal trials have been carried out in patients with AMD. The extract from *Ginkgo biloba* has been used as a traditional Chinese medicine for many years and it might have antioxidant properties (Ref. 177). However, the addition of *Ginkgo biloba* experimentally does not appear to reduce the formation of toxic products from the photo-oxidation of A2E in cultured RPE cells (Refs 49, 66). Resveratrol, which is found in red wine, reduces oxidative damage and proliferation of RPE cells in vitro (Ref. 178). Its beneficial action may be mediated via inhibition of NF-κB or its anti-inflammatory properties (Ref. 179). Quercetin, a flavinoid, also appears to protect cultured RPE cells against oxidative stress (Ref. 180). It should be noted that some supplements, such as St John's Wort, an over-the-counter antidepressant medication, could actually be harmful. One of its components, hypericin, increases lipid peroxidation and decreases antioxidant levels in human cultured RPE cells exposed to photo-oxidative stress (Ref. 181).

A new antioxidant compound of interest is OT-551 (Othera Pharmaceuticals), which has the

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novelty of being administrable in topical eye drop form. A Phase II clinical study has recently been reported, evaluating the benefit of treating GA. Ten patients with bilateral GA were treated with OT-551 eye drops three times a day in one eye, with the other eye used as a control. After 2 years, there was greater loss of vision in the untreated eye (−11.3±7.6 letters) compared with the treated eye (+0.2±13.3 letters) ($P = 0.0259$), and it was well tolerated. This raises the possibility that antioxidant eye drops could be a possible means of preserving vision in patients with GA. However, the authors express caution in interpreting these results, because there was no difference in all secondary outcomes between the study and control eye (Ref. 182). A larger Phase III study would provide further answers.

Genetic manipulation

Genetic manipulation is increasingly being considered as a treatment option for many diseases. Cellular genes can be manipulated to decrease production of harmful ROS, to increase antioxidant capacity or to boost cellular repair. There are a numerous pathways through which genetic manipulation can reduce the production of ROS. NF-κB is a protein complex found in the nucleus, which controls DNA transcription (Ref. 183). In particular, it regulates the expression of genes involved in apoptosis (Ref. 184), inflammation and the immune response (Ref. 185). Abnormalities in its regulation may be an underlying factor in several diseases, including rheumatoid arthritis and inflammatory bowel disease (Ref. 186). Thioredoxin is a ubiquitous protein with antioxidant properties (Ref. 187), and might act by inhibiting NF-κB, in addition to other pathways (Ref. 188). Thioredoxin protects photoreceptor cells from photo-oxidative stress, both in vitro and in vivo in mice (Refs 189, 190). However, by reducing apoptosis, it may also be implicated in cancer pathogenesis. Indeed, thioredoxin system inhibitors are being evaluated as cancer therapy agents (Ref. 191). The Shc group of proteins act as adaptors (accessories) in many signal transduction pathways. One Shc protein, p66Shc, might have a key role in oxidative stress damage. P66Shc-deficient mice demonstrate increased resistance to oxidative stress, with a 30% increase in life span (Ref. 192). Short-interfering ribonucleic acid (siRNA) directed against p66Shc in cultured RPE cells results in reduced apoptosis from oxidative stress. This results in reduced levels of ROS and increased levels of antioxidant enzymes. Animal eyes injected with siRNA to knock down p66Shc have reduced loss of retinal function on electroretinograms when exposed to oxidative stress (Ref. 193). However, impaired expression of p66Shc can also lead to loss of apoptosis, uncontrolled proliferation and cancerous growth (Ref. 194). One of the primary sources of ROS is NADPH enzyme system, which is involved in glycolysis within the cytoplasm and in the electron transport chain within mitochondria. siRNA against a subunit of NAPDH (p22phox) delivered in the subretinal space in a mouse eye through an adeno-associated virus vector inhibits CNV formation by downregulation of NADPH (Ref. 195).

Increasing cellular protection by the introduction of genes encoding antioxidant enzymes is an attractive therapeutic model. Positive results of antioxidant gene therapy have been reported in mice. ROS were generated in the eyes of mice by inducing retinal ischaemia (by increasing intraocular pressure for 2 h) and then allowing immediate reperfusion; 48 h preceding this, one eye was injected intravitreally with plasmids containing the *Sod2* and *Cat* (catalase) genes. The fellow eye was used as a control. The pretreated eyes demonstrated significant reduction in ROS production and endothelial cell apoptosis within the retina (Ref. 196). In another study, RPE cells in vitro transfected with adenovirus carrying the catalase gene were protected from damage from administration of H_2O_2. In vivo, mice eyes injected with the same vector and exposed to photo-oxidative stress demonstrate increased catalase activity in RPE cells, which also appear to confer protection to neighbouring photoreceptors (Ref. 197). Furthermore, direct upregulation of GPX expression in RPE cells appears to protect them from oxidative damage; additionally, this protection is conferred in transgenic mice overexpressing GPX4 (an isomer of GPX). However, this same study also found that RPE cells overexpressing SOD1 or SOD2 might sustain increased oxidative damage, suggesting that a level of homeostasis is required (Ref. 142).

Other genes can be upregulated to increase protection against oxidative damage.

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Upregulation of phase 2 genes, which encode enzymes involved in drug and toxin metabolism, might have an antioxidant protective effect (Ref. 198). Sulforaphane, found in *Ginkgo biloba*, broccoli and a wide range of vegetables, may exert its beneficial effects in this way (Refs 199, 200, 201, 202). Pharmacological induction of phase 2 genes is under investigation for cancer therapy (Refs 201, 202) and could offer a novel approach for the prevention of AMD. Furthermore, transgenic mice modified to express increased levels of glial-cell-line-derived neurotrophic factor (GDNF) sustain less retinal apoptosis when exposed to various models of oxidative damage, suggesting that gene therapy with GDNF might be protective (Ref. 203).

Finally, genes can be manipulated to increase repair of damage sustained from oxidative stress. *BCL2* is a gene that has important functions, including repair of mtDNA and promotion of cell survival. When this gene is transfected in vitro in human RPE cells, there is up to 50% less mitochondrial dysfunction after exposure to oxidative stress from H_2O_2 (Ref. 204).

Other potential avenues of treatment

There are many other compounds with antioxidant properties that could form the basis of future therapies in AMD.

Nanotechnology, or the use of particles with diameters less than 1 μm, is an emerging science. Applications include delivery of gene therapy by particles small enough to pass through nuclear membranes. Currently, there are limited reports of the use of nanotechnology in reducing oxidative stress within the retina. Engineered cerium oxide nanoparticles (nanoceria particles) can act as scavengers of ROS, and reduce ROS production in vitro in rat retinas (and reduce visual loss in vivo), under conditions of photo-oxidative stress (Ref. 205). Flupirtine, a neuroprotectant and antioxidant, decreases lipid peroxidation and protects photoreceptor cells from oxidative damage when injected intraocularly in rats (Ref. 206). The terpenoids, or isoprenoids, are polymers of isoprene units (five-carbon hydrocarbon) and include many organic compounds such as carotene and vitamins A, K and E. Derivatives of one such terpinoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), appear to have an antioxidant effect on cultured RPE and photoreceptor cells, by inhibiting the phosphatase and tensin homologue (PTEN) protein, and by reducing apoptosis (Ref. 207). However, other papers report that CDDO derivatives increase ROS production and apoptosis, and as a result are being evaluated as a potential treatment for cancer (Ref. 208). Anti-CEP adduct therapy may also be effective. CEP induces VEGF-independent new vessel growth (see above), which may be reduced by anti-CEP antibodies (Ref. 127).

The endocannabinoid system (ECS) consists of a group of lipids and receptors that have a wide range of roles in the body (Ref. 209). Post-mortem eyes of patients with AMD have increased levels of endocannabinoids in the choroid and (less so) in the retina (Ref. 210). Cultured human RPE cells express cannabinoid receptors, and these are upregulated under conditions of oxidative stress. Furthermore, cannabinoid receptor agonists protect RPE cells from oxidative damage (Ref. 211). The potential clinical benefits of modulating the ECS have not been explored in relation to the eye, but trials have studied the potential benefits in reducing cardiovascular risk, in neurodegenerative diseases, in pain relief and in obesity; however, psychiatric side effects have limited progress (Refs 209, 212). Erythropoietin (EPO) is a hormone produced by the kidneys in response to hypoxia, with a primary role in the regulation of red blood cell levels (Ref. 213). EPO also has an antioxidant effect (Ref. 214) and is antiapoptotic (Ref. 215). A receptor to EPO has been found in the retina (Ref. 216). Cultured RPE cells are protected from oxidative damage when treated with EPO (Ref. 217), and subretinal EPO injections in mice appear to be neuroprotective (Ref. 218). Furthermore, patients with wet AMD have higher serum EPO levels (Ref. 219).

Caloric restriction is the only proven means of reducing the effects of ageing, as shown in a variety of species, including rodents, dogs and monkeys. Reduced oxidative stress is one proposed mechanism by which caloric restriction works; others include reduced metabolic rate, altered gene expression and enhanced DHA repair (Ref. 220). Ageing rats exhibit retinal changes, including reduced retinal cell density, increased protein insolubility and reduced levels of the antioxidants GSH and ascorbic acid. Rats fed a gradually increasing calorie-restricted diet demonstrate a reduced decline of these parameters with age. The

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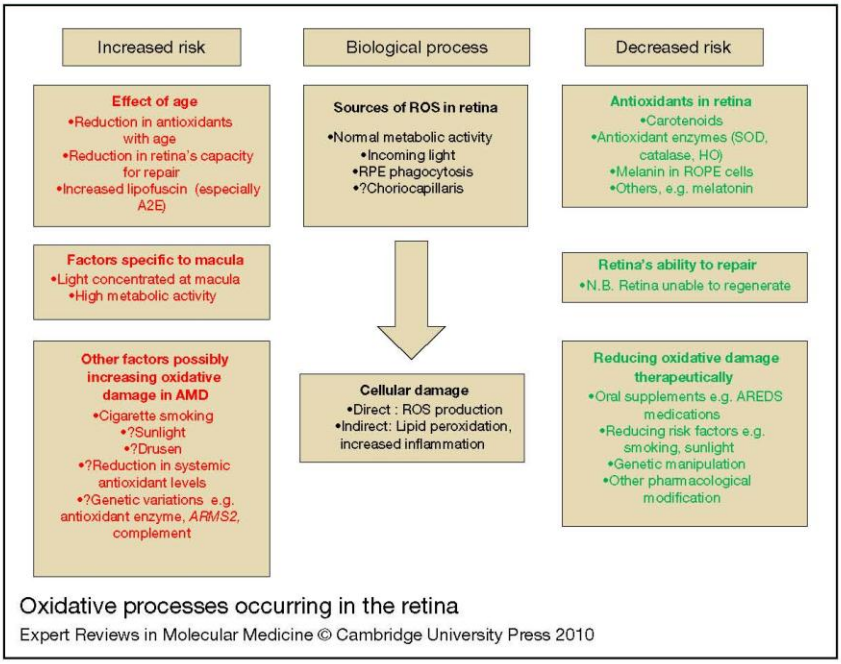

Figure 4. Oxidative processes occurring in the retina. Reactive oxygen species (ROS) are normally produced in the retina and retinal pigment epithelial (RPE) as a result of normal cellular metabolic activity. In addition, incoming light itself is a potent generator of oxidative stress, as is RPE phagocytosis of outer segments. ROS may also be produced in the choriocapillaris from the effects of light. ROS can cause retinal cellular damage directly, or indirectly by peroxidation of polyunsaturated fatty acids, found in especially high levels in retinal cell membranes. These products of lipid peroxidation are in turn toxic. Furthermore, ROS may predispose to increased inflammation, for example by activating the complement cascade, which in turn may cause further cellular damage. Normally, the adverse effects of ROS are kept under control by antioxidants in the retina, including antioxidant enzymes [e.g. superoxide dismutase (SOD), catalase, haem oxygenase (HO)], carotenoids, melanin and others such as the hormone melatonin. Any cellular damage from ROS not mopped up by the antioxidant system is repaired by the retina. Increasing age may increase the potential for oxidative damage in the retina, with a reduction in antioxidant enzyme levels, a reduction in the retina's capacity for repair and increased build-up of a toxic fluorescent substance called lipofuscin in RPE and photoreceptor cells. The macula in particular is highly susceptible to oxidative damage because of the increased levels of incoming light centred on the macula and the consequent high level of metabolic activity. Other factors that may increase oxidative stress and are associated with age-related macular degeneration (AMD) include smoking, exposure to sunlight, drusen formation, systemic antioxidant levels and genetic variations. The risk of oxidative damage may be therapeutically decreased by oral antioxidant supplementation and by reducing the exposure to risk factors such as smoking and excess sunlight. Future therapeutic options include genetic manipulation and pharmacological modification.

authors of this study propose that the increase in protein insolubility is due to oxidative modification, which is reduced by caloric reduction (Ref. 221). However, rhesus monkeys on caloric restriction (reduction of 30%) for up to 19 years did not demonstrate any significant

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difference in prevalence of drusenoid maculopathy compared with control monkeys (on a normal diet) (Ref. 222). No human studies are evident in the literature as yet.

Many avenues of research have been explored to find suitable antioxidant therapeutic agents. The most practical and readily available intervention, diet supplementation, is relatively cheap and simple to implement; ongoing clinical studies will serve to increase our understanding of which supplements might be most beneficial. Perhaps the most promising therapeutic option is gene manipulation, which might offer more effective and longer-lasting protection from the effects of oxidative damage.

Conclusion

We have highlighted the important role of oxidative damage in the pathogenesis of AMD and the potential benefits of antioxidants. This is summarised in Figure 4. However, oxidative damage is likely to be only one of several pathways involved in the pathogenesis of a multifactorial disease such as AMD. Further studies are required to investigate the possible roles of the potential therapies as listed above, especially because some might have oncogenic potential. Nevertheless, future treatments based on protecting against antioxidant damage are likely to be part of a multifaceted approach, encompassing a deeper understanding of the disease processes underlying AMD.

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References

1 Resnikoff, S. et al. (2004) Global data on visual impairment in the year 2002. Bulletin of the World Health Organization 82, 844-851
2 de Jong, P.T.V.M. (2006) Age-related macular degeneration. New England Journal of Medicine 355, 1474-1485
3 Friedman, D.S. et al. (2004) Prevalence of age-related macular degeneration in the United States. Archives of Ophthalmology 122, 564-572
4 Brown, D.M. et al. (2006) Ranibizumab versus verteporfin for neovascular age-related macular degeneration. New England Journal of Medicine 355, 1432-1444
5 Rosenfeld, P.J. et al. (2006) Ranibizumab for neovascular age-related macular degeneration. New England Journal of Medicine 355, 1419-1431
6 Schmitz-Valckenberg, S. et al. (2010) Combined confocal scanning laser ophthalmoscopy and spectral-domain optical coherence tomography imaging of reticular drusen associated with age related macular degeneration. Ophthalmology 117, 1169-1176
7 Klein, R. et al. (2008) The epidemiology of retinal reticular drusen. American Journal of Ophthalmology 145, 317-326
8 Knudtson, M.D. et al. (2004) Location of lesions associated with age-related maculopathy over a 10-year period: the Beaver Dam Eye Study. Investigative Ophthalmology and Visual Science 45, 2135-2142
9 Lotery, A. and Trump, D. (2007) Progress in defining the molecular biology of age related macular degeneration. Human Genetics 122, 219-236
10 Augood, C.A. et al. (2006) Prevalence of age-related maculopathy in older Europeans: the European Eye Study (EUREYE). Archives of Ophthalmology 124, 529-535
11 Muller, F.L. et al. (2007) Trends in oxidative aging theories. Free Radical Biology and Medicine 43, 477-503
12 Chance, B., Sies, H. and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs. Physiological Reviews 59, 527-605
13 Golden, T.R. and Melov, S. (2001) Mitochondrial DNA mutations, oxidative stress, and aging. Mechanisms of Ageing and Development 122, 1577-1589
14 Hayflick, L. and Moorhead, P.S. (1961) The serial cultivation of human diploid cell strains. Experimental Cell Research 25, 585-621
15 Yuan, H., Kaneko, T. and Matsuo, M. (1995) Relevance of oxidative stress to the limited replicative capacity of cultured human diploid cells: the limit of cumulative population doublings increases under low concentrations of oxygen and decreases in response to aminotriazole. Mechanisms of Ageing and Development 81, 159-168
16 Adelfalk, C. et al. (2001) Accelerated telomere shortening in Fanconi anemia fibroblasts–a longitudinal study. FEBS Letters 506, 22-26
17 Rubio, M.A., Davalos, A.R. and Campisi, J. (2004) Telomere length mediates the effects of telomerase on the cellular response to genotoxic stress. Experimental Cell Research 298, 17-27

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

18 Ballinger, S.W. et al. (1999) Hydrogen peroxide causes significant mitochondrial DNA damage in human RPE cells. Experimental Eye Research 68, 765-772
19 Cai, J. et al. (2000) Oxidative damage and protection of the RPE. [Review] [138 Refs]. Progress in Retinal and Eye Research 19, 205-221
20 Susin, S.A. et al. (1999) Mitochondrial release of caspase-2 and -9 during the apoptotic process. Journal of Experimental Medicine 189, 381-394
21 Yang, J. et al. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 275, 1129-1132
22 Lundberg, W.O. (1958) Lipids of biologic importance; peroxidation products and inclusion compounds of lipids. American Journal of Clinical Nutrition 6, 601-603
23 Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. Journal of Gerontology 11, 298-300
24 Organisciak, D.T. et al. (1998) Light history and age-related changes in retinal light damage. Investigative Ophthalmology and Visual Science 39, 1107-1116
25 Yamashita, H. et al. (1992) Light-induced retinal damage in mice. Hydrogen peroxide production and superoxide dismutase activity in retina. Retina 12, 59-66
26 van Kuijk, F.J. and Buck, P. (1992) Fatty acid composition of the human macula and peripheral retina. Investigative Ophthalmology and Visual Science 33, 3493-3496
27 Roehlecke, C. et al. (2009) The influence of sublethal blue light exposure on human RPE cells. Molecular Vision 15, 1929-1938
28 Miceli, M.V., Liles, M.R. and Newsome, D.A. (1994) Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. Experimental Cell Research 214, 242-249
29 Jiang, S. et al. (2005) Oxidant-induced apoptosis in human retinal pigment epithelial cells: dependence on extracellular redox state. Investigative Ophthalmology and Visual Science 46, 1054-1061
30 Glotin, A.L. et al. (2008) Prematurely senescent ARPE-19 cells display features of age-related macular degeneration. Free Radical Biology and Medicine 44, 1348-1361
31 Yu, A.L. et al. (2009) Subtoxic oxidative stress induces senescence in retinal pigment epithelial cells via TGF-beta release. Investigative Ophthalmology and Visual Science 50, 926-935
32 Sanvicens, N. et al. (2004) Oxidative stress-induced apoptosis in retinal photoreceptor cells is mediated by calpains and caspases and blocked by the oxygen radical scavenger CR-6. Journal of Biological Chemistry 279, 39268-39278
33 Fuchshofer, R. et al. (2009) Hypoxia/reoxygenation induces CTGF and PAI-1 in cultured human retinal pigment epithelium cells. Experimental Eye Research 88, 889-899
34 Pemp, B. et al. (2010) Effects of antioxidants (AREDS medication) on ocular blood flow and endothelial function in an endotoxin-induced model of oxidative stress in humans. Investigative Ophthalmology and Visual Science 51, 2-6
35 Gottsch, J.D. et al. (1990) Hematogenous photosensitization. A mechanism for the development of age-related macular degeneration. Investigative Ophthalmology and Visual Science 31, 1674-1682
36 Yang, D. et al. (2009) Association of superoxide anions with retinal pigment epithelial cell apoptosis induced by mononuclear phagocytes. Investigative Ophthalmology and Visual Science 50, 4998-5005
37 Chen, M., Forrester, J.V. and Xu, H. (2007) Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. Experimental Eye Research 84, 635-645
38 Thurman, J.M. et al. (2009) Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. Journal of Biological Chemistry 284, 16939-16947
39 Hollyfield, J.G. et al. (2008) Oxidative damage-induced inflammation initiates age-related macular degeneration. Nature Medicine 14, 194-198
40 Zhang, B. et al. (2009) Anti-inflammatory and antioxidant effects of SERPINA3 K in the retina. Investigative Ophthalmology and Visual Science 50, 3943-3952
41 Kliffen, M. et al. (1997) Increased expression of angiogenic growth factors in age-related maculopathy. British Journal of Ophthalmology 81, 154-162
42 Kannan, R. et al. (2006) Stimulation of apical and basolateral VEGF-A and VEGF-C secretion by oxidative stress in polarized retinal pigment epithelial cells. Molecular Vision 12, 1649-1659
43 Sari, A. et al. (2009) Effects of intravitreal bevacizumab in repeated doses: an experimental study. Retina 29, 1346-1355
44 Young, A.J. and Lowe, G.M. (2001) Antioxidant and prooxidant properties of carotenoids. Archives of Biochemistry and Biophysics 385, 20-27

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

45 Packer, L. (1993) Antioxidant action of carotenoids in vitro and in vivo and protection against oxidation of human low-density lipoproteins. Annal of the New York Academy of Sciences 691, 48-60
46 Lim, B.P. et al. (1992) Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. Biochimica et Biophysica Acta 1126, 178-184
47 Chucair, A.J. et al. (2007) Lutein and zeaxanthin protect photoreceptors from apoptosis induced by oxidative stress: relation with docosahexaenoic acid. Investigative Ophthalmology and Visual Science 48, 5168-5177
48 Nakajima, Y. et al. (2009) Zeaxanthin, a retinal carotenoid, protects retinal cells against oxidative stress. Current Eye Research 34, 311-318
49 Lakshminarayana, R. et al. (2008) Possible degradation/biotransformation of lutein in vitro and in vivo: isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (atmospheric pressure chemical ionization). Free Radical Biology and Medicine 45, 982-993
50 Khachik, F., Bernstein, P.S. and Garland, D.L. (1997) Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. Investigative Ophthalmology and Visual Science 38, 1802-1811
51 Behndig, A. et al. (1998) Superoxide dismutase isoenzymes in the human eye. Investigative Ophthalmology and Visual Science 39, 471-475
52 Liles, M.R., Newsome, D.A. and Oliver, P.D. (1991) Antioxidant enzymes in the aging human retinal pigment epithelium. Archives of Ophthalmology 109, 1285-1288
53 Sreekumar, P.G. et al. (2005) Protection from oxidative stress by methionine sulfoxide reductases in RPE cells. Biochemical and Biophysical Research Communications 334, 245-253
54 Kutty, R.K. et al. (1995) Induction of heme oxygenase 1 in the retina by intense visible light: suppression by the antioxidant dimethylthiourea. Proceedings of the National Academy of Sciences of the United States of America 92, 1177-1181
55 Melo, P. et al. (2010) Oxidative stress response in the adult rat retina and plasma after repeated administration of methamphetamine. Neurochemistry International 56, 431-436
56 Peters, S. et al. (2006) Melanin protects choroidal blood vessels against light toxicity. Zeitschrift fur Naturforschung Section C Journal of Biosciences 61, 427-433
57 Liang, F.Q. et al. (2004) Melatonin protects human retinal pigment epithelial (RPE) cells against oxidative stress. Experimental Eye Research 78, 1069-1075
58 Ryan, S.J. (2006) Retina (4th edn), Elsevier/Mosby, Philadelphia, PA, USA
59 Iwasaki, M. and Inomata, H. (1988) Lipofuscin granules in human photoreceptor cells. Investigative Ophthalmology and Visual Science 29, 671-679
60 Ng, K.P. et al. (2008) Retinal pigment epithelium lipofuscin proteomics. Molecular and Cellular Proteomics 7, 1397-1405
61 Nilsson, S.E. et al. (2003) Aging of cultured retinal pigment epithelial cells: oxidative reactions, lipofuscin formation and blue light damage. Documenta Ophthalmologica 106, 13-16
62 Wassell, J. et al. (1999) The photoreactivity of the retinal age pigment lipofuscin. Journal of Biological Chemistry 274, 23828-23832
63 Ng, K.P. et al. (2008) Retinal pigment epithelium lipofuscin proteomics. Molecular and Cellular Proteomics 7, 1397-1405
64 Schutt, F. et al. (2003) Proteins modified by malondialdehyde, 4-hydroxynonenal, or advanced glycation end products in lipofuscin of human retinal pigment epithelium. Investigative Ophthalmology and Visual Science 44, 3663-3668
65 Shamsi, F.A. and Boulton, M. (2001) Inhibition of RPE lysosomal and antioxidant activity by the age pigment lipofuscin. Investigative Ophthalmology and Visual Science 42, 3041-3046
66 Sparrow, J.R. et al. (2003) A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. Journal of Biological Chemistry 278, 18207-18213
67 Sparrow, J.R., Nakanishi, K. and Parish, C.A. (2000) The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. Investigative Ophthalmology and Visual Science 41, 1981-1989
68 Sparrow, J.R. and Cai, B. (2001) Blue light-induced apoptosis of A2E-containing RPE: involvement of caspase-3 and protection by Bcl-2. Investigative Ophthalmology and Visual Science 42, 1356-1362
69 Zhou, J. et al. (2009) Complement activation by bisretinoid constituents of RPE lipofuscin. Investigative Ophthalmology and Visual Science 50, 1392-1399
70 Wang, Z., Dillon, J. and Gaillard, E.R. (2006) Antioxidant properties of melanin in retinal pigment epithelial cells. Photochemistry and Photobiology 82, 474-479

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

71 Castorina, C. et al. (1992) Lipid peroxidation and antioxidant enzymatic systems in rat retina as a function of age. Neurochemical Research 17, 599-604
72 Zareba, M. et al. (2006) Effects of photodegradation on the physical and antioxidant properties of melanosomes isolated from retinal pigment epithelium. Photochemistry and Photobiology 82, 1024-1029
73 Rozanowski, B. et al. (2008) The phototoxicity of aged human retinal melanosomes. Photochemistry and Photobiology 84, 650-657
74 Feigl, B., Stewart, I. and Brown, B. (2007) Experimental hypoxia in human eyes: implications for ischaemic disease. Clinical Neurophysiology 118, 887-895
75 Nork, T.M. et al. (2006) Measurement of regional choroidal blood flow in rabbits and monkeys using fluorescent microspheres. Archives of Ophthalmology 124, 860-868
76 Wing, G.L., Blanchard, G.C. and Weiter, J.J. (1978) The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. Investigative Ophthalmology and Visual Science 17, 601-607
77 Bone, R.A. et al. (1997) Distribution of lutein and zeaxanthin stereoisomers in the human retina. Experimental Eye Research 64, 211-218
78 Rapp, L.M., Maple, S.S. and Choi, J.H. (2000) Lutein and zeaxanthin concentrations in rod outer segment membranes from perifoveal and peripheral human retina. Investigative Ophthalmology and Visual Science 41, 1200-1209
79 Bertram, K.M. et al. (2009) Molecular regulation of cigarette smoke induced-oxidative stress in human retinal pigment epithelial cells: implications for age-related macular degeneration. American Journal of Physiology – Cell Physiology 297, C1200-C1210
80 Jia, L. et al. (2007) Acrolein, a toxicant in cigarette smoke, causes oxidative damage and mitochondrial dysfunction in RPE cells: protection by (R)-alpha-lipoic acid. Investigative Ophthalmology and Visual Science 48, 339-348
81 Kalariya, N.M. et al. (2009) Cadmium-induced apoptotic death of human retinal pigment epithelial cells is mediated by MAPK pathway. Experimental Eye Research 89, 494-502
82 Klein, R. et al. (1993) The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. American Journal of Epidemiology 137, 190-200
83 Smith, W., Mitchell, P. and Leeder, S.R. (1996) Smoking and age-related maculopathy. The Blue Mountains Eye Study. Archives of Ophthalmology 114, 1518-1523
84 Vingerling, J.R. et al. (1996) Age-related macular degeneration and smoking. The Rotterdam Study. Archives of Ophthalmology 114, 1193-1196
85 Tomany, S.C. et al. (2004) Sunlight and the 10-year incidence of age-related maculopathy: the Beaver Dam Eye Study. Archives of Ophthalmology 122, 750-757
86 West, S.K. et al. (1989) Exposure to sunlight and other risk factors for age-related macular degeneration. Archives of Ophthalmology 107, 875-879
87 Taylor, H.R. et al. (1990) Visible light and risk of age-related macular degeneration. Transactions of the American Ophthalmological Society 88, 163-173
88 Cruickshanks, K.J., Klein, R. and Klein, B.E. (1993) Sunlight and age-related macular degeneration. The Beaver Dam Eye Study. Archives of Ophthalmology 111, 514-518
89 Darzins, P., Mitchell, P. and Heller, R.F. (1997) Sun exposure and age-related macular degeneration. An Australian case-control study. Ophthalmology 104, 770-776
90 Fletcher, A.E. et al. (2008) Sunlight exposure, antioxidants, and age-related macular degeneration. Archives of Ophthalmology 126, 1396-1403
91 Bruban, J. et al. (2009) Amyloid-beta(1-42) alters structure and function of retinal pigmented epithelial cells. Aging Cell 8, 162-177
92 Crabb, J.W. et al. (2002) Drusen proteome analysis: an approach to the etiology of age-related macular degeneration. Proceedings of the National Academy of Sciences of the United States of America 99, 14682-14687
93 Frank, R.N., Amin, R.H. and Puklin, J.E. (1999) Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration. American Journal of Ophthalmology 127, 694-709
94 Decanini, A. et al. (2007) Changes in select redox proteins of the retinal pigment epithelium in age-related macular degeneration. American Journal of Ophthalmology 143, 607-615
95 Totan, Y. et al. (2009) Oxidative macromolecular damage in age-related macular degeneration. Current Eye Research 34, 1089-1093
96 Everekliogl u, C. et al. (2003) Nitric oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration. Documenta Ophthalmologica 106, 129-136

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

97 Totan, Y. et al. (2001) Plasma malondialdehyde and nitric oxide levels in age related macular degeneration. British Journal of Ophthalmology 85, 1426-1428
98 Tsai, D.C. et al. (2006) Different plasma levels of vascular endothelial growth factor and nitric oxide between patients with choroidal and retinal neovascularization. Ophthalmologica 220, 246-251
99 Rochtchina, E. et al. (2007) Elevated serum homocysteine, low serum vitamin B12, folate, and age-related macular degeneration: the Blue Mountains Eye Study. American Journal of Ophthalmology 143, 344-346
100 Seddon, J.M. et al. (2006) Evaluation of plasma homocysteine and risk of age-related macular degeneration. American Journal of Ophthalmology 141, 201-203
101 Coral, K. et al. (2006) Plasma homocysteine and total thiol content in patients with exudative age-related macular degeneration. Eye (London) 20, 203-207
102 Ates, O. et al. (2009) Decreased serum paraoxonase 1 activity and increased serum homocysteine and malondialdehyde levels in age-related macular degeneration. Tohoku Journal of Experimental Medicine 217, 17-22
103 Nowak, M. et al. (2005) Homocysteine, vitamin B12, and folic acid in age-related macular degeneration. European Journal of Ophthalmology 15, 764-767
104 Vine, A.K. et al. (2005) Biomarkers of cardiovascular disease as risk factors for age-related macular degeneration. Ophthalmology 112, 2076-2080
105 Kamburoglu, G. et al. (2006) Plasma homocysteine, vitamin B12 and folate levels in age-related macular degeneration. Graefes Archive for Clinical and Experimental Ophthalmology 244, 565-569
106 Axer-Siegel, R. et al. (2004) Association of neovascular age-related macular degeneration and hyperhomocysteinemia. American Journal of Ophthalmology 137, 84-89
107 Heuberger, R.A. et al. (2002) Relation of blood homocysteine and its nutritional determinants to age-related maculopathy in the third National Health and Nutrition Examination Survey. American Journal of Clinical Nutrition 76, 897-902
108 Wu, K.H. et al. (2007) Circulating inflammatory markers and hemostatic factors in age-related maculopathy: a population-based case-control study. Investigative Ophthalmology and Visual Science 48, 1983-1988
109 Eye Disease Case-Control Study Group. (1993) Antioxidant status and neovascular age-related macular degeneration. Archives of Ophthalmology 111, 104-109
110 Michikawa, T. et al. (2009) Serum antioxidants and age-related macular degeneration among older Japanese. Asia Pacific Journal of Clinical Nutrition 18, 1-7
111 Mares-Perlman, J.A. et al. (1995) Serum antioxidants and age-related macular degeneration in a population-based case-control study. Archives of Ophthalmology 113, 1518-1523
112 Cardinault, N. et al. (2005) Lycopene but not lutein nor zeaxanthin decreases in serum and lipoproteins in age-related macular degeneration patients. Clinica Chimica Acta 357, 34-42
113 Simonelli, F. et al. (2002) Serum oxidative and antioxidant parameters in a group of Italian patients with age-related maculopathy. Clinica Chimica Acta 320, 111-115
114 Prashar, S. et al. (1993) Antioxidant enzymes in RBCs as a biological index of age related macular degeneration. Acta Ophthalmologica 71, 214-218
115 Yildirim, O. et al. (2004) Changes in antioxidant enzyme activity and malondialdehyde level in patients with age-related macular degeneration. Ophthalmologica 218, 202-206
116 Cohen, S.M. et al. (1994) Low glutathione reductase and peroxidase activity in age-related macular degeneration. British Journal of Ophthalmology 78, 791-794
117 Baskol, G. et al. (2006) Serum paraoxonase 1 activity and lipid peroxidation levels in patients with age-related macular degeneration. Ophthalmologica 220, 12-16
118 De, La Paz, M.A., Zhang, J. and Fridovich, I. (1996) Red blood cell antioxidant enzymes in age-related macular degeneration. British Journal of Ophthalmology 80, 445-450
119 Delcourt, C. et al. (1999) Associations of antioxidant enzymes with cataract and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. Ophthalmology 106, 215-222
120 Newsome, D.A. et al. (1986) Macular degeneration and elevated serum ceruloplasmin. Investigative Ophthalmology and Visual Science 27, 1675-1680
121 Schmid-Kubista, K.E. et al. (2009) Daytime levels of melatonin in patients with age-related macular degeneration. Acta Ophthalmologica 87, 89-93
122 Suzuki, M. et al. (2007) Oxidized phospholipids in the macula increase with age and in eyes with age-related macular degeneration. Molecular Vision 13, 772-778

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

123 Choudhary, S. et al. (2005) Toxicity and detoxification of lipid-derived aldehydes in cultured retinal pigmented epithelial cells. Toxicology and Applied Pharmacology 204, 122-134
124 Fliesler, S.J. and Anderson, R.E. (1983) Chemistry and metabolism of lipids in the vertebrate retina. Progress in Lipid Research 22, 79-131
125 Gu, X. et al. (2003) Oxidatively truncated docosahexaenoate phospholipids: total synthesis, generation, and peptide adduction chemistry. Journal of Organic Chemistry 68, 3749-3761
126 Gu, X. et al. (2003) Carboxyethylpyrrole protein adducts and autoantibodies, biomarkers for age-related macular degeneration. Journal of Biological Chemistry 278, 42027-42035
127 Ebrahem, Q. et al. (2006) Carboxyethylpyrrole oxidative protein modifications stimulate neovascularization: implications for age-related macular degeneration.[Erratum appears in Proc Natl Acad Sci U S A. 2006 Oct 17;103(42):15722]. Proceedings of the National Academy of Sciences of the United States of America 103, 13480-13484
128 Gu, J. et al. (2009) Assessing susceptibility to age-related macular degeneration with proteomic and genomic biomarkers. Molecular and Cellular Proteomics 8, 1338-1349
129 Ikeda, T. et al. (2001) Paraoxonase gene polymorphisms and plasma oxidized low-density lipoprotein level as possible risk factors for exudative age-related macular degeneration. American Journal of Ophthalmology 132, 191-195
130 Javadzadeh, A. et al. (2007) Enhanced susceptibility of low-density lipoprotein to oxidation in wet type age-related macular degeneration in male patients. Saudi Medical Journal 28, 221-224
131 Ethen, C.M. et al. (2007) Age-related macular degeneration and retinal protein modification by 4-hydroxy-2-nonenal. Investigative Ophthalmology and Visual Science 48, 3469-3479
132 Johnson, F. and Giulivi, C. (2005) Superoxide dismutases and their impact upon human health. Molecular Aspects of Medicine 26, 340-352
133 Online Mendelian Inheritance in Man, OMIM (TM). McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD). World Wide Web URL:http://www.ncbi.nlm.nih.gov/omim/
134 Kimura, K. et al. (2000) Genetic association of manganese superoxide dismutase with exudative age-related macular degeneration. American Journal of Ophthalmology 130, 769-773
135 Gotoh, N. et al. (2008) Manganese superoxide dismutase gene (SOD2) polymorphism and exudative age-related macular degeneration in the Japanese population. American Journal of Ophthalmology 146, 146-147
136 Kondo, N. et al. (2009) SOD2 gene polymorphisms in neovascular age-related macular degeneration and polypoidal choroidal vasculopathy. Molecular Vision 15, 1819-1826
137 Esfandiary, H. et al. (2005) Association study of detoxification genes in age related macular degeneration. British Journal of Ophthalmology 89, 470-474
138 Dong, A. et al. (2009) Oxidative stress promotes ocular neovascularization. Journal of Cellular Physiology 219, 544-552
139 Imamura, Y. et al. (2006) Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. Proceedings of the National Academy of Sciences of the United States of America 103, 11282-11287
140 Hashizume, K. et al. (2008) Retinal dysfunction and progressive retinal cell death in SOD1-deficient mice. American Journal of Pathology 172, 1325-1331
141 Kasahara, E. et al. (2005) SOD2 protects against oxidation-induced apoptosis in mouse retinal pigment epithelium: implications for age-related macular degeneration. Investigative Ophthalmology and Visual Science 46, 3426-3434
142 Lu, L. et al. (2009) Increased expression of glutathione peroxidase 4 strongly protects retina from oxidative damage. Antioxidants and Redox Signaling 11, 715-724
143 Jakobsdottir, J. et al. (2005) Susceptibility genes for age-related maculopathy on chromosome 10q26. American Journal of Human Genetics 77, 389-407
144 Rivera, A. et al. (2005) Hypothetical LOC387715 is a second major susceptibility gene for age-related macular degeneration, contributing independently of complement factor H to disease risk. Human Molecular Genetics 14, 3227-3236
145 Fritsche, L.G. et al. (2008) Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nature Genetics 40, 892-896
146 Wang, G. et al. (2009) Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria. Investigative Ophthalmology and Visual Science 50, 3084-3090

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

147 Schmidt, S. et al. (2006) Cigarette smoking strongly modifies the association of LOC387715 and age-related macular degeneration. American Journal of Human Genetics 78, 852-864
148 Jones, M.M. et al. (2007) Mitochondrial DNA haplogroups and age-related maculopathy. Archives of Ophthalmology 125, 1235-1240
149 SanGiovanni, J.P. et al. (2009) Mitochondrial DNA variants of respiratory complex I that uniquely characterize haplogroup T2 are associated with increased risk of age-related macular degeneration. PLoS One 4, e5508
150 Swaroop, A. et al. (2009) Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annual Review of Genomics and Human Genetics 10, 19-43
151 Klein, R.J. et al. (2005) Complement factor H polymorphism in age-related macular degeneration. Science 308, 385-389
152 Haines, J.L. et al. (2005) Complement factor H variant increases the risk of age-related macular degeneration. Science 308, 419-421
153 Edwards, A.O. et al. (2005) Complement factor H polymorphism and age-related macular degeneration. Science 308, 421-424
154 Yates, J.R. et al. (2007) Complement C3 variant and the risk of age-related macular degeneration. New England Journal of Medicine 357, 553-561
155 Gold, B. et al. (2006) Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nature Genetics 38, 458-462
156 Ennis, S. et al. (2009) Support for the involvement of complement factor I in age-related macular degeneration. European Journal of Human Genetics 18, 15-16
157 Fagerness, J.A. et al. (2009) Variation near complement factor I is associated with risk of advanced AMD. European Journal of Human Genetics 17, 100-104
158 Hageman, G.S. et al. (2006) Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Annals of Medicine 38, 592-604
159 Hughes, A.E. et al. (2006) A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. Nature Genetics 38, 1173-1177
160 Ennis, S. et al. (2008) Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. Lancet 372, 1828-1834
161 Meyers, S.M., Ostrovsky, M.A. and Bonner, R.F. (1993) A model of spectral filtering to reduce photochemical damage in age-related macular degeneration. Transactions of the American Ophthalmological Society 102, 83-93
162 Khan, J.C. et al. (2006) Smoking and age related macular degeneration: the number of pack years of cigarette smoking is a major determinant of risk for both geographic atrophy and choroidal neovascularisation. British Journal of Ophthalmology 90, 75-80
163 Sundelin, S.P. and Nilsson, S.E. (2001) Lipofuscin-formation in retinal pigment epithelial cells is reduced by antioxidants. Free Radical Biology and Medicine 31, 217-225
164 Chong, E.W. et al. (2007) Dietary antioxidants and primary prevention of age related macular degeneration: systematic review and meta-analysis. BMJ 335, 755-758
165 Age-Related Eye Disease Study Research Group. (2001) A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8 [Erratum appears in Arch Ophthalmol. 2008 Sep;126(9):1251]. Archives of Ophthalmology 119, 1417-1436
166 Vanderbilt University, USA. Antioxidant systems and age-related macular degeneration. In ClinicalTrials.gov [Internet], National Library of Medicine (US), Bethesda, MD, USA, 2000 [cited 2010 July 07], available from: http://clinicaltrials.gov/ct2/show/NCT00668213. NLM Identifier: NCT00668213
167 Omenn, G.S. et al. (1996) Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. Journal of National Cancer Institute 88, 1550-1559
168 National Eye Institute (NEI). Age-related eye disease study 2 (AREDS2). In ClinicalTrials.gov [Internet], National Library of Medicine (US), Bethesda, MD, USA, 2000 [cited 2010 March 20], available from: http://clinicaltrials.gov/ct2/show/NCT00345176. NLM Identifier: NCT00345176
169 Centre de Recherche en Nutrition Humaine Rhone-Alpe, France. Superoxide dismutase (SOD) as antioxidant treatment of age related macular degeneration (ARMD). In ClinicalTrials.gov [Internet], National Library of Medicine (US), Bethesda, MD, USA, 2000 [cited 2010 July 07],

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

available from: http://clinicaltrials.gov/ct2/show/NCT00800995. NLM Identifier: NCT00800995
170 Zhang, B. and Osborne, N.N. (2006) Oxidative-induced retinal degeneration is attenuated by epigallocatechin gallate. Brain Research 1124, 176-187
171 National Eye Institute (NEI), USA. Effects of antioxidants on human macular pigments. In ClinicalTrials.gov [Internet], National Library of Medicine (US), Bethesda, MD, USA, 2000 [cited 2010 July 07], available from http://clinicaltrials.gov/ct2/show/NCT00718653. NLM Identifier: NCT00718653
172 Li, L. et al. (2010) Supplementation with lutein or lutein plus green tea extracts does not change oxidative stress in adequately nourished older adults. Journal of Nutritional Biochemistry 21, 544-549
173 Singh, S. and Aggarwal, B.B. (1995) Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. Journal of Biological Chemistry 270, 24995-25000
174 Scapagnini, G. et al. (2006) Curcumin activates defensive genes and protects neurons against oxidative stress. Antioxidants and Redox Signaling 8, 395-403
175 Abe, Y., Hashimoto, S. and Horie, T. (1999) Curcumin inhibition of inflammatory cytokine production by human peripheral blood monocytes and alveolar macrophages. Pharmacological Research 39, 41-47
176 Mandal, M.N. et al. (2009) Curcumin protects retinal cells from light-and oxidant stress-induced cell death. Free Radical Biology and Medicine 46, 672-679
177 Dubey, A.K. et al. (2004) Ginkgo biloba – an appraisal. [Review] [44 Refs]. Kathmandu University Medical Journal 2, 225-229
178 King, R.E., Kent, K.D. and Bomser, J.A. (2005) Resveratrol reduces oxidation and proliferation of human retinal pigment epithelial cells via extracellular signal-regulated kinase inhibition. Chemico-Biological Interactions 151, 143-149
179 Kumar, A. and Sharma, S.S. (2010) NF-kappaB inhibitory action of resveratrol: a probable mechanism of neuroprotection in experimental diabetic neuropathy. Biochemical and Biophysical Research Communications 394, 360-365
180 Kook, D. et al. (2008) The protective effect of quercetin against oxidative stress in the human RPE in vitro. Investigative Ophthalmology Visual Science 49, 1712-1720
181 Wielgus, A.R. et al. (2007) Phototoxicity in human retinal pigment epithelial cells promoted by hypericin, a component of St. John's wort. Photochemistry and Photobiology 83, 706-713
182 Wong, W.T. et al. (2010) Treatment of geographic atrophy by the topical administration of OT-551: results of a phase II clinical trial. Investigative Ophthalmology Visual Science 2010 Jun 23 [Epub ahead of print]
183 Wan, F. and Lenardo, M.J. (2010) The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives. Cell Research 20, 24-33
184 Dutta, J. et al. (2006) Current insights into the regulation of programmed cell death by NF-kappaB. Oncogene 25, 6800-6816
185 Hatada, E.N., Krappmann, D. and Scheidereit, C. (2000) NF-kappaB and the innate immune response. Current Opinion in Immunology 12, 52-58
186 Mankan, A.K. et al. (2009) NF-kappaB regulation: the nuclear response. Journal of Cellular and Molecular Medicine 13, 631-643
187 Nordberg, J. and Arner, E.S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radical Biology and Medicine 31, 1287-1312
188 Berndt, C., Lillig, C.H. and Holmgren, A. (2007) Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. AJP – Heart and Circulatory Physiology 292, H1227-H1236
189 Wang, X.W. et al. (2008) Thioredoxin-like 6 protects retinal cell line from photooxidative damage by upregulating NF-kappaB activity. Free Radical Biology and Medicine 45, 336-344
190 Tanito, M. et al. (2002) Cytoprotective effect of thioredoxin against retinal photic injury in mice. Investigative Ophthalmology and Visual Science 43, 1162-1167
191 Arnér, E.S.J. and Holmgren, A. (2006) The thioredoxin system in cancer. Seminars in Cancer Biology 16, 420-426
192 Migliaccio, E. et al. (1999) The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature 402, 309-313
193 Wu, Z. et al. (2006) Reduction of p66Shc suppresses oxidative damage in retinal pigmented epithelial cells and retina. Journal of Cellular Physiology 209, 996-1005
194 Capitani, N. et al. (2010) Impaired expression of p66Shc, a novel regulator of B-cell survival, in

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

chronic lymphocytic leukemia. Blood 115, 3726-3736
195 Li, Q. et al. (2008) Downregulation of p22phox in retinal pigment epithelial cells inhibits choroidal neovascularization in mice. Molecular Therapy: The Journal of the American Society of Gene Therapy 16, 1688-1694
196 Chen, B. et al. (2009) Delivery of antioxidant enzyme genes to protect against ischemia/reperfusion-induced injury to retinal microvasculature. Investigative Ophthalmology and Visual Science 50, 5587-5595
197 Rex, T.S. et al. (2004) Adenovirus-mediated delivery of catalase to retinal pigment epithelial cells protects neighboring photoreceptors from photo-oxidative stress. Human Gene Therapy 15, 960-967
198 Gao, X. and Talalay, P. (2004) Induction of phase 2 genes by sulforaphane protects retinal pigment epithelial cells against photooxidative damage. Proceedings of the National Academy of Sciences of the United States of America 101, 10446-10451
199 del, V.C. et al. (2008) Demonstration by redox fluorometry that sulforaphane protects retinal pigment epithelial cells against oxidative stress. Investigative Ophthalmology and Visual Science 49, 2606-2612
200 Liu, X.P. et al. (2007) Extract of Ginkgo biloba induces phase 2 genes through Keap1-Nrf2-ARE signaling pathway. Life Sciences 80, 1586-1591
201 Talalay, P. (2000) Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 12, 5-11
202 Talalay, P., Dinkova-Kostova, A.T. and Holtzclaw, W.D. (2003) Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Advances in Enzyme Regulation 43, 121-134
203 Dong, A. et al. (2007) Increased expression of glial cell line-derived neurotrophic factor protects against oxidative damage-induced retinal degeneration. Journal of Neurochemistry 103, 1041-1052
204 Godley, B.F. et al. (2002) Bcl-2 overexpression increases survival in human retinal pigment epithelial cells exposed to H(2)O(2). Experimental Eye Research 74, 663-669
205 Chen, J. et al. (2006) Rare earth nanoparticles prevent retinal degeneration induced by intracellular peroxides. Nature Nanotechnology 1, 142-150
206 Fawcett, R.J. and Osborne, N.N. (2007) Flupirtine attenuates sodium nitroprusside-induced damage to retinal photoreceptors, in situ. Brain Research Bulletin 73, 278-288
207 Pitha-Rowe, I. et al. (2009) Synthetic triterpenoids attenuate cytotoxic retinal injury: cross-talk between Nrf2 and PI3 K/AKT signaling through inhibition of the lipid phosphatase PTEN. Investigative Ophthalmology and Visual Science 50, 5339-5347
208 Petronelli, A., Pannitteri, G. and Testa, U. (2009) Triterpenoids as new promising anticancer drugs. Anticancer Drugs 20, 880-892
209 Onaivi, E.S. (2009) Cannabinoid receptors in brain: pharmacogenetics, neuropharmacology, neurotoxicology, and potential therapeutic applications. International Review of Neurobiology 88, 335-369
210 Matias, I. et al. (2006) Changes in endocannabinoid and palmitoylethanolamide levels in eye tissues of patients with diabetic retinopathy and age-related macular degeneration. Prostaglandins, Leukotrienes, and Essential Fatty Acids 75, 413-418
211 Wei, Y., Wang, X. and Wang, L. (2009) Presence and regulation of cannabinoid receptors in human retinal pigment epithelial cells. Molecular Vision 15, 1243-1251
212 Butler, H. and Korbonits, M. (2009) Cannabinoids for clinicians: the rise and fall of the cannabinoid antagonists. European Journal of Endocrinology 161, 655-662
213 Lacombe, C. and Mayeux, P. (1998) Biology of erythropoietin. Haematologica 83, 724-732
214 Katavetin, P. et al. (2007) Antioxidative effects of erythropoietin. Kidney International. Supplement S10-S15
215 Chung, H. et al. (2009) Neuroprotective role of erythropoietin by antiapoptosis in the retina. Journal of Neuroscience Research 87, 2365-2374
216 Shah, S.S., Tsang, S.H. and Mahajan, V.B. (2009) Erythropoetin receptor expression in the human diabetic retina. BMC Research Notes 2, 234
217 Wang, Z.Y. et al. (2009) Erythropoietin protects retinal pigment epithelial cells from oxidative damage. Free Radical Biology and Medicine 46, 1032-1041
218 Rex, T.S. et al. (2009) Neuroprotection of photoreceptors by direct delivery of erythropoietin to the retina of the retinal degeneration slow mouse. Experimental Eye Research 89, 735-740
219 Yodoi, Y. et al. (2007) Circulating hematopoietic stem cells in patients with neovascular age-related macular degeneration. Investigative Ophthalmology and Visual Science 48, 5464-5472

Accession information: doi:10.1017/S146239941000164X; Vol. 12; e34; October 2010

220 Mattison, J.A. et al. (2007) Dietary restriction in aging nonhuman primates. Interdiscip Top Gerontol 35, 137-158
221 Li, D., Sun, F. and Wang, K. (2003) Caloric restriction retards age-related changes in rat retina. Biochemical and Biophysical Research Communications 309, 457-463
222 Gouras, P. et al. (2008) Drusenoid maculopathy in rhesus monkeys (Macaca mulatta): effects of age and gender. Graefes Archive for Clinical and Experimental Ophthalmology 246, 1395-1402
223 Kanda, A., Chen, W., Othman, M. et al. (2007) A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. Proceedings of the National Academy of Sciences of the United States of America 104, 16227-16232

Further reading, resources and contacts

Reviews

Swaroop, A. et al. (2009) Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annual Review of Genomics and Human Genetics 10, 19-43
A summary of the current literature regarding the pathogenesis of AMD.

Muller, F.L. et al. (2007) Trends in oxidative aging theories. Free Radical Biology and Medicine 43, 477-503
Background of the role of oxidative stress in ageing in general.

Brennan, L.A. and Kantorow, M. (2009) Mitochondrial function and redox control in the aging eye: role of MsrA and other repair systems in cataract and macular degenerations. Experimental Eye Research 88, 195-203
Greater detail of the oxidative processes in the ageing eye.

Boulton, M. et al. (2001) Retinal photodamage. Journal of Photochemistry and Photobiology B 64, 144-161
Greater detail of photo-oxidation.

Cai, J. et al. (2000) Oxidative damage and protection of the RPE. [Review] [138 refs]. Progress in Retinal and Eye Research 19, 205-221
Greater detail of the oxidative process in relation to the RPE cell.

Websites

Online Mendelian Inheritance in Man (OMIM) is a comprehensive database of gene-specific information: http://www.ncbi.nlm.nih.gov/omim

A database of current clinical trials can be found at: http://www.clinicaltrials.gov

Features associated with this article

Figures

Figure 1. Changes in the retina in dry and wet age-related macular degeneration (AMD).
Figure 2. Fundus photographs depicting age-related macular degeneration (AMD).
Figure 3. The antioxidant system and sources of reactive oxygen species in the body.
Figure 4. Oxidative processes occurring in the retina.

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5.4 Abstract of presentation given at ARVO 2011 + travel award

Investigative Ophthalmology & Visual Science

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QUICK SEARCH: Author: Keyword(s): Go Year: Vol: Page:

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1233

Prevalence Of AMD In A Liver Transplant Cohort

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Commercial Relationships: Sam Khandhadia, None; **Andrew J. Lotery**, None

Support: TFC Frost Charitable Trust

Abstract

Purpose: A cohort of liver transplanted patients was recruited, to investigate whether systemic gene therapy in the form of liver transplant can influence the risk of developing age-related macular degeneration (AMD). Of note, the liver is the predominant site of complement protein production in the body. This includes complement factor H (CFH), a regulator of the alternate complement pathway. Mutations in the CFH gene, have been strongly associated with AMD. The primary study hypothesis is that systemic replacement of a mutated CFH gene with a wild-type copy, via liver transplant, can reduce the risk of developing AMD. The prevalence of AMD in this cohort, is presented.

Methods: Patients over 55 years old of Caucasian origin with a history of liver transplantation at least 5 years previously were recruited at 4 specialist liver transplant centres in the UK, over an 18 month period. Dilated fundus examination and photos were taken for AMD grading via a standard scale (Age-Related Eye Disease Study AREDS grading). Bloods were also taken for recipient genotyping, and donor tissue retrieved for donor genotyping.

Results: 197 patients with gradable eyes have been recruited to date. The mean age (+/- 1 SD) was 66.8 +/- 5.9 years, and the mean duration post liver transplant 11.8 +/- 4.5 years. The prevalence of AMD in either eye (grades 2-5) was 52.7% (see figure 1)

Conclusions: This is the first report of the prevalence of AMD in a liver transplant cohort. AMD seems to be more prevalent in this cohort than in the normal population. This may be the result of systemic illness. Alternately receipt of a liver with varying CFH gene status to that originally in the recipient may influence the risk of developing AMD.

Travel Grant Award Notification!

grants@arvo.org [grants@arvo.org]

Sent: 09 February 2011 17:33

To: Khandhadia Sam (SOUTHAMPTON UNIVERSITY HOSPITALS NHS TRUST)

Dear Sam Khandhadia,

Congratulations!

On behalf of the Association for Research in Vision and Ophthalmology (ARVO), I am pleased to inform you that you have been selected to receive an ARVO Foundation for Eye Research/Pauline and Oswald Lapp Travel Grant in the amount of $1,100.00. These funds are to provide partial travel support to attend the ARVO Annual Meeting, May 1 - 5, in Fort Lauderdale, Florida.

Please DO NOT REGISTER for the Meeting! Your complimentary registration will be processed within the few weeks and you will receive an e-mail receipt. If you have previously registered, you will automatically be issued a refund.

To accept this grant YOU MUST REPLY to this e-mail by Thursday, FEBRUARY 24.

If for any reason you cannot accept this grant, including withdrawal of your abstract or if you have been awarded a different grant to attend this Meeting, please let us know immediately at grants@arvo.org. The grant will become null and void if you do not attend and present at the ARVO Annual Meeting.

You will be reimbursed following the meeting for actual expenses incurred up to the amount of this award. Qualifying expenses include:

- Round-trip economy airfare (maximum $1,500 international)
- Ground transportation to/from airports and airport parking (ARVO does not reimburse for rental cars.)
- Hotel room and taxes for up to 6 nights (maximum $200 per night)
- Per diem of $60 per day (up to 7 days, including travel days)for meals and incidentals; receipts are NOT required for these expenses.

Prior to the meeting, we will forward additional information to you concerning photo times and name badges.

We anticipate that you will find attending the ARVO Annual Meeting will be a most worthwhile experience and will serve as a catalyst for future research contributions in eye research.

If you have any questions, please contact Andrew Dobos, adobos@arvo.org.

5.5 Presentation at Royal College of Ophthalmology Conference, 2011

12.45 - 1.30: LUNCH
View the DVD & Poster Exhibitions

1.30 - 3.00 HALL 9
ACADEMIC OPHTHALMOLOGY & TRANSLATIONAL RESEARCH
Professor Andrew Lotery, Consultant Ophthalmologist
Southampton Eye Unit &
Professor Andrew Dick, Consultant Ophthalmologist,
Bristol Eye Hospital

Afternoon session 1.30 – 3.00 pm
Professor Andrew Lotery

1.30 – 1.45
Ocular Surface Impression Cytology: What else does it have to offer?
Mr. Geraint Williams, Wellcome Trust Clinical Fellow
University of Birmingham

1.45 – 2.00
AMD in a liver transplant cohort
Mr. Sam Khandhadia, Research Registrar in Ophthalmology
Southampton General Hospital

2.00 – 2.15
Isolation and Propagation of Stem Cell-rich Sites of the Conjunctiva
Miss Rosalind Stewart, Clinical Research Fellow
University of Liverpool

2.15 – 2.30
Cu2+ selective chelation with trientine prevents neurovascular changes in diabetic rat retinas
Dr. Marta Ugarte, NIHR Clinical Lecturer in Ophthalmology
University of Manchester

2.30 – 3.00
Translational Research Changes Lives
Professor Peng Khaw, Consultant Ophthalmologist
NIHR Biomedical Research Centre in Ophthalmology at Moorfields Eye Centre and UCL Institute of Ophthalmology

1.30 - 3.00 HALL 1
OPHTHALMIC EMERGENCIES
Dr. Carrie MacEwen, Consultant Ophthalmologist
Ninewells Hospital & Medical School, Dundee

Sudden vascular visual loss
Mr. Mike Burdon, Consultant Ophthalmologist
Selly Oak Hospital, Birmingham

Lacerating injuries
Dr. Vikas Chadha, Consultant Ophthalmologist
Gartnavel General Hospital, Glasgow

Corneal emergencies
Mr. Peter McDonnell, Consultant Ophthalmologist
Birmingham & Midland Eye Centre

Orbital angst
Mr. Brian Leatherbarrow, Consultant Ophthalmologist
Manchester Royal Eye Hospital

1.30 - 3.15 HALL 5
MANAGING PAEDIATRIC CORNEAL DISEASES
Mr. Ken Nischal, Consultant Ophthalmologist
Great Ormond Street Hospital for Children

Congenital corneal opacification – time for a re-think
Mr. William Moore, Consultant Ophthalmologist
Great Ormond Street Hospital for Children

Congenital corneal opacification – A lifetime of hardwork
Professor Stuart Brown, Professor of Ophthalmology
University California San Diego, USA

The role of automated, onlay and overlay lamellar keratoplasty in children
Mr. Sheraz Daya, Consultant Ophthalmologist
Queen Victoria Hospital, East Grinstead

Corneal dystrophies in children look different
Mr. Susmito Biswas, Consultant Ophthalmologist
Manchester Royal Eye Hospital

Alternatives to PKP in Paediatric Corneal Opacification
Mr. Ken Nischal

2.00 - 3.30 HALL 6
£ RETINAL IMAGING COURSE PART 2
DISCUSSIONS & DEMONSTRATIONS
Mr. Paulo Stanga, Consultant Ophthalmologist
Manchester Royal Eye Hospital &
Mr. James Talks, Consultant Ophthalmologist
Royal Victoria Infirmary, Newcastle upon Tyne &
Ms. Jane Gray, Head of Ophthalmic Imaging Department
Manchester Royal Eye Hospital

New imaging techniques and clinical cases have been added to last year's programme and handouts with the slides of each presentation will be provided.

A series of educational retinal cases will be available on laptops to be worked through by the delegates with the session presenters.

Delegates will be allocated into groups and the presenters will rotate between the groups to achieve one-to-one interaction.

There will be hands-on OCT and Wide-angle Fluorescein Angiography and Fundus Autofluorescence Imaging equipment demonstrations by Optos UK and Topcon UK.

By the end of the symposium delegates should have not only increased their knowledge in the interpretation of the results obtained with the presented imaging techniques but also be able to image patients themselves.

Places are limited and priced at £45 for both parts. Please check availability with registration staff

5.6 Ethical approval for project

NHS

National Research Ethics Service

SOUTHAMPTON & SOUTH WEST HAMPSHIRE RESEARCH ETHICS COMMITTEE (B)

1st Floor, Regents Park Surgery
Park Street, Shirley
Southampton
Hampshire
SO15 4RJ

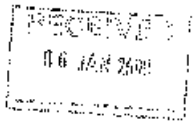
Tel: 023 8033 2400
023 8033 3462
Fax: 023 8033 4110

Email: sotha.SWHREC@nhs.net

RK/sta

02 January 2009

Professor Andrew Lotery
Professor of Ophthalmology
University of Southampton
MP506, Clinical Neurosciences Div
Southampton General Hospital
Southampton
SO16 6YD



Dear Professor Lotery

Full title of study: **Determining the Association with a Genetic Mutation in the Complement Factor H Gene and Age-Related Macular Degeneration in a Liver Transplant Patient Series**

REC reference number: **08/H0504/191**

Thank you for your letter of 15 December 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Alternate Vice-Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. We will write to you again as soon as the Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.reforum.nhs.uk.

The following changes are required to the documentation supplied and revised copies submitted for information.

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

5.7 Example of patient invitation letter

University Hospitals Birmingham NHS
NHS Foundation Trust

PATIENT INVITATION LETTER

Study title: "Role of CFH mutations in liver and eye disease"

Ref: 08/H0504/191

Dear Sir / Madam,

We would like to invite you to participate in a research project we are carrying out with eye doctors from the University of Southampton. You have been sent this letter because you are attending our liver unit following a liver transplant.

The project is investigating whether a liver transplant can in some cases increase or decrease the risk of a patient developing a common eye condition called age-related macular degeneration. Please note that liver transplant itself will not necessarily put you at an increased risk of an eye condition.

If you kindly agree to take part, then this research project will take place either at one of your routine visits to the liver outpatients department, or on a separate day (if convenient to you), and will take about 30-45 minutes altogether (the exact day and location will be confirmed later)

As part of the research:

1. We will ask you some questions about your general health.
2. We will perform an eye examination
3. We will take a photograph (+ scan, if available) of the back of your eyes.
4. We will take a blood sample from you.

We may ask you to have a repeated eye examination in 2 years time

We will need to put in drops to examine the back of your eyes, and this may blur your vision for a few hours afterwards. As a result you will not be able to drive for up to 2-4 hours after, till your vision returns back to normal.

If your English is not adequate, it would be very useful to bring an English-speaking relative or friend with you to help you.

Please take time to read the enclosed information sheet. If you decide to take part in this study, please could you send the reply slip back to us in the enclosed stamped addressed envelope. One of the research team will subsequently get in touch with you to supply you with more information on

when and where the research clinic may take place. The research team will consist of doctors and nurses from the University of Southampton and University Hospitals Birmingham NHS Trust.

Yours sincerely,

Professor David Adams
Liver Unit
University Hospitals Birmingham NHS Foundation Trust
Queen Elizabeth Hospital
Birmingham
B15 2TH

5.8 Example of patient information sheet

University Hospitals Birmingham NHS
NHS Foundation Trust

PATIENT INFORMATION SHEET

STUDY TITLE: Role of CFH mutations in liver and eye disease (Ref: protocol version 3, 05 Dec 09)

CHIEF INVESTIGATOR: Professor Andrew Lotery, Southampton Eye Unit, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD. Tel: 02380 795049

LOCAL INVESTIGATOR: Professor David Adams, Liver Unit, University Hospitals Birmingham NHS Foundation Trust, Queen Elizabeth Hospital, Birmingham, B15 2TH

We would like to invite you to take part in a research study. Before you decide, you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?

- We hope to increase the understanding of a common cause of visual problems in the elderly called Age-Related Macular Degeneration (ARMD), and whether or not some liver transplants can be protective against ARMD.
- A person's liver has many beneficial functions. One of these is to produce proteins to control the level of inflammation in the body. These proteins are produced by genes.
- Some people have changes in these genes, which might cause more inflammation in parts of the body, including the eye. It is possible that this might be a cause of ARMD.
- Recently a change in one such gene called Complement Factor H (CFH) has been found to be more common in patients with ARMD. We would like to find out if someone who originally has changes in the CFH gene can be protected from developing ARMD, if they happen to receive a normal copy of this gene from a liver transplant.
- We would like to look at patient's eyes for any sign of ARMD, and look for any changes in the CFH gene and other genes related to ARMD from DNA samples.
- This may suggest that we may be able to prevent ARMD in future with a new form of treatment replacing a changed gene in the liver with a normal one.
- Please note that this does not mean you are necessarily at increased risk of developing ARMD or any other eye condition, just because you have had a liver transplant.

Why have I been invited to take part in this study?

- You have been chosen to take part in this study because you have had a liver transplant at least 5 years ago, and are over 55 years old. We hope to select about 420 patients altogether to take part in this study.
- Please note this does NOT mean you necessarily have an eye condition, although part of the research involves looking at the back of your eye (which may reveal a condition – see below).

Do I have to take part?

- It is up to you. If you do not want to take part then you will continue to receive the usual medical care from your doctor. You will not jeopardise this in any way.

What will happen to me if I take part?

- We would like to carry out the following. This could be done on the same day as your routine check-up at the liver out-patients clinic, or on another day if convenient for you. The research clinics will not necessarily be in the liver clinic, but will be at the same hospital (the exact day and location will be confirmed later). There will be a maximum of 2 visits.

VISIT 1

1. **Medical history**: We would like to ask you some questions about your general health, previous medical history, and any eye problems.
2. **Eye examination**: We would like to examine your eyes using a special microscope. To do this, we would like to put some drops into your eyes to dilate your pupils to allow us to look at the back of your eyes. If you have had an eye examination before, you may have had these drops put in.
3. **Eye photo**: We would like to take a photo of the retina at the back of your eye using a special eye camera.
4. **Eye scan**. If available, we would also like to take pictures using an eye scanner, called an OCT (optical coherence tomogram), to obtain detailed pictures of the back of your eye. The OCT scan is a safe, non-invasive imaging system already in wide usage worldwide, and is not known to pose any risk to patients.
5. **Blood test**: We would like to take about 6 teaspoons of blood, to obtain a sample of your DNA and identify any changes in genes related to ARMD. We would also measure some proteins in the blood which can go up in inflammation, to see if there is any link with the genes being analysed. You should be able to have these blood tests at the same time as your routine ones, to save you being bled twice.

VISIT 2 – about 2 years later

1. If possible, we may contact you to repeat the above in about 2 years time (but we will probably not need to take another blood sample)

<u>What other information do we require for the study?</u>

- **Donor DNA:** We would like to obtain DNA from the person who donated your liver. We will try to obtain this from previously stored samples at the time of the transplant. If this is not available then we can obtain this from a previous biopsy from your transplanted liver. If this also is not available, then we would like your permission to use a small residual sample from a routine liver biopsy which you might have in the future as a part of your normal care. Please note this study does **NOT REQUIRE** you to have an additional biopsy. We would like to analyse this for changes in genes related to ARMD.

- **Previous eye records:** We would also like to contact your opticians or eye doctor for any previous eye records before your liver transplant, so we can get an idea of what your eyes were like before the transplant.

How much of my time will the study take?

- The research will take about 30-45 minutes of your time per visit, on a maximum of 2 visits.

Expenses and payments

- We are unable to offer any expenses or payments for taking part in the study.

Will taking part in the study affect my daily life? Is there anything I am not allowed to do while taking part in the study?

- We will not be asking you to make any other changes to your medical care, and you should continue your day-to-day activities as normal.

What are the possible benefits of taking part?

- Your eyes will be examined by a trained ophthalmologist.
- In general the study will increase understanding of ARMD and may also offer the potential for the development of effective preventative therapy although it is unlikely this will be of direct benefit to you.

What are the possible disadvantages and risks of taking part?

- If it is found that you have a mutated gene, then this may also suggest you are more likely to develop ARMD in the future, and also may imply a genetic predisposition in other family members. This information could be linked back to you as there will be a record kept that links you to this blood sample. However, this information will be kept confidentially by Professor Lotery. If you wish to discuss this further, then please phone Professor Lotery's secretary on 023 8079 5049. Genetic counselling can be offered if required.
- The risks of providing a blood sample are mild bruising of the skin and some discomfort from the needle.
- The dilating drops may blur your near vision for 2-4 hours. Unfortunately this means you will be unable to drive for this time, until your vision returns to normal. We can arrange to see you a few hours before your outpatient appointment in the liver unit, so that by the time you finish your appointment with the liver team, your vision should return back to normal, and you should be able to drive then. NB although the drops take 2-4 hours to wear off, we only need to spend 30-45 minutes with you at the research clinic.

- You may also be sensitive to bright light during this time as well. Other side effects are very uncommon. However if you experience any side effects from the eye examination (such as pain or prolonged reduced vision) then please contact your local eye casualty department for further advice.

What happens when the research study stops?

- You will continue to receive the usual medical care from your doctor.
- The DNA and serum/plasma from the blood sample you have given will be kept with others like it so that Professor Lotery can continue to study macular degeneration for many years to come. However you can ask us to remove your sample at any time. Your DNA and serum/plasma sample may also be used for other unrelated studies in the future, but only if ethical approval has been given.
- We may contact you in the future regarding other research studies.

What will happen in the unlikely event that I might lose the ability to consent during the study?

- No further data will be collected
- We would like to still retain the tissue and personal data collected already and use this confidentially in connection with this project. We also may use this information for further research in future, for projects which are ethically approved.

Will my taking part in the study be kept confidential?

- Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.
- Your blood samples, photographs and clinical information will be kept in an anonymised state, linked only to your personal information by a record kept separately in a file, in a locked secure room on site. A copy will also be kept in a locked filing cabinet in Professor Lotery's office at Southampton General Hospital. This information will only be accessible to the research team. The sample may be shared with scientists outside Professor Lotery's laboratory for other projects, but all identifying information will be removed from the sample before it leaves the laboratory.
- You may have your personal information removed from this file at any time by contacting the investigators.

What will happen if I don't want to carry on with the study?

- You will continue to receive the usual medical care from your doctor. If you wish to withdraw, then we would like to use any information collected

so far. Please let us know if you do not wish this to be the case. Any extra information collected by us can be destroyed securely if you wish.

Will my taking part in this study be kept confidential?

- All information which is collected about you during the course of the research will be kept strictly confidential. Your confidentiality will be safeguarded both during and after the study. Our procedures for handling, processing, storage and destruction of your data match the Data Protection Act of 1998.
- Your data will be collected on paper initially. This will be kept in a locked room only accessible by the study personnel, at your hospital, and at Southampton General Hospital. This data will be transferred electronically onto a computer with restricted access, and will be password-protected.
- Confidential data may be transmitted electronically, but only via securely encrypted email which has national approval for transmission of confidential patient information
- Your data will be accessible only to the study personnel, and to certain Research Departments for monitoring the quality of the study. These include the Research Governance Office of the University of Southampton (the sponsor), and the Research and Development Department of your local NHS trust. No-one else will have access to your data.
- Please note that you will not be identified in any report/publication.
- Your data will be retained for a minimum of 5 years.

Will my GP know I am taking part in this study?

- We do not anticipate that there will be any relevant information that your GP will need to know regarding this study and so as not to overburden them with paperwork we will not contact them unless you specifically ask us to do so.

What if I am already taking part in another study?

- You may not be able to take part in this study if you are involved in another study. Please let us know.

What will happen to the results of the research study?

- We aim to analyse the results of the research and publish them in leading eye research journals. The results are likely to be published about three years from now and should be accessible from Professor Lotery's secretary on 02380 795049. However you will not personally be identified in any publication although anonymous photographs of your retina may be shown.

Who is organising and funding the research?

- This research is being organised by a research group at the University of Southampton, and is being carried out as part of a PhD research project. The chief investigator is Professor Andrew Lotery. This research has been funded by a research grant from Frimley Park Hospital NHS Trust. Further funding may be received in future from charities or government organisations.

Who has reviewed the study?

- All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given a favourable opinion by Southampton and South West Hampshire Research Ethics Committee.

Where can I obtain further information?

- For specific information about this research project, and for advice as to whether you should participate, please approach your doctor at the liver clinic. Alternately, please contact Professor Lotery's secretary on 02380 795049, who will put you in contact with one of the research team. If no one answers, please leave a message, and someone will get back to you.
- **For more information on age related macular degeneration:** www.rcophth.ac.uk/docs/college/patientinfo/UnderstandingAgeRelatedMacularDegeneration.pdf
- **For independent advice on research in general:** Please refer to the NHS Research Ethics Service website: www.nres.npsa.nhs.uk/public/index.htm
- **For independent advice on this particular research study:** You may contact the University of Southampton's Research Governance Office on 02380 598849, or write to: The Research Governance Manager, Research Governance Office, University of Southampton, Highfield, Southampton SO17 1BJ.
- You may also contact your own hospital's Patient Advice and Liaison Service (PALS).

Who can I contact in an emergency

For any emergencies out-of-hours:

- If eye-related: Please call your local eye casualty department
- If not eye-related: Please call your GP, or NHS Direct on 0845 4647

What if there is a problem? Who can I contact if I am unhappy or have concerns with the study.

- Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed.
- If you have concerns about any aspect of the way you have been approached or treated during the course of this study, you should ask to speak to the researchers who will do their best to answer your questions (via Professor Lotery's secretary on 023 8079 5049). You can also write to the chief investigator. The address is: Professor Andrew Lotery, Southampton Eye Unit, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD.
- If you remain unhappy and wish to complain formally, you may contact the University of Southampton's Research Governance Office on 02380 598849, or write to: The Research Governance Manager, Research Governance Office, University of Southampton, Highfield, Southampton SO17 1BJ.
- You may also contact your own hospital's Patient Advice and Liaison Service (PALS).
- We are legally bound to tell you the following: "If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. "

How long do I have to decide in I want to take part in the study?

- You have as long as you wish to decide. However we would be grateful if you could contact us after a few days of receiving this information sheet if you wish to take part.

What do I have to do if I decide I want to take part in the study?

- Please tick the box on the enclosed reply letter and send back in the enclosed stamped-addressed envelope.
- Alternately you may contact Professor Lotery via his secretary on 023 8079 5049.
- One of the research team will subsequently get in touch with you to supply you with more information on when and where the research clinic may take place. NB you may not necessarily be seen at your next clinic appointment. If you do not hear from one of the research team before this appointment, please assume that we are not able to see you at that visit.

Thank you for reading this information leaflet and for considering taking part in this study. You may keep this leaflet with you.

5.9 Example of informed consent form

University Hospitals Birmingham NHS
NHS Foundation Trust

CONSENT FORM (staged)

Study title: "Role of CFH mutations in liver and eye disease"

Patient sticker

Patient Identification Number for this study: _____

Chief Investigator: Professor Andrew Lotery, Southampton Eye Unit, Tremona Road, Southampton SO16 6YD Tel: 02380 795049

Local Investigator: Professor David Adams, Liver Unit, University Hospitals Birmingham NHS Foundation Trust, Queen Elizabeth Hospital, Birmingham, B15 2TH

PART A: Consent for the current study

(samples to be destroyed on study completion unless part B completed)

PLEASE INITIAL THE BOXES IF YOU AGREE WITH EACH SECTION:

1. I confirm that I have read and understand the information sheet dated 05 December 2009 (version 4) for the above study and have been given a copy to keep. I have been able to ask questions about the study and I understand why the research is being done and any risks involved. ☐

2. I agree to give a sample of blood for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without my medical treatment or legal rights being affected. ☐

3. I agree to allow collection of a sample of previously stored tissue / DNA from the liver donor, or a residual sample from previous / future routine liver biopsies from myself. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without my medical treatment or legal rights being affected. ☐

4. I give permission for sections of my medical notes to be looked at by responsible individuals where it is relevant to this study. I expect that my medical notes will be treated confidentially at all times. ☐

5. I give permission for my previous opticians / eye doctor to be contacted to obtain previous records of my eye examinations ☐

6. I understand that I will not benefit financially if this research leads to the development of a new treatment or test. ☐

7. I know how to contact the research team if I need to. ☐

8. I agree to take part in the above study ☐

Name of Patient	Signature	Date

Researcher	Signature	Date

When completed, 1 for patient; 1 (orginal) for researcher site file; 1 to be kept in hospital medical notes

PART B: Samples for storage and use in possible future studies - Linked anonymised samples

9. I give permission for my sample and the information gathered about me to be stored by Professor Andrew Lotery at the University of Southampton Eye Laboratory, Southampton General Hospital, for possible use in future projects, as described in the information sheet. I understand that some of these projects may be carried out by other researchers. I understand that future studies will be reviewed and approved by a Research Ethics Committee prior to my sample being used, and that I can alter these decisions at any stage by letting the research team know.

   a. I give permission for the sample to be used for research about eye disease ☐

   b. I give permission for the sample to be used for other unrelated research studies the precise nature of which will depend upon future scientific advances. ☐

10. I want / do not want (delete as applicable) to be told the results of any future test which may have health implications for me. ☐

11. I give permission for sections of my medical notes to be looked at by responsible individuals where it is relevant to such future study. I expect that my medical notes will be treated confidentially at all times. ☐

12. I agree to be contacted in future regarding other studies ☐

Name of Patient	Signature	Date
Researcher	Signature	Date

When completed, 1 for patient; 1 (original) for researcher site file; 1 to be kept in hospital medical notes.

5.10 Example of proforma

PROFORMA

Study title:
"ROLE OF CFH MUTATIONS IN LIVER AND EYE DISEASE"

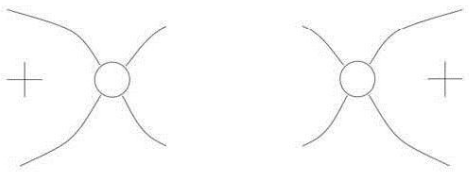
REC ref: 08/H0504/191

CI: Professor Andrew Lotery,
Consultant Ophthalmologist,
Southampton General Hospital

STUDY ID: LB

INITIALS:

Date	
Site	BIRMINGHAM
Gender	Male / Female
Age	
Year of transplant operation	
Hospital where transplant carried out (pls circle / list)	Birmingham Other: _____
Type of transplant (pls circle)	Complete / Partial
Reason for liver transplant (pls circle / list)	Hepatitis B Other: Hepatitis C Alcoholic liver disease Autoimmune Primary Biliary Cirrhosis Primary Sclerosing Cholangitis Hepatocellular carcinoma
Any other transplants? (please circle)	None / Previous liver transplant / Previous kidney transplant / Other Details:
Any previous liver biopsies at Southampton?	Yes. When approx was last one: _____ No. Done where: _____
Medical history (pls circle / list)	High blood pressure Other: High cholesterol Diabetes Angina / Heart attack Mini strokes (TIAs) or full strokes Colitis

Medications (pls circle / list)	Cyclosporin Other: Mycophenolate Azathioprine Prednisolone Sirolimus / Tacrolimus Ursodeoxycholic acid Statin Aspirin
Any eye problems (besides glasses), including operations	
Smoking status (pls circle / list)	Never / Current / Ex (gave up _____ years ago) If current / ex: No of years smoked for : _____ Average no per day: _____
Optician / previous eye clinic details	Name + address of opticians / eye specialist who has checked your eyes (ideally before the liver transplant, otherwise the one closest to this date)
FOR RESEARCHER TO FILL IN:	
Slit lamp examination ☐ Not done	R: L: 
Photos taken	☐ Taken
Bloods	☐ Taken / Form given to patient
Other	
Height	
Weight	
BP	

References

1. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. Bull World Health Organ 2004;82:844-51.

2. Seddon JM, Reynolds R, Yu Y, et al. Risk models for progression to advanced age-related macular degeneration using demographic, environmental, genetic, and ocular factors. Ophthalmology 2011;118:2203-11.

3. Khandhadia S, Cipriani V, Yates JR, Lotery AJ. Age-related macular degeneration and the complement system. Immunobiology 2012;217:127-46.

4. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. Science 2005;308:385-9.

5. Haines JL, Hauser MA, Schmidt S, et al. Complement factor H variant increases the risk of age-related macular degeneration. Science 2005;308:419-21.

6. Edwards AO, Ritter R, III, Abel KJ, et al. Complement factor H polymorphism and age-related macular degeneration. Science 2005;308:421-4.

7. Halme J, Sachse M, Vogel H, et al. Primary human hepatocytes are protected against complement by multiple regulators. Mol Immunol 2009;46:2284-9.

8. Schulz TF, Schwable W, Stanley KK, Dierich MP. Use of a high-efficiency expression vector to isolate cDNA clones for factor H and map their positions within the molecule. Mol Immunol 1986;23:1243-8.

9. Hageman GS, Anderson DH, Johnson LV, et al. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci U S A 2005;102:7227-32.

10. Strachan T, Read AP. Human Molecular Genetics. 4th ed. New York: Garland Science; 2011.

11. National Center for Biotechnology Information (NCBI). A Science Primer. www.ncbi.nlm.nih.gov/About/primer/snps.html. 2007

12. International HapMap Project. What Is The Hapmap. http://hapmap.ncbi.nlm.nih.gov/whatishapmap.html.en. 2011

13. Kanda A, Chen W, Othman M, et al. A variant of mitochondrial protein LOC387715/ARMS2, not HTRA1, is strongly associated with age-related macular degeneration. Proc Natl Acad Sci U S A 2007;104:16227-32.

14. Kim S, Misra A. SNP genotyping: technologies and biomedical applications. Annu Rev Biomed Eng 2007;9:289-320.

15. Seland JH, Vingerling JR, Augood CA, et al. Visual impairment and quality of life in the older European population, the EUREYE study. Acta Ophthalmol 2011;89:608-13.

16. Klein R, Cruickshanks KJ, Nash SD, et al. The prevalence of age-related macular degeneration and associated risk factors. Arch Ophthalmol 2010;128:750-8.

17. Friedman DS, O'Colmain BJ, Munoz B, et al. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004;122:564-72.

18. de Jong PT. Age-related macular degeneration. N Engl J Med 2006;355:1474-85.

19. Klein R, Klein BE, Knudtson MD, et al. Prevalence of age-related macular degeneration in 4 racial/ethnic groups in the multi-ethnic study of atherosclerosis. Ophthalmology 2006;113:373-80.

20. Klein R, Klein BE, Jensen SC, Meuer SM. The five-year incidence and progression of age-related maculopathy: the Beaver Dam Eye Study. Ophthalmology 1997;104:7-21.

21. Snell RS, Lemp MA. Clinical anatomy of the eye. 2nd ed. Malden, MA: Blackwell Science; 1998.

22. Bressler NM, Bressler SB, Fine SL. Age-Related Macular Degeneration: Non-neovascular Early AMD, Intermediate AMD, and Geographic Atrophy. In: Ryan SJ, ed. Retina. Philadelphia, Pa.: Elsevier/Mosby, 2006:1017-74.

23. Boulton M, Dayhaw-Barker P. The role of the retinal pigment epithelium: topographical variation and ageing changes. Eye 2001;15:3-9.

24. Khandhadia S, Lotery A. Oxidation and age-related macular degeneration: insights from molecular biology. Expert Rev Mol Med 2010;12:e34.

25. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. Surv Ophthalmol 1995;39:367-74.

26. Klaver CC, Assink JJ, van LR, et al. Incidence and progression rates of age-related maculopathy: the Rotterdam Study. Invest Ophthalmol Vis Sci 2001;42:2237-41.

27. Augood CA, Vingerling JR, de Jong PT, et al. Prevalence of age-related maculopathy in older Europeans: the European Eye Study (EUREYE). Arch Ophthalmol 2006;124:529-35.

28. Ferris FL, III, Wilkinson CP, Bird A, et al. Clinical classification of age-related macular degeneration. Ophthalmology 2013;120:844-51.

29. Wang L, Clark ME, Crossman DK, et al. Abundant lipid and protein components of drusen. PLoS One 2010;5:e10329.

30. Owen CG, Jarrar Z, Wormald R, et al. The estimated prevalence and incidence of late stage age related macular degeneration in the UK. Br J Ophthalmol 2012;96:752-6.

31. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8.[Erratum appears in Arch Ophthalmol. 2008 Sep;126(9):1251]. Arch Ophthalmol 2001;119:1417-36.

32. Montgomery MP, Kamel F, Pericak-Vance MA, et al. Overall diet quality and age-related macular degeneration. Ophthalmic Epidemiol 2010;17:58-65.

33. Augood C, Chakravarthy U, Young I, et al. Oily fish consumption, dietary docosahexaenoic acid and eicosapentaenoic acid intakes, and associations with neovascular age-related macular degeneration. Am J Clin Nutr 2008;88:398-406.

34. Cho E, Seddon JM, Rosner B, et al. Prospective study of intake of fruits, vegetables, vitamins, and carotenoids and risk of age-related maculopathy. Arch Ophthalmol 2004;122:883-92.

35. Knudtson MD, Klein R, Klein BE, et al. Location of lesions associated with age-related maculopathy over a 10-year period: the Beaver Dam Eye Study. Invest Ophthalmol Vis Sci 2004;45:2135-42.

36. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432-44.

37. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419-31.

38. Amselem L, Diaz-Llopis M, Felipe A, et al. Clinical magnification and residual refraction after implantation of a double intraocular lens system in patients with macular degeneration. J Cataract Refract Surg 2008;34:1571-7.

39. Giansanti F, Eandi CM, Virgili G. Submacular surgery for choroidal neovascularisation secondary to age-related macular degeneration. Cochrane Database Syst Rev 2009;CD006931.

40. Smith W, Assink J, Klein R, et al. Risk factors for age-related macular degeneration: Pooled findings from three continents. Ophthalmology 2001;108:697-704.

41. Swaroop A, Chew EY, Rickman CB, Abecasis GR. Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annu Rev Genomics Hum Genet 2009;10:19-43.

42. Yoshida S, Yashar BM, Hiriyanna S, Swaroop A. Microarray analysis of gene expression in the aging human retina. Invest Ophthalmol Vis Sci 2002;43:2554-60.

43. Katz ML, Robison WG, Jr. Age-related changes in the retinal pigment epithelium of pigmented rats. Exp Eye Res 1984;38:137-51.

44. Wing GL, Blanchard GC, Weiter JJ. The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. Invest Ophthalmol Vis Sci 1978;17:601-7.

45. Iwasaki M, Inomata H. Lipofuscin granules in human photoreceptor cells. Invest Ophthalmol Vis Sci 1988;29:671-9.

46. Ng KP, Gugiu B, Renganathan K, et al. Retinal pigment epithelium lipofuscin proteomics. Molecular & Cellular Proteomics 2008;7:1397-405.

47. Rudnicka AR, Jarrar Z, Wormald R, et al. Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis. Ophthalmology 2012;119:571-80.

48. Espinosa-Heidmann DG, Marin-Castano ME, Pereira-Simon S, et al. Gender and estrogen supplementation increases severity of experimental choroidal neovascularization. Experimental Eye Research 2005;80:413-23.

49. Vanderbeek BL, Zacks DN, Talwar N, et al. Racial differences in age-related macular degeneration rates in the United States: a longitudinal analysis of a managed care network. Am J Ophthalmol 2011;152:273-82.

50. Kawasaki R, Yasuda M, Song SJ, et al. The prevalence of age-related macular degeneration in Asians: a systematic review and meta-analysis. Ophthalmology 2010;117:921-7.

51. Klein R, Klein BE, Linton KL, DeMets DL. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. Am J Epidemiol 1993;137:190-200.

52. Smith W, Mitchell P, Leeder SR. Smoking and age-related maculopathy. The Blue Mountains Eye Study. Arch Ophthalmol 1996;114:1518-23.

53. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. Arch Ophthalmol 1996;114:1193-6.

54. Chakravarthy U, Wong TY, Fletcher A, et al. Clinical risk factors for age-related macular degeneration: a systematic review and meta-analysis. BMC Ophthalmol 2010;10:31.

55. Casparis H, Lindsley K, Kuo IC, et al. Surgery for cataracts in people with age-related macular degeneration. Cochrane Database Syst Rev 2012;6:CD006757.

56. Wieberdink RG, Ho L, Ikram MK, et al. Age-related macular degeneration and the risk of stroke: the Rotterdam study. Stroke 2011;42:2138-42.

57. Ikram MK, Mitchell P, Klein R, et al. Age-related macular degeneration and long-term risk of stroke subtypes. Stroke 2012;43:1681-3.

58. Duan Y, Mo J, Klein R, et al. Age-related macular degeneration is associated with incident myocardial infarction among elderly Americans. Ophthalmology 2007;114:732-7.

59. Wong TY, Tikellis G, Sun C, et al. Age-related macular degeneration and risk of coronary heart disease: the Atherosclerosis Risk in Communities Study. Ophthalmology 2007;114:86-91.

60. Adams MK, Chong EW, Williamson E, et al. 20/20--Alcohol and age-related macular degeneration: the Melbourne Collaborative Cohort Study. Am J Epidemiol 2012;176:289-98.

61. Gold B, Merriam JE, Zernant J, et al. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 2006;38:458-62.

62. Hughes AE, Orr N, Esfandiary H, et al. A common CFH haplotype, with deletion of CFHR1 and CFHR3, is associated with lower risk of age-related macular degeneration. Nat Genet 2006;38:1173-7.

63. Fagerness JA, Maller JB, Neale BM, et al. Variation near complement factor I is associated with risk of advanced AMD. Eur J Hum Genet 2009;17:100-4.

64. Ennis S, Jomary C, Mullins R, et al. Association between the SERPING1 gene and age-related macular degeneration: a two-stage case-control study. Lancet 2008;372:1828-34.

65. Neale BM, Fagerness J, Reynolds R, et al. Genome-wide association study of advanced age-related macular degeneration identifies a role of the hepatic lipase gene (LIPC). Proc Natl Acad Sci U S A 2010;107:7395-400.

66. Chen W, Stambolian D, Edwards AO, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. Proc Natl Acad Sci U S A 2010;107:7401-6.

67. Klaver CC, Kliffen M, van Duijn CM, et al. Genetic association of apolipoprotein E with age-related macular degeneration. Am J Hum Genet 1998;63:200-6.

68. Yu Y, Bhangale TR, Fagerness J, et al. Common variants near FRK/COL10A1 and VEGFA are associated with advanced age-related macular degeneration. Hum Mol Genet 2011;20:3699-709.

69. Fritsche LG, Loenhardt T, Janssen A, et al. Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 2008;40:892-6.

70. Wang G, Spencer KL, Court BL, et al. Localization of age-related macular degeneration-associated ARMS2 in cytosol, not mitochondria. Invest Ophthalmol Vis Sci 2009;50:3084-90.

71. Ambati J, Fowler BJ. Mechanisms of age-related macular degeneration. Neuron 2012;75:26-39.

72. Ambati J, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. Nat Rev Immunol 2013;13:438-51.

73. Muller FL, Lustgarten MS, Jang Y, et al. Trends in oxidative aging theories. Free Radic Biol Med 2007;43:477-503.

74. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527-605.

75. Golden TR, Melov S. Mitochondrial DNA mutations, oxidative stress, and aging. Mech Ageing Dev 2001;122:1577-89.

76. Susin SA, Lorenzo HK, Zamzami N, et al. Mitochondrial release of caspase-2 and -9 during the apoptotic process. J Exp Med 1999;189:381-94.

77. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275:1129-32.

78. Gu X, Sun M, Gugiu B, et al. Oxidatively truncated docosahexaenoate phospholipids: total synthesis, generation, and Peptide adduction chemistry. Journal of Organic Chemistry 2003;68:3749-61.

79. Johnson LV, Ozaki S, Staples MK, et al. A potential role for immune complex pathogenesis in drusen formation. Exp Eye Res 2000;70:441-9.

80. Johnson LV, Leitner WP, Staples MK, Anderson DH. Complement activation and inflammatory processes in Drusen formation and age related macular degeneration. Exp Eye Res 2001;73:887-96.

81. Mullins RF, Russell SR, Anderson DH, Hageman GS. Drusen associated with aging and age-related macular degeneration contain proteins common to

extracellular deposits associated with atherosclerosis, elastosis, amyloidosis, and dense deposit disease. FASEB J 2000;14:835-46.

82. Sparrow JR, Vollmer-Snarr HR, Zhou J, et al. A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. Journal of Biological Chemistry 2003;278:18207-13.

83. Zhou J, Kim SR, Westlund BS, Sparrow JR. Complement activation by bisretinoid constituents of RPE lipofuscin. Investigative Ophthalmology & Visual Science 2009;50:1392-9.

84. Holtkamp GM, Kijlstra A, Peek R, de Vos AF. Retinal pigment epithelium-immune system interactions: cytokine production and cytokine-induced changes. Prog Retin Eye Res 2001;20:29-48.

85. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 2003;22:1-29.

86. Frank RN. Growth factors in age-related macular degeneration: pathogenic and therapeutic implications. Ophthalmic Res 1997;29:341-53.

87. Tsutsumi-Miyahara C, Sonoda KH, Egashira K, et al. The relative contributions of each subset of ocular infiltrated cells in experimental choroidal neovascularisation. Br J Ophthalmol 2004;88:1217-22.

88. Baudouin C, Peyman GA, Fredj-Reygrobellet D, et al. Immunohistological study of subretinal membranes in age-related macular degeneration. Jpn J Ophthalmol 1992;36:443-51.

89. Ebrahem Q, Renganathan K, Sears J, et al. Carboxyethylpyrrole oxidative protein modifications stimulate neovascularization: Implications for age-related macular degeneration.[Erratum appears in Proc Natl Acad Sci U S A. 2006 Oct 17;103(42):15722]. Proceedings of the National Academy of Sciences of the United States of America 2006;103:13480-4.

90. Imamura Y, Noda S, Hashizume K, et al. Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. Proceedings of the National Academy of Sciences of the United States of America 2006;103:11282-7.

91. Giuffre G. Main posterior watershed zone of the choroid. Variations of its position in normal subjects. Doc Ophthalmol 1989;72:175-80.

92. Straubhaar M, Orgul S, Gugleta K, et al. Choroidal laser Doppler flowmetry in healthy subjects. Arch Ophthalmol 2000;118:211-5.

93. Metelitsina TI, Grunwald JE, Dupont JC, et al. Foveolar choroidal circulation and choroidal neovascularization in age-related macular degeneration. Invest Ophthalmol Vis Sci 2008;49:358-63.

94. Starita C, Hussain AA, Pagliarini S, Marshall J. Hydrodynamics of ageing Bruch's membrane: implications for macular disease. Exp Eye Res 1996;62:565-72.

95. Francois J, De Laey JJ, Cambie E, et al. Neovascularization after argon laser photocoagulation of macular lesions. Am J Ophthalmol 1975;79:206-10.

96. Pruett RC, Weiter JJ, Goldstein RB. Myopic cracks, angioid streaks, and traumatic tears in Bruch's membrane. Am J Ophthalmol 1987;103:537-43.

97. Mullins RF, Olvera MA, Clark AF, Stone EM. Fibulin-5 distribution in human eyes: relevance to age-related macular degeneration. Exp Eye Res 2007;84:378-80.

98. Stone EM, Braun TA, Russell SR, et al. Missense variations in the fibulin 5 gene and age-related macular degeneration. N Engl J Med 2004;351:346-53.

99. Spraul CW, Lang GE, Grossniklaus HE, Lang GK. Histologic and morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in postmortem eyes with age-related macular degeneration and histologic examination of surgically excised choroidal neovascular membranes. Surv Ophthalmol 1999;44 Suppl 1:S10-S32.

100. Sheraidah G, Steinmetz R, Maguire J, et al. Correlation between lipids extracted from Bruch's membrane and age. Ophthalmology 1993;100:47-51.

101. Cherepanoff S, McMenamin P, Gillies MC, et al. Bruch's membrane and choroidal macrophages in early and advanced age-related macular degeneration. Br J Ophthalmol 2010;94:918-25.

102. Steen B, Sejersen S, Berglin L, et al. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1998;39:2194-200.

103. Janssen A, Hoellenriegel J, Fogarasi M, et al. Abnormal vessel formation in the choroid of mice lacking tissue inhibitor of metalloprotease-3. Invest Ophthalmol Vis Sci 2008;49:2812-22.

104. Guo L, Hussain AA, Limb GA, Marshall J. Age-dependent variation in metalloproteinase activity of isolated human Bruch's membrane and choroid. Invest Ophthalmol Vis Sci 1999;40:2676-82.

105. Curcio CA, Johnson M, Rudolf M, Huang JD. The oil spill in ageing Bruch membrane. Br J Ophthalmol 2011;95:1638-45.

106. Klein ML, Mauldin WM, Stoumbos VD. Heredity and age-related macular degeneration. Observations in monozygotic twins. Arch Ophthalmol 1994;112:932-7.

107. Mann SS, Rutishauser-Arnold Y, Peto T, et al. The symmetry of phenotype between eyes of patients with early and late bilateral age-related macular degeneration (AMD). Graefes Arch Clin Exp Ophthalmol 2011;249:209-14.

108. Lopez PF, Sippy BD, Lambert HM, et al. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1996;37:855-68.

109. Saint-Geniez M, Kurihara T, Sekiyama E, et al. An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris. Proc Natl Acad Sci U S A 2009;106:18751-6.

110. Kaneko H, Dridi S, Tarallo V, et al. DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. Nature 2011;471:325-30.

111. Wood P. Understanding immunology. 2nd ed. Harlow: Pearson /Prentice Hall; 2006.

112. Zhou W, Marsh J.E., Sacks S.H. Intrarenal synthesis of complement. http://www.nature.com/ki/journal/v59/n4/fig_tab/4492147f1.html. 2011

113. Janssen BJ, Huizinga EG, Raaijmakers HC, et al. Structures of complement component C3 provide insights into the function and evolution of immunity. Nature 2005;437:505-11.

114. Ripoche J, Day AJ, Harris TJ, Sim RB. The complete amino acid sequence of human complement factor H. Biochem J 1988;249:593-602.

115. Pangburn MK. Host recognition and target differentiation by factor H, a regulator of the alternative pathway of complement. Immunopharmacology 2000;49:149-57.

116. Zipfel PF, Skerka C, Hellwage J, et al. Factor H family proteins: on complement, microbes and human diseases. Biochem Soc Trans 2002;30:971-8.

117. Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where, when and why? Clin Exp Immunol 1997;107:1-7.

118. Schmeling A, Correns A, Staffa G, Geserick G. Changes in the phenotype of polymorphic plasma proteins after liver transplantation - new data and medico-legal consequences. Int J Legal Med 1999;112:364-7.

119. Gasque P, Fontaine M, Morgan BP. Complement expression in human brain. Biosynthesis of terminal pathway components and regulators in human glial cells and cell lines. J Immunol 1995;154:4726-33.

120. Cunha-Vaz JG. The blood-retinal barriers system. Basic concepts and clinical evaluation. Exp Eye Res 2004;78:715-21.

121. Chen H, Liu B, Lukas TJ, Neufeld AH. The aged retinal pigment epithelium/choroid: a potential substratum for the pathogenesis of age-related macular degeneration. PLoS One 2008;3:e2339.

122. Booij JC, van SS, Swagemakers SM, et al. Functional annotation of the human retinal pigment epithelium transcriptome. BMC Genomics 2009;10:164.

123. Chen M, Forrester JV, Xu H. Synthesis of complement factor H by retinal pigment epithelial cells is down-regulated by oxidized photoreceptor outer segments. Experimental Eye Research 2007;84:635-45.

124. Mandal MN, Ayyagari R. Complement factor H: spatial and temporal expression and localization in the eye. Invest Ophthalmol Vis Sci 2006;47:4091-7.

125. Coffey PJ, Gias C, McDermott CJ, et al. Complement factor H deficiency in aged mice causes retinal abnormalities and visual dysfunction. Proc Natl Acad Sci U S A 2007;104:16651-6.

126. Lundh von LP, Kam JH, Bainbridge J, et al. Complement factor h is critical in the maintenance of retinal perfusion. Am J Pathol 2009;175:412-21.

127. Faber C, Williams J, Juel HB, et al. Complement factor H deficiency results in decreased neuroretinal expression of Cd59a in aged mice. Invest Ophthalmol Vis Sci 2012;53:6324-30.

128. Lommatzsch A, Hermans P, Weber B, Pauleikhoff D. Complement factor H variant Y402H and basal laminar deposits in exudative age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 2007;245:1713-6.

129. Lommatzsch A, Hermans P, Muller KD, et al. Are low inflammatory reactions involved in exudative age-related macular degeneration? Morphological and immunhistochemical analysis of AMD associated with basal deposits. Graefes Arch Clin Exp Ophthalmol 2008;246:803-10.

130. Nozaki M, Raisler BJ, Sakurai E, et al. Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A 2006;103:2328-33.

131. Li M, Atmaca-Sonmez P, Othman M, et al. CFH haplotypes without the Y402H coding variant show strong association with susceptibility to age-related macular degeneration. Nat Genet 2006;38:1049-54.

132. Francis PJ, Schultz DW, Hamon S, et al. Haplotypes in the complement factor H (CFH) gene: associations with drusen and advanced age-related macular degeneration. PLoS One 2007;2:e1197.

133. Seddon JM, Reynolds R, Maller J, et al. Prediction model for prevalence and incidence of advanced age-related macular degeneration based on genetic, demographic, and environmental variables. Invest Ophthalmol Vis Sci 2009;50:2044-53.

134. Gibson J, Cree A, Collins A, et al. Determination of a gene and environment risk model for age-related macular degeneration. Br J Ophthalmol 2010;94:1382-7.

135. Kopplin LJ, Igo RP, Jr., Wang Y, et al. Genome-wide association identifies SKIV2L and MYRIP as protective factors for age-related macular degeneration. Genes Immun 2010;11:609-21.

136. Yates JR, Sepp T, Matharu BK, et al. Complement C3 variant and the risk of age-related macular degeneration. N Engl J Med 2007;357:553-61.

137. Dinu V, Miller PL, Zhao H. Evidence for association between multiple complement pathway genes and AMD. Genet Epidemiol 2007;31:224-37.

138. Age-Related Eye Disease Study Research Group. The Age-Related Eye Disease Study system for classifying age-related macular degeneration from stereoscopic color fundus photographs: the Age-Related Eye Disease Study Report Number 6. Am J Ophthalmol 2001;132:668-81.

139. Seddon JM, George S, Rosner B, Klein ML. CFH gene variant, Y402H, and smoking, body mass index, environmental associations with advanced age-related macular degeneration. Hum Hered 2006;61:157-65.

140. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. Nat Genet 2013;45:433-9.

141. Sivaprasad S, Adewoyin T, Bailey TA, et al. Estimation of systemic complement C3 activity in age-related macular degeneration. Arch Ophthalmol 2007;125:515-9.

142. Machalinska A, Dziedziejko V, Mozolewska-Piotrowska K, et al. Elevated plasma levels of C3a complement compound in the exudative form of age-related macular degeneration. Ophthalmic Res 2009;42:54-9.

143. Reynolds R, Hartnett ME, Atkinson JP, et al. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. Invest Ophthalmol Vis Sci 2009;50:5818-27.

144. Hecker LA, Edwards AO, Ryu E, et al. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. Hum Mol Genet 2010;19:209-15.

145. Scholl HP, Charbel IP, Walier M, et al. Systemic complement activation in age-related macular degeneration. PLoS One 2008;3:e2593.

146. Hakobyan S, Harris CL, Tortajada A, et al. Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: application to assessing risk of age-related macular degeneration. Invest Ophthalmol Vis Sci 2008;49:1983-90.

147. Parker C. Eculizumab for paroxysmal nocturnal haemoglobinuria. Lancet 2009;373:759-67.

148. Loyet KM, Good J, Sturgeon L, et al. Anti-Factor D Fab Specifically Inhibits the Alternative Complement Pathway: In vitro Characterization and in vivo Effects Following Administration to Cynomolgus Monkeys. ARVO Meeting Abstracts 2010;51:2980.

149. Mayer G, Jenne A. Aptamers in research and drug development. BioDrugs 2004;18:351-9.

150. van Lookeren Campagne M, Katschke KJ, Jr., Wu P, et al. Targeting the Alternative Pathway of Complement in AMD With a Factor D Selective Antibody. ARVO Meeting Abstracts 2010;51:4511.

151. Kim SJ, Kim J, Lee J, et al. Intravitreal human complement factor H in a rat model of laser-induced choroidal neovascularisation. Br J Ophthalmol 2013;97:367-70.

152. Potentia Pharmaceuticals. Safety of Intravitreal POT-4 Therapy for Patients With Neovascular Age Related Macular Degeneration (AMD) (ASaP). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT00473928. 2011

153. Kaushal S, Grossi F, Francois C, et al. Complement C3 inhibitor POT-4: Clinical Safety of Intravitreal Administration. ARVO Meeting Abstracts 2009;50:5010.

154. Potentia Pharmaceuticals. Evaluation of AL-78898A in Exudative Age-Related Macular Degeneration (AMD). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT01157065. 2011

155. University of Miami. Complement Inhibition With Eculizumab for the Treatment of Non-Exudative Macular Degeneration (AMD) (COMPLETE). In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT00935883. 2011

156. Ophthotech Corporation. ARC1905 (ANTI-C5 APTAMER) Given Either In Combination Therapy With Lucentis® 0.5 mg/Eye In Subjects With Neovascular Age-Related Macular Degeneration. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT00709527. 2011

157. Cousins SW, Ophthotech Study Group. Targeting Complement Factor 5 in Combination with Vascular Endothelial Growth Factor (VEGF) Inhibition for

Neovascular Age Related Macular Degeneration (AMD): Results of a Phase 1 Study. ARVO Meeting Abstracts 2010;51:1251.

158. Ophthotech Corporation. Study of ARC1905 (Anti-C5 Aptamer) in Subjects With Dry Age-related Macular Degeneration. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT00950638. 2011

159. Novartis Pharmaceuticals. Intravitreal LFG316 in Patients With Advanced Age-related Macular Degeneration. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT01255462. 2011

160. Genentech. A Study of Safety, Tolerability, and Evidence of Activity of FCFD4514S Administered Monthly or Every Other Month to Patients With Geographic Atrophy. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT01229215. 2011

161. Genentech. A Study of the Safety, Tolerability, Pharmacokinetics, and Immunogenicity of Intravitreal Injections of FCFD4514S in Patients With Geographic Atrophy. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 April 01]. http://clinicaltrials.gov/ct2/show/NCT00973011. 2011

162. Bora NS, Kaliappan S, Jha P, et al. Complement activation via alternative pathway is critical in the development of laser-induced choroidal neovascularization: role of factor B and factor H. J Immunol 2006;177:1872-8.

163. Klein ML, Schultz DW, Edwards A, et al. Age-related macular degeneration. Clinical features in a large family and linkage to chromosome 1q. Arch Ophthalmol 1998;116:1082-8.

164. Seddon JM, Reynolds R, Rosner B. Associations of smoking, body mass index, dietary lutein, and the LIPC gene variant rs10468017 with advanced age-related macular degeneration. Mol Vis 2010;16:2412-24.

165. Flicek P, Ahmed I, Amode MR, et al. Ensembl 2013. Nucleic Acids Res 2013;41:D48-D55.

166. Despriet DD, Klaver CC, Witteman JC, et al. Complement factor H polymorphism, complement activators, and risk of age-related macular degeneration. JAMA 2006;296:301-9.

167. Thakkinstian A, Han P, McEvoy M, et al. Systematic review and meta-analysis of the association between complement factor H Y402H polymorphisms and age-related macular degeneration. Hum Mol Genet 2006;15:2784-90.

168. Hageman GS, Hancox LS, Taiber AJ, et al. Extended haplotypes in the complement factor H (CFH) and CFH-related (CFHR) family of genes protect against age-related macular degeneration: characterization, ethnic distribution and evolutionary implications. Ann Med 2006;38:592-604.

169. Abrera-Abeleda MA, Nishimura C, Smith JL, et al. Variations in the complement regulatory genes factor H (CFH) and factor H related 5 (CFHR5) are associated with membranoproliferative glomerulonephritis type II (dense deposit disease). J Med Genet 2006;43:582-9.

170. Mullins RF, Aptsiauri N, Hageman GS. Structure and composition of drusen associated with glomerulonephritis: implications for the role of complement activation in drusen biogenesis. Eye (Lond) 2001;15:390-5.

171. Bergeron-Sawitzke J, Gold B, Olsh A, et al. Multilocus analysis of age-related macular degeneration. Eur J Hum Genet 2009;17:1190-9.

172. Herbert AP, Uhrin D, Lyon M, et al. Disease-associated sequence variations congregate in a polyanion recognition patch on human factor H revealed in three-dimensional structure. J Biol Chem 2006;281:16512-20.

173. Skerka C, Lauer N, Weinberger AA, et al. Defective complement control of factor H (Y402H) and FHL-1 in age-related macular degeneration. Mol Immunol 2007;44:3398-406.

174. Lauer N, Mihlan M, Hartmann A, et al. Complement regulation at necrotic cell lesions is impaired by the age-related macular degeneration-associated factor-H His402 risk variant. J Immunol 2011;187:4374-83.

175. Ormsby RJ, Ranganathan S, Tong JC, et al. Functional and structural implications of the complement factor H Y402H polymorphism associated with age-related macular degeneration. Invest Ophthalmol Vis Sci 2008;49:1763-70.

176. Clark SJ, Perveen R, Hakobyan S, et al. Impaired binding of the age-related macular degeneration-associated complement factor H 402H allotype to Bruch's membrane in human retina. J Biol Chem 2010;285:30192-202.

177. Kelly U, Yu L, Kumar P, et al. Heparan sulfate, including that in Bruch's membrane, inhibits the complement alternative pathway: implications for age-related macular degeneration. J Immunol 2010;185:5486-94.

178. Mold C, Gewurz H, Du Clos TW. Regulation of complement activation by C-reactive protein. Immunopharmacology 1999;42:23-30.

179. Herbert AP, Deakin JA, Schmidt CQ, et al. Structure shows that a glycosaminoglycan and protein recognition site in factor H is perturbed by age-related macular degeneration-linked single nucleotide polymorphism. J Biol Chem 2007;282:18960-8.

180. Laine M, Jarva H, Seitsonen S, et al. Y402H polymorphism of complement factor H affects binding affinity to C-reactive protein. J Immunol 2007;178:3831-6.

181. Sjoberg AP, Trouw LA, Clark SJ, et al. The factor H variant associated with age-related macular degeneration (His-384) and the non-disease-associated form bind differentially to C-reactive protein, fibromodulin, DNA, and necrotic cells. J Biol Chem 2007;282:10894-900.

182. Yu J, Wiita P, Kawaguchi R, et al. Biochemical analysis of a common human polymorphism associated with age-related macular degeneration. Biochemistry 2007;46:8451-61.

183. Okemefuna AI, Nan R, Miller A, et al. Complement factor H binds at two independent sites to C-reactive protein in acute phase concentrations. J Biol Chem 2010;285:1053-65.

184. Johnson PT, Betts KE, Radeke MJ, et al. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. Proc Natl Acad Sci U S A 2006;103:17456-61.

185. Mullins RF, Dewald AD, Streb LM, et al. Elevated membrane attack complex in human choroid with high risk complement factor H genotypes. Exp Eye Res 2011;93:565-7.

186. Loyet KM, Deforge LE, Katschke KJ, Jr., et al. Activation of the Alternative Complement Pathway in Vitreous is Controlled by Genetics in Age-Related Macular Degeneration. Invest Ophthalmol Vis Sci 2012;53:6628-37.

187. Ufret-Vincenty RL, Aredo B, Liu X, et al. Transgenic mice expressing variants of complement factor H develop AMD-like retinal findings. Invest Ophthalmol Vis Sci 2010;51:5878-87.

188. Zee RY, Diehl KA, Ridker PM. Complement factor H Y402H gene polymorphism, C-reactive protein, and risk of incident myocardial infarction, ischaemic stroke, and venous thromboembolism: a nested case-control study. Atherosclerosis 2006;187:332-5.

189. Mooijaart SP, Koeijvoets KM, Sijbrands EJ, et al. Complement Factor H polymorphism Y402H associates with inflammation, visual acuity, and cardiovascular mortality in the elderly population at large. Exp Gerontol 2007;42:1116-22.

190. Despriet DD, Klaver CC, van Duijn CC, Janssens AC. Predictive value of multiple genetic testing for age-related macular degeneration. Arch Ophthalmol 2007;125:1270-1.

191. Lyzogubov VV, Tytarenko RG, Jha P, et al. Role of ocular complement factor H in a murine model of choroidal neovascularization. Am J Pathol 2010;177:1870-80.

192. Kerr H, Richards A. Complement-mediated injury and protection of endothelium: lessons from atypical haemolytic uraemic syndrome. Immunobiology 2012;217:195-203.

193. Noris M, Caprioli J, Bresin E, et al. Relative role of genetic complement abnormalities in sporadic and familial aHUS and their impact on clinical phenotype. Clin J Am Soc Nephrol 2010;5:1844-59.

194. Geerdink LM, Westra D, van Wijk JA, et al. Atypical hemolytic uremic syndrome in children: complement mutations and clinical characteristics. Pediatr Nephrol 2012;27:1283-91.

195. Giordano M, Castellano G, Messina G, et al. Preservation of renal function in atypical hemolytic uremic syndrome by eculizumab: a case report. Pediatrics 2012;130:e1385-e1388.

196. Neumann HP, Salzmann M, Bohnert-Iwan B, et al. Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. J Med Genet 2003;40:676-81.

197. Caprioli J, Castelletti F, Bucchioni S, et al. Complement factor H mutations and gene polymorphisms in haemolytic uraemic syndrome: the C-257T, the A2089G and the G2881T polymorphisms are strongly associated with the disease. Hum Mol Genet 2003;12:3385-95.

198. Remuzzi G, Ruggenenti P, Codazzi D, et al. Combined kidney and liver transplantation for familial haemolytic uraemic syndrome. Lancet 2002;359:1671-2.

199. Kumar PJ, Clark ML. Kumar & Clark clinical medicine. 6th ed ed. Edinburgh: Elsevier Saunders; 2005.

200. Adam R, McMaster P, O'Grady JG, et al. Evolution of liver transplantation in Europe: report of the European Liver Transplant Registry. Liver Transpl 2003;9:1231-43.

201. Saland JM, Emre SH, Shneider BL, et al. Favorable long-term outcome after liver-kidney transplant for recurrent hemolytic uremic syndrome associated with a factor H mutation. American Journal of Transplantation 2006;6:1948-52.

202. Jalanko H, Peltonen S, Koskinen A, et al. Successful liver-kidney transplantation in two children with aHUS caused by a mutation in complement factor H. American Journal of Transplantation 2008;8:216-21.

203. Saland JM, Shneider BL, Bromberg JS, et al. Successful split liver-kidney transplant for factor H associated hemolytic uremic syndrome. Clinical Journal of The American Society of Nephrology: CJASN 2009;4:201-6.

204. Haller W, Milford DV, Goodship TH, et al. Successful isolated liver transplantation in a child with atypical hemolytic uremic syndrome and a mutation in complement factor H. American Journal of Transplantation 2010;10:2142-7.

205. Wilson C, Torpey N, Jaques B, et al. Successful simultaneous liver-kidney transplant in an adult with atypical hemolytic uremic syndrome associated with a mutation in complement factor H. Am J Kidney Dis 2011;58:109-12.

206. Cheong HI, Lee BS, Kang HG, et al. Attempted treatment of factor H deficiency by liver transplantation. Pediatric Nephrology 2004;19:454-8.

207. Remuzzi G, Ruggenenti P, Colledan M, et al. Hemolytic uremic syndrome: a fatal outcome after kidney and liver transplantation performed to correct

factor h gene mutation. American Journal of Transplantation 2005;5:1146-50.

208. Brown JH, Tellez J, Wilson V, et al. Postpartum aHUS secondary to a genetic abnormality in factor H acquired through liver transplantation. Am J Transplant 2012;12:1632-6.

209. University of Leicester. Polymerase chain reaction. http://www2.le.ac.uk/departments/emfpu/genetics/explained/pcr. 2013

210. Qiagen Ltd HG. High Resolution Melt Design and Analysis - Rotorgene 6000 Protocol. www.qiagen.com. 2006

211. Liew M, Pryor R, Palais R, et al. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem 2004;50:1156-64.

212. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001;291:1304-51.

213. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977;74:5463-7.

214. Qiagen Ltd. HG. DyeEx Handbook. www.qiagen.com/literature/render.aspx?id=154. 2012

215. CU Cancer Center DNA Sequencing & Analysis Service UoCAMCACU. DNA Sequencing Troubleshooting guide. http://dnasequencingcore.ucdenver.edu/pdf-Files/ABI%20SequenceScannerManual.pdf. 2010

216. Thermo Fisher Scientific Inc WU. ELISA assay development handbook. www.piercenet.com/files/16014567_AssayDevHB_Intl.pdf. 2011

217. Mabtech Inc. NSS. The ELISA Method. www.mabtech.com/main/Page.asp?PageId=26&PageName=About+ELISA. 2011

218. Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E and beta carotene for age-related cataract and vision loss: AREDS report no. 9.[Erratum appears in Arch Ophthalmol. 2008 Sep;126(9):1251]. Arch Ophthalmol 2001;119:1439-52.

219. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology 1992;99:933-43.

220. Barbone F, Bovenzi M, Cavallieri F, Stanta G. Cigarette smoking and histologic type of lung cancer in men. Chest 1997;112:1474-9.

221. Kumar PJ, Clark ML. Kumar & Clark's clinical medicine. 7th ed ed. Edinburgh: Saunders/Elsevier; 2009.

222. Longmore JM. Oxford handbook of clinical medicine. 8th ed. Oxford: Oxford University Press; 2010.

223. Levey AS, Coresh J, Balk E, et al. National Kidney Foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Ann Intern Med 2003;139:137-47.

224. Levey AS, Bosch JP, Lewis JB, et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction

equation. Modification of Diet in Renal Disease Study Group. Ann Intern Med 1999;130:461-70.

225. Beutler E, Gelbart T, Kuhl W. Interference of heparin with the polymerase chain reaction. Biotechniques 1990;9:166.

226. Lahiri DK, Schnabel B. DNA isolation by a rapid method from human blood samples: effects of MgCl2, EDTA, storage time, and temperature on DNA yield and quality. Biochem Genet 1993;31:321-8.

227. Lachmann PJ. Preparing serum for functional complement assays. J Immunol Methods 2010;352:195-7.

228. Dessauer A, Rother U, Rother K. Freeze-thaw activation of the complement attack phase: I. Separation of two steps in the formation of the active C--56 complex. Acta Pathol Microbiol Immunol Scand Suppl 1984;284:75-81.

229. Age-Related Eye Disease Study Research Group. The Age-Related Eye Disease Study (AREDS): design implications. AREDS report no. 1. Control Clin Trials 1999;20:573-600.

230. Klein R, Davis MD, Magli YL, et al. The Wisconsin age-related maculopathy grading system. Ophthalmology 1991;98:1128-34.

231. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.

232. Goverdhan SV, Howell MW, Mullins RF, et al. Association of HLA class I and class II polymorphisms with age-related macular degeneration. Investigative Ophthalmology & Visual Science 46(5):1726-34, 2005.

233. Goverdhan SV, Hannan S, Newsom RB, et al. An analysis of the CFH Y402H genotype in AMD patients and controls from the UK, and response to PDT treatment. Eye (Lond) 2008;22:849-54.

234. Karger BL, Guttman A. DNA sequencing by CE. Electrophoresis 2009;30 Suppl 1:S196-S202.

235. McKibbin M, Ali M, Bansal S, et al. CFH, VEGF and HTRA1 promoter genotype may influence the response to intravitreal ranibizumab therapy for neovascular age-related macular degeneration. Br J Ophthalmol 2012;96:208-12.

236. Thermo Fisher Scientific Inc WU. Nanodrop ND-1000 User Manual. www.nanodrop.com/Library/nd-1000-v3.7-users-manual-8.5x11.pdf. 2007

237. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 2000;132:365-86.

238. Fernandez-Suarez XM, Schuster MK. Using the ensembl genome server to browse genomic sequence data. Curr Protoc Bioinformatics 2010;Chapter 1:Unit1.

239. National Center for Biotechnology Information (US). SNP FAQ Archive [Internet]. Bethesda (MD); 2010.

240. Dreszer TR, Karolchik D, Zweig AS, et al. The UCSC Genome Browser database: extensions and updates 2011. Nucleic Acids Res 2012;40:D918-D923.

241. Kibbe WA. OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res 2007;35:W43-W46.

242. Hardy GH. Mendelian proportions in a mixed population. Science 1908;28:49-50.

243. Weinberg W. Über den Nachweis der Vererbung beim Menschen. Jahreshefte des Vereins für vaterländische Naturkunde in Württemberg 1908;368-82.

244. Wigginton JE, Cutler DJ, Abecasis GR. A note on exact tests of Hardy-Weinberg equilibrium. Am J Hum Genet 2005;76:887-93.

245. Stothard P. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 2000;28:1102, 1104.

246. Bioline Reagents Ltd. LUK. SensiMix SYBR No-ROX Kit protocol. www.bioline.com/documents/product_inserts/SensiMix%20SYBR%20No-ROX%20Kit.pdf#zoom=130. 2012

247. Qiagen Ltd. HG. QIAquick Spin Handbook. www.qiagen.com/literature/render.aspx?id=103715. 2012

248. Applied Biosystems PUK. BigDye® Terminator v1.1 Cycle Sequencing Kit. http://products.invitrogen.com/ivgn/product/4337450. 2010

249. Khokhar SK, Mitui M, Leos NK, et al. Evaluation of Maxwell(R) 16 for automated DNA extraction from whole blood and formalin-fixed paraffin embedded (FFPE) tissue. Clin Chem Lab Med 2012;50:267-72.

250. Qiagen HG. QIAamp DNA FFPE Tissue Handbook. http://www.qiagen.com/literature/render.aspx?id=200374. 2010

251. Hakobyan S, Tortajada A, Harris CL, et al. Variant-specific quantification of factor H in plasma identifies null alleles associated with atypical hemolytic uremic syndrome. Kidney Int 2010;78:782-8.

252. Ng IO, Chan KL, Shek WH, et al. High frequency of chimerism in transplanted livers. Hepatology 2003;38:989-98.

253. Qiagen HG. Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution. http://www.qiagen.com/literature/render.aspx?id=201668. 2011

254. Qiagen HG. Qiagen REPLI-g Mini Kit. http://www.qiagen.com/literature/render.aspx?id=401. 2011

255. Paez JG, Lin M, Beroukhim R, et al. Genome coverage and sequence fidelity of phi29 polymerase-based multiple strand displacement whole genome amplification. Nucleic Acids Res 2004;32:e71.

256. Tindall KR, Kunkel TA. Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry 1988;27:6008-13.

257. Qiagen HG. Purification of REPLI-g amplified DNA using the QIAamp DNA Mini Kit. http://www.qiagen.com/literature/protocols/pdf/rg14.pdf. 2011

258. Gibson J, Hakobyan S, Cree AJ, et al. Variation in complement component C1 inhibitor in age-related macular degeneration. Immunobiology 2012;217:251-5.

259. Beckton DaCoFLU. C4a ELISA protocol. www.bdbiosciences.com/external_files/pm/doc/manuals/live/web_enabled/00-81014-550947-D.pdf. 2011

260. Hycult Biotech Inc. UTN. C5a ELISA protocol. www.hycultbiotech.com/doc509.dx. 2011

261. Hycult Biotech Inc. UTN. C3a ELISA protocol. http://www.hycultbiotech.com/doc498.dx. 2011

262. Hycult Biotech Inc. UTN. TCC ELISA protocol. www.hycultbiotech.com/doc1290.dx. 2011

263. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010;11:785-97.

264. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159-74.

265. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81:559-75.

266. Field AP. Discovering statistics using SPSS. 3rd ed. Los Angeles: SAGE; 2009.

267. Bomback AS, Smith RJ, Barile GR, et al. Eculizumab for dense deposit disease and C3 glomerulonephritis. Clin J Am Soc Nephrol 2012;7:748-56.

268. McGeer PL, Sibley J. Sparing of age-related macular degeneration in rheumatoid arthritis. Neurobiol Aging 2005;26:1199-203.

269. Qin X, Gao B. The complement system in liver diseases. Cell Mol Immunol 2006;3:333-40.

270. Sun S, Guo Y, Zhao G, et al. Complement and the alternative pathway play an important role in LPS/D-GalN-induced fulminant hepatic failure. PLoS One 2011;6:e26838.

271. Lindgren S, Laurell AB, Eriksson S. Complement components and activation in primary biliary cirrhosis. Hepatology 1984;4:9-14.

272. Qin S, Zhou Y, Lok AS, et al. SRM targeted proteomics in search for biomarkers of HCV-induced progression of fibrosis to cirrhosis in HALT-C patients. Proteomics 2012;12:1244-52.

273. Yang JD, Seol SY, Leem SH, et al. Genes associated with recurrence of hepatocellular carcinoma: integrated analysis by gene expression and methylation profiling. J Korean Med Sci 2011;26:1428-38.

274. Bykov I, Junnikkala S, Pekna M, et al. Effect of chronic ethanol consumption on the expression of complement components and acute-phase proteins in liver. Clin Immunol 2007;124:213-20.

275. Silva AS, Teixeira AG, Bavia L, et al. Plasma levels of complement proteins from the alternative pathway in patients with age-related macular degeneration are independent of Complement Factor H Tyr(402)His polymorphism. Mol Vis 2012;18:2288-99.

276. Metcalf S, Pandha HS, Morgan R. Antiangiogenic effects of zoledronate on cancer neovasculature. Future Oncol 2011;7:1325-33.

277. Honda S, Nagai T, Kondo N, et al. Therapeutic effect of oral bisphosphonates on choroidal neovascularization in the human eye. J Ophthalmol 2010;Epub 2010 Jul 25.

278. Funabashi KS, Barcelos D, Visona I, et al. DNA extraction and molecular analysis of non-tumoral liver, spleen, and brain from autopsy samples: The effect of formalin fixation and paraffin embedding. Pathol Res Pract 2012;208:584-91.

279. Ferguson AE, Cohn RJ, Ashton LJ. Use of formalin-fixed paraffin-embedded tumor tissue as a DNA source in molecular epidemiological studies of pediatric CNS tumors. Diagn Mol Pathol 2012;21:105-13.

280. Liang CW, Lee YS, Marino-Enriquez A, et al. The utility and limitation of single nucleotide polymorphism analysis on whole genome amplified mesenchymal tumour DNA in formalin fixed tumour samples. Pathology 2012;44:33-41.