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REVIEW

Progress in defining the molecular biology of age related macular degeneration

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Abstract

Age related macular degeneration (AMD) is an extremely prevalent complex genetic disorder. Its incidence rises exponentially in the elderly to a frequency of 1 in 2 in the general population by age 85. It affects approximately 25 million people and is the commonest cause of irreversible visual loss in the Western world. It is therefore a major public health problem. However, until recently its aetiology was unknown. Our understanding of both the molecular biology of AMD and the relevant clinical treatments has progressed dramatically in the last 2 years. Two genes of large effect have beeen identified which together contribute to over 70% of the population attributable risk of AMD. Treatments which inhibit expression of vascular endothelial growth factor have been developed which can rescue vision in the "wet" form of the disease. The association of complement factor H with AMD highlights the importance of the alternative complement pathway in the developemnt of AMD whilst the pathophysiology of the serine protease HTRA1 is now under intensive study. This review will give an insight into these developments and will summarise our current knowledge of the molecular biology of AMD.

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Background

Age related macular degeneration (AMD) is one of the most common genetic disorders worldwide, with an age dependent prevalence which rises exponentially in the elderly to a frequency of 1 in 2 in the general population by age 85. It affects approximately 25 million people worldwide and is the commonest cause of irreversible visual loss in the Western world (Tielsch et al. 1995). Catastrophic loss of central vision occurs as AMD affects the macular region of the eye (Fig. 1). This is the central area of the retina containing the fovea, the only region of the retina where the density of photoreceptors is sufficient to permit fine vision. Hence any disruption of this small area can lead to severe loss of central vision. For the individual, severe visual loss from AMD is equivalent in terms of reduction in quality of life to having severe prostatic cancer with uncontrollable pain (Brown et al. 2005). For the nation, the economic burden to the United Kingdom of AMD has been estimated to be as much as 101.1 million Euros (Bonastre et al. 2002). AMD has been judged on the basis of a spectrum of measures of patient disability to be the third most disabling disease in the US population after diabetes and cancer (SST 2004; Brody et al. 2001; Rovner et al. 2002; Royal College of Ophthalmologists 2002).

Age related macular degeneration has a multifactorial aetiology with both genetic and environmental factors influencing the likelihood of an individual developing the condition. There have been important recent developments in understanding some of the genetic basis for AMD which has, in turn, increased our understanding of the underlying molecular events leading to its development. This article will give an overview of AMD, its genetic basis and the current understanding of its molecular pathology.

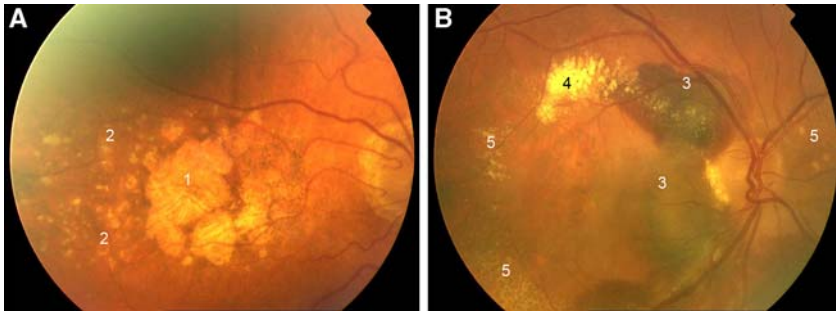

Fig. 1 **a** Retina of the right eye of a patient, homozygous for the Y402H mutation in CFH, with geographic atrophy (dry AMD) (*1*) affecting the central macula region of the retina. Drusen which are the hallmark of AMD are seen in the periphery (*2*). **b** Retina of the right eye of a patient, also homozygous for the Y402H mutation in CFH, with a large area of sub-retinal haemorrhage (*3*) and intra-retinal exudation of protein (*4*) in the central macula due to the complication of choroidal neovascularisation (wet AMD). Drusen are again seen in the periphery of the eye (*5*). Both patients are legally blind

Structure of the retina

Age related macular degeneration pathology affects the outer retina leading to a loss of central vision. The retina is a ten-layered tissue consisting of three layers of neurones (Fig. 2) (Kolb 2003). The light sensitive photoreceptors (rods and cones) form the outer layer surrounded by the photoreceptor matrix and forming close contacts with the retinal epithelial cells (RPE). Rods and cones have outer and inner segments connected by a connecting cilium with phototransduction occurring in the outer segments. These are adapted accordingly with rod outer segments (OS) consisting of many discs and cone OS consists of a highly folded membrane providing a large surface area for photo-

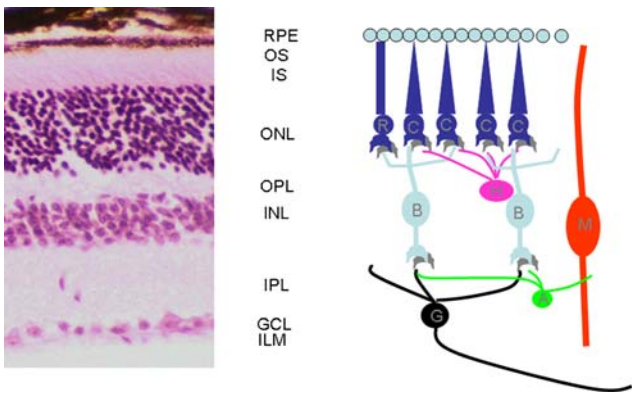

Fig. 2 Structure of the retina. The *left panel* shows a retinal section which has been H and E (haematoxylin and eosin) stained to show the cellular layers and the *right panel* is a cartoon depicting the structure and the different cell types: *C* cone photoreceptor, *R* rod photoreceptor, *B* bipolar cell, *M* Muller cell, *H* horizontal cell, *A* amacrine cell, *G* ganglion cell). The retinal layers are indicated: *RPE* retinal pigment epithelium, *OS* outer segment photoreceptors, *IS* inner segment, photoreceptors, *OPL* outer plexiform layer, *ONL* outer nuclear layer, *IPL* inner plexiform layer, *INL* inner nuclear layer, *GCL* ganglion cell layer, *ILM* inner limiting membrane

transduction. The tips of these cells are shed, phagocytosed by RPE cells and replaced at the base of the photoreceptors.

The basement membrane of RPE cells provides the first layer of a specialised extracellular matrix structure called Bruch's membrane which consists of five layers: a central elastic layer sandwiched between two collagen layers which lie between the basement membrane of the RPE cells and the choriocapillaris. Bruch's membrane can become thickened as a consequence of AMD.

Ultrastructurally the most common change is debris on both sides of the elastic layer (Feeney-Burns and Ellersieck 1985). The choriocapillaris or choroidal circulation is a vascular plexus which provides oxygen and nutrition to the RPE cells and photoreceptors and so there is diffusion through Bruch's membrane to these structures or more generally the outer retina. There is a blood retinal barrier whereby undesirable substances are prevented from entering the retina by first the tight junctions which exist between the endothelial cells of the capillary wall of the retinal vessels (the inner blood retinal barrier) and second by the tight junctions between RPE cells (the outer retinal barrier) (Cunha-Vaz 1976; Shiose and Oguri 1969). The outer retinal barrier thus is a barrier between the uvea and the retina. RPE cells support the photoreceptors with a number of functions including the engulfing and phagocytosis of up to 10% of the photoreceptor outer segments each day, regeneration of retinal (the vitamin A derivative which is the chromophore for visual pigments), maintenance of the interphoreceptor matrix and Bruch's membrane, absorption of scattered light via interaction with melanin and transport of ions and fluids from the choriocapillaris. Over time the phagocytosis of photoreceptor outer segments results in the accumulation of lipofuscin within the RPE cells and on Bruch's membrane. In addition, structural connections exist between the RPE and Bruch's membrane (Chen et al. 2003; McLaughlin et al. 2003). This is important as drusen also

form between the RPE basement membrane and Bruch's membrane thereby indicating the importance of this anatomical site for AMD pathogenesis.

AMD classification and disease phenotypes

One of the first descriptions of age related macular degeneration was in 1884 by Nettleship who used the term central guttate senile choroiditis (Nettleship 1884). Subsequently the disease was known as senile macular degeneration (Haab 1885) and now is described as age related macular degeneration to reflect the age related changes seen in the retina. In an attempt to harmonise phenotype among studies a classification system for AMD was defined by the International Age-related Maculopathy Epidemiological Study Group which proposed a classification and grading system for an early stage called age related maculopathy (ARM) and a late stage known as AMD. Classification was based on detailed assessment of colour fundus photographs where ten sub-fields of the macula were identified using a grid placed over retinal transparencies and graded according to drusen morphology and the degree of hypo- and hyper-pigmentation of the retina. In total there were 74 classification statements for these retinal features (Bird et al. 1995). Subsequently a simplified system has been developed by the age related eye disease study which is widely used by clinicians (AREDS 2001). In the AREDS system similar features are graded but this then leads to a four step level of AMD severity from mild AMD (grade 1) to end stage AMD with geographic atrophy or choroidal neovascularisation in the center of the macula (grade 4). Recently this has been further simplified to produce a five point clinical scoring system which can predict rate of progression of AMD (Ferris et al. 2005).

Clinically, the hallmark of the disease are drusen (Fig. 1). These are localized deposits lying between the basement membrane of the retinal pigment epithelium and Bruch's membrane. Drusen are visible with an ophthalmoscope as yellow white deposits beneath the retina and are typically located in the central macula. They may fade with time, leaving atrophy of the retina and RPE. They are classified as hard or soft depending on their appearance. Molecular studies of AMD have been aided by histopathological studies of drusen in donor eyes.

Drusen

Drusen have a complex protein, lipid and lipoprotein composition including complement factors (C1q, C3a and C5a), complement regulators (complement factor H, clusterin, vitronectrin), immunoglobulins, acute phase proteins, amyloid B (component of Alzheimer's plaques), tissue inhibitor of matrix metalloproteinases-3, phospholipids, cholesterol esters, glycoprotein moieties and apolipoproteins B and E (Anderson et al. 2004; Hageman et al. 1999; Johnson et al. 2001; Mullins et al. 1997, 2000, 2001; Nozaki et al. 2006; Russell et al. 2000). These components (particularly immunoglobulins and complement factors) suggest an inflammatory component in AMD pathogenesis which is consistent with descriptions of chronic inflammatory cells associated with atrophic RPE and neovascular lesions. This was suggested by Hageman and colleagues (2001) who hypothesized that drusen occur as a consequence of a localised inflammatory response following RPE injury. This is supported by the pathology occurring in membranoproliferative glomerulonephritis type II (MPGN-II) or dense deposit disease (OMIM 609814) in which patients develop renal disease and drusen which are similar in composition to those in AMD (Appel et al. 2005; Brera-Abeleda et al. 2006; Dragon-Durey et al. 2004; Duvall-Young et al. 1989; Leys et al. 1991; Mullins et al. 2001). Complement mediated immune system damage occurs in the kidney with deposition of complement factors, immunoglobulins and other proteins. This rare disorder occurs as a consequence of low serum levels of complement factor H (CFH), the main regulator of the alternate complement pathway. It is due in a proportion of cases to inactivating mutations in the gene encoding complement factor H.

Drusen in AMD

To summarise AMD has two distinctive phenotypes. "Dry AMD" also known as geographic atrophy (GA) or non-exudative AMD and "Wet AMD" or neovascular, exudative or disciform AMD. The dry form is characterised by any sharply delineated round or oval area of hypopigmentation or depigmentation or apparent absence of the RPE in which choroidal vessels are more visible than in surrounding areas (Fig. 1a). Wet AMD is characterised by the presence of choroidal neovascularisation (CNV) (Fig. 1b). This (CNV) produces sub-retinal haemorrhage, fibrovascular pigment epithelial detachments, classic or occult patterns of dye leakage on fluorescein angiography and usually leads to a fibrotic disciform scarring with resultant severe loss of vision. Studies report different frequencies of dry and wet AMD (Augood et al. 2006; Friedman et al. 2004; Klaver et al. 2001; Klein et al. 1999; Mitchell et al. 1995; Seddon and Chen 2004; Vingerling et al. 1995). These range from 85% for dry AMD and 15% for wet AMD to a higher prevalence of wet AMD c.f. dry AMD. These differences may be real or reflect different definitions for what constitutes dry AMD as opposed to normal aging change in the eye. Defining the phenotype has been a challenge for genetic

studies i.e. do you lump dry and wet AMD into one cohort or split into separate phenotypes of dry and wet?

Risk factors

The most consistent risk factors associated with AMD are increasing age and cigarette smoking. Cigarette smoking increases the risk of AMD two- to four-fold (AREDS 2000; Christen et al. 1996; DeAngelis et al. 2007; Evans 2001; Hyman and Neborsky 2002; Khan et al. 2006b; Schmidt et al. 2005; Seddon et al. 1996, 2006a; Smith et al. 2001). AMD is commoner in white populations cf. hispanic and black populations (Cruickshanks et al. 1997). Diet is also important with positive associations of total fat intake with AMD (Cho et al. 2001; Mares-Perlman et al. 1995; Seddon et al. 1994, 2001, 2003a). A high body mass index is associated with both dry and wet AMD (Seddon et al. 2003a). Inconsistent risk factors include hypertension (AREDS 2000; Delcourt et al. 2001; Klein et al. 1997, 2003a; Macular Photocoagulation Study Group 1997), high cholesterol levels (Klein et al. 2003a, 2003b; Mares-Perlman et al. 1995; Smith et al. 2000; van et al. 2003) and sunlight exposure (Darzins et al. 1997; Khan et al. 2006a; Taylor et al. 1992). There is some evidence that statin intake may reduce the risk of developing AMD but no prospective studies have been done to confirm these initial observations from cross-sectional and cohort studies (Guymer et al. 2005; Hall et al. 2001; Klein et al. 2003c; McCarty et al. 2001; McGwin et al. 2003; van et al. 2003). The AREDS study has shown that supplementation with high doses of antioxidants zinc, vitamin C, vitamin E and β-carotene provide a protective effect on progression to advanced AMD (AREDS 2001). A summary of genes associated with AMD is summarised in Table 1. Possible reasons for conflicting findings for some genes include different prevalence of mutations in different ethnic groups and populations; errors in phenotyping such as phenocopies in the cases and undiagnosed AMD in control groups; environmental influences such as smoking not being controlled for and poor study design e.g. not analysing genes identically in both case and control groups.

Animal models of AMD

There are now several descriptions of mouse models of AMD, each of which exhibit some of the features of human AMD with the most recent, a SOD1 knock out mouse model perhaps most closely resembling the human AMD phenotype. The mouse has drusen of a similar composition to drusen in AMD, thickened Bruch's membrane, choroidal neovascularisation and signs of oxidative damage to RPE cells (Imamura et al. 2006). This model was generated to investigate the role of oxidative stress in the pathogenesis of AMD. Three superoxide dismutase (SOD) isoenzymes have a role in the antioxidant systems in the retina: SOD1 in the cytosol, SOD2 in the mitochondrial matrix, and secreted SOD3 (Behndig et al. 1998) with SOD1 expressed at the highest levels. Oxidative stress has been implicated in AMD for a number of years with the widespread evidence that smoking increases the risk of its development (AREDS 2000; DeAngelis et al. 2007; Evans 2001; Hyman and Neborsky 2002; Khan et al. 2006b; Schmidt et al. 2005; Seddon et al. 1996, 2006a; Smith et al. 2001) and treatment with antioxidants can slow its progression (AREDS 2001). The authors suggest that since only SOD1 has been knocked out of these mice (SOD2 and 3 are still present) then this enzyme may play an important role in protecting RPE from oxidative damage and that oxidative stress is a primary cause of age-related retinal degeneration (Behndig et al. 1998).

Another mouse model which has many of the features of human AMD was generated by targeted knock in of human apolipoprotein E (Apo E) alleles. Mice were generated expressing one of the three Apo E human alleles E2, E3 or E4 and maintained on a high fat cholesterol rich diet (Malek et al. 2005) to model the combined effects of age, a lipid rich diet and expression of each of the APO E alleles. Epidemiological studies had indicated an association between Apo E and AMD (Klaver et al. 1998a; Schmidt et al. 2002; Souied et al. 1998) and both cholesterol and APO E, its transporter, are constituents of drusen (Mullins et al. 2000). The results indicated that APO E alleles alone resulted in only mild retinal changes with ageing but both Apo E E2 and Apo E E4 in combination with a high fat, high cholesterol diet resulted in AMD-like disease with the more severe pathology occurring in the E4 expressing mice. These mice had drusen like deposits, a thickened Bruch's membrane and RPE changes including atrophy. Some mice developed choroidal neovascularisation. These results indicate both the importance of a high fat, high cholesterol diet as a risk factor for AMD but also the expression of the E4 allele. It would therefore be interesting to revisit the conflicting epidemiological studies (Klein et al. 2003a; Klein et al. 2003b; Mares-Perlman et al. 1995; Smith et al. 2000; van et al. 2003) which did not find a consistent link with cholesterol and see if the association could be strengthened if APO E genotype is considered in the analysis. Previous genetic association studies suggested an association with the E2 allele rather than the E4 allele (Baird et al. 2004b; Schmidt et al. 2002; Simonelli et al. 2001). The authors suggest this discrepancy may have arisen as the mice were all homozygous for the alleles whereas the humans in the studies were almost all heterozygous, the effects of diet were not studied in the AMD risk studies and the difference

Table 1 Summary of genetic studies in AMD

Gene name	Variants	Odds ratio for risk of AMD	Association *P* value	Positive studies	Cases	Controls	Function of gene	References
Genes with only positive findings								
Complement factors B and C2	Haplotypes H1 (increased risk) Haplotypes H7 H10 (decreased risk)	1.32 0.45 0.36	0.0013 <0.0001 <0.0001	1	900	400	Complement regulators	(Gold et al. 2006)
CST3	Genotype B/B	2.97	0.037	1	167	517	Cysteine protease inhibitor	(Zurdel et al. 2002)
CX3CR1	I249 and M280	3.57	0.04	1	85	105	Chemokine receptor	(Tuo et al. 2004)
FBLN5	Seven missense variants	Not stated	<0.01	1	402	429	Extracellular-matrix protein	(Stone et al. 2004)
TLR4	D299G	2.65	0.025	1	667	439	Toll-like receptor	(Zareparsi et al. 2005b)
VEGF	Five SNPs	4.13	0.002	2	399	159	Vascular endothelial growth factor	(Haines et al. 2006)
	Haplotype analysis	18.24	0.0074		45	94		(Churchill et al. 2006)
LRP6	Five SNPs	1.76	0.004	1	399	159	Interacts with APOE	(Haines et al. 2006)
MMP9	Promoter ca repeat	3	<0.0005	1	107	223	Degrades extracellular matrix	(Fiotti et al. 2005)
HTRA1	rs11200638	10	10^{-11}	4	>1,000	>1,000	Serine protease	(Cameron et al. 2007; DeWan et al. 2006; Yang et al. 2006; Yoshida et al. 2007)
HLA	Cw0701	1.85	0.036	1	100	92	Regulate immune response	(Goverdhan et al. 2005)
	B 4001	0.39	0.027					
	DRB11301	0.31	0.009					

Gene name	Variants		Association *P* value	Number of studies	Cases	Controls	Function of the gene	References
Genes with conflicting findings								
APOE	e4	Positive studies	$P < 0.01$	5	>1,000	>1,000	Cholesterol transport to liver	(Baird et al. 2004b; Schmidt et al. 2002; Simonelli et al. 2001; Souied et al. 1998; Zareparsi et al. 2004)
		Negative studies	NS	6	>1,000	>1,000		(Conley et al. 2005; Gotoh et al. 2004; Pang et al. 2000; Schmidt et al. 2005; Schultz et al. 2003a; Wong et al. 2006)
ABCA4	Multiple missense changes	Positive studies	$P < 0.0001$	>2	>1,000	>1,000	Outwardly directed flippase for *N*-retinylidene-PE in photoreceptors	(Allikmets 2000; Allikmets et al. 1997a)
		Negative studies	NS	5	>1,000	>1,000		(De La Paz et al. 1999; Guymer et al. 2001; Souied et al. 2000; Stone et al. 1998; Webster et al. 2001)

Table 1 continued

Gene name	Variants		Association *P* value	Number of studies	Cases	Controls	Function of the gene	References
CFH	Y402H	Positive studies	$P < 10^{-11}$	>7	>1,000	>1,000	Key regulator of alternative complement pathway	(Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005; Lau et al. 2006; Simonelli et al. 2006; Zareparsi et al. 2005a)
		Negative studies	NS	2	213	212	Negative in 2 studies of Japanese patients	(Gotoh et al. 2006; Uka et al. 2006)
HEMICENTIN-1	Gln5346Arg	Positive studies	Not stated	1	One large family (27 members)	174	Fibulin gene	(Schultz et al. 2003b)
		Negative studies	NS		>1,000	>1,000		(Abecasis et al. 2004; Conley et al. 2005; Hayashi et al. 2004; Iyengar et al. 2004; McKay et al. 2004; Seitsonen et al. 2006; Stone et al. 2004)
PON1	Leu-Met54	Positive study	0.009	1	72	140	Prevents low-density lipoprotein oxidation	(Ikeda et al. 2001)
	Gln-Arg192		0.0127	1	72	140		
		Negative study	NS	1	94	95		(Baird et al. 2004a; Esfandiary et al. 2005)
		Negative study	NS	1	62	115		[28]
ELOVL4	Met299Val		<0.0001	1	196	120	Mutated in Stargardt like autosomal dystrophy	(Conley et al. 2005)
			NS		245	243		(Zhang et al. 2001)
			NS		778	551		(Ayyagari et al. 2001)
ACE	Alu(+/+)	0.22	0.004		173	189	Angiotensin converting enzyme	(Hamdi et al. 2002)
			NS		338 families			(Conley et al. 2005)
			NS		196	120		(Conley et al. 2005)
			NS		162 families			(Haines et al. 2006)
			NS		399	159		(Haines et al. 2006)
SOD2		10.14	0.0005		102	200	Intramitochondrial free radical scavenging enzyme	(Kimura et al. 2000)
			0.71		94	95		(Esfandiary et al. 2005)

might have arisen due to differences in human/mouse physiology. The model remains useful as a means of investigating APOE in the development of the AMD phenotype. The authors suggest the E4 allele may firstly lead to high cholesterol levels with a resulting inflammatory cascade and in addition since E4 has the lowest antioxidant activity of the three alleles might promote increased lipid peroxidation (Smith et al. 1998) which is implicated in AMD.

Another possible interpretation of the conflicting APO E studies (Table 1) is that there is no evidence of a strong association between the APO E gene and early ARM in middle-aged persons, suggesting that APO E is not a major determinant of the early stages of ARM in younger people (Wong et al. 2006) but that the APOE epsilon2 allele is associated with a significant increased risk of late ARM development, whereas the epsilon4 allele may confer some protection (Tikellis et al. 2007).

The knock out mouse models of the gene for monocyte chemoattractant protein 1 (MCP1/Ccl2) or the MCP1 receptor C-C chemokine receptor 2 (Ccr2) are also good models of AMD with aging of the mouse (Ambati et al. 2003) showing a phenotype similar to AMD after 18 months of age with complement and IgG deposition in RPE and choroid. It is hypothesized that the phenotype develops as a consequence of impaired macrophage recruitment leading to accumulation of C5 and IgG thus inducing vascular endothelial growth factor (VEGF) production and ultimately development of CNV, implicating macrophage dysfunction in the development of AMD.

Genetic studies of AMD

Genetic studies of AMD are difficult as AMD is a disease of the elderly. While it can occur from age 50, more commonly, patients present in their 70 years or older. Therefore the late onset of the disease limits the size of study pedigrees and straight forward linkage studies are difficult to achieve. Nevertheless a variety of genetic epidemiology studies have confirmed a significant genetic component to the disease. Familial aggregation studies have demonstrated a higher rate of AMD in first degree relatives or in proband sibling pairs cf. proband–spouse pairs e.g. Klaver et al. demonstrated that the first degree relatives of cases are three times more likely to develop exudative AMD than controls (Klaver et al. 1998b). They estimated that the population-attributable risk related to genetic factors was 23%. In addition a sibling analysis of 564 families showed significant correlations among siblings for AMD. Statistical modelling rejected the hypothesis that these effects were due to a random environmental major effect and suggested a single major gene could account for between 89 and 97% of the variability due to a major effect (Heiba et al. 1994).

Twin studies of AMD have been performed in both selected cases (Gottfredsdottir et al. 1999; Klein et al. 1994; Meyers et al. 1995; Meyers and Zachary 1988) and also in large population based surveys (Hammond et al. 2002; Seddon et al. 2005). These studies consistently show a greater than 90% concordance among monozygotic twins (Gottfredsdottir et al. 1999; Hammond et al. 2002; Klein et al. 1994; Meyers et al. 1995; Seddon et al. 2005) and a heritability score of 45% was demonstrated in one study focusing on ARM. The most heritable phenotypes being soft drusen >125 microns and > 20 hard drusen (Hammond et al. 2002).

Candidate gene studies of juvenile macular dystrophies

Juvenile macular dystrophies share some phenotypic characteristics with AMD. Following the great success in positional cloning of these genes, many have been studied in AMD to investigate the possibility of an association of AMD with sequence variants within these genes. The results however have been negative and are summarised in Table 1. In addition, a genome wide scan using a non-parametric affected pedigree method in 364 families and 386 markers (Weeks et al. 2000) did not find any evidence of linkage between the loci of RDS, VMD2, ABCA4, TIMP3 or EFEMP1 thus supporting the results of candidate gene screening described below. Of particular interest are the results from the investigation of VMD2, mutated in Best disease, ABCA4, mutated in Stargardt disease and EFEMP1 mutated in Malattia Leventinese.

Best disease

A few missense changes have been found in AMD patients in the VMD2 gene but the frequency of these changes did not reach statistical significance in several studies when compared to the frequency of such mutations found in controls (Allikmets et al. 1999; Lotery et al. 2000) e.g. Lotery et al. identified 5 missense changes in 321 AMD patients but this was not statistically different from the control frequency of 0 in 192 controls (Lotery et al. 2000). However, this suggests that a small fraction of patients with the clinical diagnosis of AMD may actually have a late-onset variant of Best disease.

Stargardt disease

The ABCA4 is mutated in autosomal recessive Stargardt disease (Allikmets et al. 1997b). Stargardt disease is probably the commonest juvenile maculopathy, present at a

population frequency of 1 in 10,000 (Blacharski 1988). An initial assessment of the ABCA4 gene in AMD suggested that it was mutated in 16% of patients with AMD (Allikmets et al. 1997a). However, subsequent studies could not replicate these findings (Guymer et al. 2001; Rivera et al. 2000; Souied et al. 2000; Stone et al. 1998; Webster et al. 2001) and in addition if this was a true prevalence then the population based frequency of ABCA4 related Stargardt disease would be expected to be six times its observed frequency (Dryja et al. 1998). Part of the difficulty in mutation scanning this gene is its large size and high allelic diversity in the general population (Webster et al. 2001). The majority of the general population have at least one variation from the consensus sequence (Webster et al. 2001) and therefore for this gene in particular it is difficult to assign pathogenic significance to sequence variation alone as polymorphism within the gene is so frequent. Therefore the significance of this gene in AMD is still unresolved.

Malattia leventinese

In contrast to the allelic diversity of the ABCA4 gene (Webster et al. 2001) only one missense change, ARG345TRP has been described in the EFEMP1 gene mutated in two similar retinal dystrophies, Malattia Leventinese and Doyne Honeycomb Retinal Dystrophy (Stone et al. 1999). No association with AMD has been found. However, this gene also known as fibulin 3 did result in the fibulin gene family being studied as potential candidate genes for AMD. This subsequently resulted in the association of FBLN5 with AMD (Stone et al. 2004). Recently it has been suggested that similar pathological processes may be present in both AMD and EFEMP1 (Marmorstein 2004).

Recent developments

Two landmark recent discoveries are the association of complement factor H (CFH) and HTRA1 with AMD. In addition, VEGF has been identified as a crucial rate limiting step in the development of CNV. Inhibition of VEGF is currently the most effective treatment for wet AMD.

CFH

Despite the difficulties in pursuing linkage analysis in AMD several studies found evidence of a susceptibility locus at Chromosome 1q32 (Abecasis et al. 2004; Iyengar et al. 2004; Klein et al. 1998; Majewski et al. 2003; Seddon et al. 2003b; Weeks et al. 2001). Subsequent analysis of this region led to the identification of an association between AMD and the Y402H allele of the complement factor H gene. The first reports came from four groups (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005) in 2005 and the findings have since been confirmed by many studies in different populations (Klein et al. 2005; Lau et al. 2006; Simonelli et al. 2006; Zareparsi et al. 2005a). Susceptibility to AMD increases with a CFH SNP 1277T > C which results in a substitution of histidine for tyrosine at codon 402 (Y402H). This has an odds ratio for AMD of 11.02 in homozygous individuals compared to noncarriers of this polymorphism (Despriet et al. 2006). Estimations of the risk of developing AMD by age 95 are 48.3% for homozygotes, 42.6% for heterozygotes, and 21.9% for noncarriers (Despriet et al. 2006) and the population attributable risk for late AMD is estimated between 24 (Schmidt et al. 2006) and 54% (Despriet et al. 2006). Environmental modifiable risk factors including smoking and body mass index appear to be acting independently of the Y402H polymorphism (DeAngelis et al. 2007; Schaumberg et al. 2007; Seddon et al. 2006b; Sepp et al. 2006).

The prevalence of AMD varies in different ethnic groups (Friedman et al. 2004; Gregor and Joffe 1978; Munoz et al. 2000; Oshima et al. 2001; Seddon et al. 2006b; Sommer et al. 1991; Varma et al. 2004; Yuzawa et al. 1997) and the frequency of the Y402H polymorphism was therefore investigated in these populations to determine whether this could account for the variation (Grassi et al. 2006). The prevalence of Y402H showed great variation between ethnic groups with a 34% frequency in the Caucasian and Somali populations and 35% in the African Americans but only a 7% frequency in the Japanese and 17% in Hispanics. The similarity in the allele frequencies between the Caucasians and African Americans is greatly at odds with the frequencies of AMD in these populations as the prevalence of late AMD is approximately five times lower in the African American group (Klein et al. 1999; Pieramici et al. 1994). This cannot be explained by a difference in lifestyle and environmental factors (Cruickshanks et al. 1997; Friedman et al. 1999; Klein et al. 1999). This suggests additional genetic factors are important in the pathogenesis of AMD which might act either independently or moderate the effects of the Y402H allele. More recently it has been suggested that other sequence variations in CFH may also be associated with AMD (Ennis et al. 2007; Maller et al. 2006).

Complement factor H originally known as beta-1H globulin, is a serum glycoprotein which is a negative regulator of the alternative complement system and is predominantly produced in the liver (Pangburn et al. 2000; Rodriguez de et al. 2004; Zipfel 2001). The complement systems (classical, alternative and lectin) recognise and react to foreign pathogens (Holers 2003). The classical and

lectin pathways recognise protein–protein or protein–carbohydrate interactions and are thus triggered. The alternative pathway has a constant low rate of activity or 'tickover' in which a cascade of proteolytic events occurs (Thurman and Holers 2006) and can be triggered into an 'amplification loop' by activation of the classical or lectin pathways therefore requiring tight control. Uncontrolled activation is damaging to tissues and is associated with a number of disease processes including asthma, lupus, glomerulonephritis, IgA nephropathy and rheumatoid arthritis (For review see (Thurman and Holers 2006).

Complement factor H acts as a key regulator of the alternative pathway by binding C3b which causes proteolytic cleavage accelerating the decay of the complex C3bBb and also acts as a cofactor for complement factor I another C3b inhibitor. It is thought to recognise polyanionic structures on cell surfaces such as sialic acid and the glycosaminoglycan (GAG) chains of proteoglycans [e.g. heparan sulfate (HS) and dermatan sulfate (DS)] preventing complement activation on host surfaces (Carreno et al. 1989). It may be that the Y402H allele interferes with the regulation function of CFH leading to uncontrolled activation of the alternative pathway contributing to AMD. CHF is a component of drusen, co-localising with C3b (Hageman et al. 2005) and is known to be expressed in many ocular tissues both during development and through adult life (Mandal and Ayyagari 2006) suggesting it controls complement activation in these tissues. In addition, inactivating mutations within the CFH gene, leading to lack of CFH expression are known to result in autosomal recessive type II membranoproliferative glomerulonephritis (dense deposit disease) (Appel et al. 2005; Brera-Abeleda et al. 2006; Dragon-Durey et al. 2004) in which patients develop drusen which are similar to those occurring in AMD (Duvall-Young et al. 1989; Leys et al. 1991; Mullins et al. 2000).

Complement factor H [reviewed in (Alexander and Quigg 2007)] consists of 20 short consensus repeats (SCRs) or complement control protein (CCP) domains which are also seen in the other members of the regulators of complement (RCA) gene family on chromosome 1q32. The three C3b-binding sites are located in SCRs 1–4, 12–14 and 19–20 (Pangburn et al. 2000; Ram et al. 1998; Sharma and Pangburn 1996; Sharma and Pangburn 1997) and heparin/sialic acid binding sites occur in SCRs 7, 13 and 19–20 (Blackmore et al. 1996; Blackmore et al. 1998; Jokiranta et al. 2005). The Y402H polymorphism lies within the seventh SCR and leads to reduced heparin binding which is likely to reflect a reduction in binding to polyanion structures on cell surfaces thus reducing the inhibition of complement activation (Clark et al. 2006). This hypothesis is supported by an investigation of CFH mutations which lead to the rare renal disease haemolytic uraemic syndrome (Herbert et al. 2006). These mutations have been shown to congregate in a polyanion recognition patch interfering with recognition of self markers which may then lead to unregulated activation of complement on cell surfaces (Herbert et al. 2006). Since the Y402H polymorphism also lies within a polyanion recognition region this suggests a similar underlying pathogenic mechanism.

The gene for complement factor H lies within a cluster of genes in chromosome 1q23 with the closely related genes CFHR1–5 spanning around 355 kb. They share a high level of sequence similarity. A recent study identified a common haplotype associated with a decreased risk of AMD which encompassed an 84 kb deletion within this cluster of genes which removed the CFHR1 and CFHR3 genes (Hughes et al. 2006). The proteins encoded by these genes were absent in the homozygotes. This cannot be explained by linkage disequilibrium with the Y402H CFH allele. The authors speculate that since there is such close similarity between CFH and these related proteins, the CFHR proteins may provide an additional level of complement regulation by competing with CFH for C3 binding. By removing the CFHR1 and CFHR3 some of this competition may be eliminated thus increasing the binding of CFH to C3 and its negative regulation of complement activity.

Mice lacking expression of the complement factor H gene have been generated in the past to study the renal disease associated with the null alleles (Pickering et al. 2002). A recent study investigated the retinal pathology in these knock-out mice. The mice had reduced visual acuity but no apparent differences in the retinal phenotype apart from an apparently deeper location of autofluorescent deposits (Moss et al. 2006). This indicates that CFH is required for the maintenance of a healthy retina and that additional studies of the Y402H polymorphism in mice would be of value.

Complement factors B and C2

Once it had been shown that abnormal complement regulation was likely to be important in susceptibility to AMD further investigation of other regulators of the alternative complement pathway revealed an association between AMD and complement factors B and C2 (Gold et al. 2006). Complement factor H, as described above, prevents uncontrolled activation of the alternative pathway by binding C3b and thus accelerating the decay of the complex C3bBb. Factor B acts to stabilise C3bBb and thus activates the pathway and factor C2 is an activator of the classical complement pathway. These genes lie within the major histocompatibility region on chromosome 6p21 only 500 bp (base pairs) apart. A common haplotype, associated with an increased risk of AMD and two protec-

tive haplotypes were identified. These results may indicate that changes in the enzyme activity of the activating factor B account for the variation with a reduction lowering complement activity giving a reduced risk of drusen formation, and the association with C2 reflecting linkage disequilibrium due to these genes lying so closely together.

Chromosome 10 AMD locus

In 2005, two groups identified a strong SNP association at the 10q26 locus with AMD. (Jakobsdottir et al. 2005) identified a strong association signal overlying three genes, PLEKHA1, LOC387715, and PRSS11 (also known as HTRA1). All non-synonymous SNPs known in this critical region were genotyped, yielding a highly significant association between PLEKHA1/LOC387715 and AMD. This association was independent of the previous association with the CFH Y402H variant. The association of either a single or a double copy of the high-risk allele within the PLEKHA1/LOC387715 locus accounted for an odds ratio of 5.0 (95% confidence interval 3.2–7.9) for AMD and a population attributable risk as high as 57%. Independently Rivera et al. (2005) also studied this region and found a significant association across a 60 kb region of high linkage disequilibrium harbouring two genes PLEKHA1 and hypothetical LOC387715. The strongest association centred over a frequent coding polymorphism, Ala69Ser, at LOC387715, strongly implicating this gene in the pathogenesis of AMD. The joint contribution of the common risk allele at LOC387715, Ala69Ser, and the CFH Y402H variant suggested a disease odds ratio of 57.6 (95% CI 37.2, 89.0) conferred by homozygosity for risk alleles at both CFH and LOC387715 when compared with the baseline non-risk genotype.

Subsequently, two other groups identified a different genetic variant at the same locus as the true association with AMD. Of interest, based on evidence of only a single cDNA sequence found in placental tissue, LOC387715 has subsequently been removed from the GenBank database.

DeWan et al. (2006) identified a novel SNP (rs11200638) in almost complete linkage disequilibrium (LD) with the previously described LOC387715 SNP rs10490924. Rs11200638 is located 512 bp upstream of the putative transcriptional start site of the gene HTRA1 and exhibited a complete LD pattern with the previously associated SNP rs10490924. Rs11200638 is a functional variant in the promoter region of HTRA1. In a whole-genome association mapping strategy in a Chinese population it was significantly associated with AMD $P < 10^{-11}$. Independently in a Caucasian population, Yang et al. (2006) similarly identified that the strongest association at the 10q26 locus was with the rs11200638 SNP $P = 1.6 \times 10^{-11}$ rather than the rs10490924 SNP $P = 1.2 \times 10^{-8}$. Thus a single mutation in the promotor region of HTRA1 appears to be the true association with AMD at the 10q26 locus.

The HTRA1 gene is biologically plausible because it encodes a member of a family of serine proteases expressed in the mouse and human retina and RPE (Oka et al. 2004; Yang et al. 2006). HTRA1 is activated under conditions of cellular stress. In addition, drusen from donor eyes of AMD patients have been found to express HTRA1 (Yang et al. 2006). It appears HTRA1 regulates the degradation of extracellular matrix proteoglycans. Conceivably, over expression of HTRA1 may alter the integrity of Bruch's membrane, favouring the invasion of choroidal capillaries across the extracellular matrix, as occurs in wet AMD. HTRA1 also binds and inhibits transforming growth factor-b (TGF-b), an important regulator of extracellular matrix deposition and angiogenesis (Oka et al. 2004).

Therefore HTRA1 may be particularly important in the development of CNV or "wet AMD" (DeWan et al. 2006). Yang et al. estimated that the population-attributable risk for HTRA1 is 49.3%. Consistent with an additive effect, the estimated population-attributable risk from a joint model with CFH Y402H (i.e., for a risk allele at either locus) is 71.4% (Yang et al. 2006).

VEGF

Vascular endothelial growth factor is a homodimeric glycoprotein and is a growth factor specific for endothelial cells (Ferrara et al. 2003). It is a critical regulator of vasculogenesis and angiogenesis, as well as a potent inducer of vascular permeability (Ferrara and Henzel 1989; Leung et al. 1989; Senger et al. 1983). Three VEGF receptors have been identified: VEGF receptor (VEGFR1) has both positive and negative angiogenic effects; VEGFR2 mediates the mitogenic, angiogenic and vascular permeability effects of VEGF-A; and VEGFR3 mediates the angiogenic effects on lymphatic vessels (Ferrara et al. 2003; Karkkainen et al. 2002).

The VEGF gene family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF-A is the target of most current anti-VEGF treatments (Carmeliet et al. 1996; Ferrara 2002). Nine major VEGF-A isoforms exist in humans: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$ (Takahashi and Shibuya 2005). These isoforms are produced by alternative exon splicing of the human VEGF-A gene (Ferrara et al. 2003; Takahashi and Shibuya 2005). $VEGF_{165}$ is the most abundantly expressed VEGF-A isoform and has a vital role in angiogenesis (Keyt et al. 1996; Soker et al. 1998).

Anti-VEGF treatments

Three anti-VEGF agents are now available in clinical practice. Pegaptanib sodium (Macugen™) is a pegylated ribonucleic acid oligonucleotide aptamer. It specifically targets the $VEGF_{165}$ isoform. Ranibizumab (Lucentis™) is a humanised antigen-binding fragment of a murine full-length monoclonal antibody (mAB) directed against human VEGF-A. Ranibizumab inhibits all VEGF-A isoforms. Bevacizumab (Avastin™) is similarly a humanised mAB against all VEGF-A isoforms approved as intravenous infusion for metastatic colorectal cancer. Off-label use of bevacizumab for neovascular AMD in small retrospective studies has shown benefits for vision and reduced macular thickening (Avery et al. 2006; Rosenfeld et al. 2005). Despite the lack of randomised clinical trial data bevacizumab has been widely used as a treatment for wet AMD. This is mainly due to its cheapness cf. the other available therapies. Randomised clinical trials of bevacizumab in comparison with ranibizumab are now being planned in both the United States and the United Kingdom.

Therapeutic outcomes

Pegaptanib has been shown to be beneficial for all subtypes of CNV secondary to AMD. This compares favourably to laser treatment with visudyne where only certain sub-types of CNV respond to treatment. In the VISION study, considerably more pegaptanib-treated patients lost <15 letters than patients who received sham injections (70 vs. 55%) at 12 months and more patients gained ≥3 lines of visual acuity (6 vs. 2%) (Gragoudas et al. 2004).

Even better visual outcomes have been reported in the MARINA and ANCHOR trials of Ranibizumab. In the phase III MARINA trial of patients with occult or minimally classic subfoveal CNV secondary to AMD at month 12 considerably more Ranibizumab-treated patients lost <15 letters than sham-injected patients (95 vs. 62%). Of the Ranibizumab-treated patients, 25 and 34% (0.3 and 0.5 mg, respectively) gained ≥15 letters compared with 5% of sham-injected patients, a significant difference (Rosenfeld et al. 2006). In the phase III ANCHOR trial of patients with predominantly classic subfoveal CNV secondary to AMD, 94% and 96% of Ranibizumab-treated patients (0.3 and 0.5 mg, respectively) lost <15 letters of visual acuity compared with 64% of photodynamic therapy-treated patients in month 12 (Brown et al. 2006). Of the Ranibizumab-treated patients, 36 and 40% (0.3 and 0.5 mg, respectively) gained ≥15 letters compared with 6% of photodynamic therapy-treated patients, a significant difference (Brown et al. 2006).

VEGF associations with AMD

Two studies have associated VEGF with AMD. Haines et al. (2006) found genetic linkage and allelic association, using two independent datasets: a family-based association dataset including 162 families and an independent case–control dataset with 399 cases and 159 fully evaluated controls. VEGF showed evidence of linkage (HLOD = 1.32) and demonstrated significant independent allelic association in both the family-based ($P = 0.001$) and case–control ($P = 0.02$) datasets.

Churchill et al. (2006) conducted a case–control study where 45 individuals with neovascular AMD and 94 age-matched controls were genotyped for 14 single nucleotide polymorphisms (SNPs) in the VEGF promoter and gene. The single SNP +674 CC genotype was significantly associated with AMD (OR = 2.40, 95%CI 1.09–5.26, $P = 0.027$). Haplotype analysis of SNPs +674, +4,618, +5,092, +9,162 and +9,512 revealed that CTCCT and TCACC were associated with AMD (OR = 15.77, 95% CI 1.91–130.24, $P = 0.0161$ and OR = 9.95, 95%CI 3.22–30.74, $P = 0.000053$, respectively).

Disease mechanisms

It has been known for some time that AMD is a common disease associated with aging and has a multifactorial aetiology with both genetic and environmental risk factors. Local inflammation has been thought to have a role in the development of AMD (Anderson et al. 2002; Hageman et al. 2001; Penfold et al. 1984, 2001) in part since the components of drusen consist of many inflammatory and acute phase proteins (Hageman et al. 1999; Johnson et al. 2000, 2001; Mullins et al. 2000) and components of the complement cascade and its regulation (Hageman et al. 1999; Johnson et al. 2001; Mullins et al. 1997, 2000, 2001; Russell et al. 2000). The recent genetic advances implicating genes encoding proteins involved in complement control underlines the importance of complement in the pathogenesis of AMD.

The Y402H polymorphism in factor H confers significant susceptibility to AMD in both homozygotes and heterozygotes. It is hypothesized that this change interferes with the negative regulatory role of CFH in the alternative complement cascade (Clark et al. 2006; Herbert et al. 2006) perhaps by interfering with recognition of polyanion structures on cell surfaces resulting in a lack of control and thus increased complement activation. Recent results describing an association with a deletion of the closely related genes CFHR1 and CFHR3 and an association with polymorphisms within the complement activator factor B support this suggested aetiology. They suggest uncontrolled activa-

tion of the alternative complement pathway at Bruch's membrane is likely to be important in the development of drusen to the pathogenesis of AMD.

There is an additional association between AMD and HTRA1 which acts as an independent risk factor to CFH. HTRA1 is activated under conditions of cellular stress and its over expression may alter the integrity of Bruch's membrane, allowing invasion of choroid (Oka et al. 2004).

Modifiable environmental risk factors such as smoking and obesity are known to act independently of the genetic risks (AREDS 2000; Cho et al. 2001; Christen et al. 1996; Cruickshanks et al. 1997; DeAngelis et al. 2007; Evans 2001; Hyman and Neborsky 2002; Khan et al. 2006b; Mares-Perlman et al. 1995; Seddon et al. 1994, 2003a). Smoking is known to lead to an increase in inflammatory mediators (Sastry and Hemontolor 1998) such as prostaglandins and leukotrines which appears to lead to additional inflammatory retina damage.

It has been suggested (Gehrs et al. 2006) that the most likely scenario is an event such as infection triggers the onset of AMD in individuals who are genetically susceptible and this with sustained activation of complement, perhaps with smoking acting as an additional trigger of inflammation leads to drusen and development of AMD. HTRA1 over expression may increase this risk and may be particularly important in susceptibility to CNV or "wet AMD" (DeWan et al. 2006).

Summary

The landscape of AMD research has changed dramatically in the last 2 years. Two genes of large effect have been identified which together contribute over 70% of the population attributable risk of AMD. The association of CFH with AMD highlights the importance of the alternative complement pathway in the development of AMD whilst the pathophysiology of HTRA1 is now under intensive study. It is somewhat surprising that the genetic variants of this disease are so limited. This is in contrast to other forms of retinal degeneration such as retinitis pigmentosa (RP) where currently over 37 genes have been identified which cause RP (http://www.sph.uth.tmc.edu/Retnet/).

Of course this is good news for patients as early genetic testing will be easier with a limited number of genetic variants to test for. Similarly drug developments which correct the defects in the CFH complement or HTRA1 pathways will be of benefit to the majority of patients who suffer from AMD. In the future we can therefore expect targeted drug therapies and personalised medicines for patients based on their underlying genotype. Thus patients affected with AMD can increasingly expect to maintain, what to most people is their most highly valued sense, their sight.

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