Unraveling a Multifactorial Late-Onset Disease: From Genetic Susceptibility to Disease Mechanisms for Age-Related Macular Degeneration

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Key Words

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Abstract

Aging-associated neurodegenerative diseases significantly influence the quality of life of affected individuals. Genetic approaches, combined with genomic technology, have provided powerful insights into common late-onset diseases, such as age-related macular degeneration (AMD). Here, we discuss current findings on the genetics of AMD to highlight areas of rapid progress and new challenges. We also attempt to integrate available genetic and biochemical data with cellular pathways involved in aging to formulate an integrated model of AMD pathogenesis.

INTRODUCTION

Many common noninfectious diseases exhibit a more severe clinical presentation in older individuals. These diseases often exhibit complex etiology and can affect different tissues and cell types, with a wide spectrum of clinical outcomes. Prominent aging-associated neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), and age-related macular degeneration (AMD), all of which can severely compromise the quality of life and have serious repercussions on both the individual and society at large. These late-onset diseases generally result from the interplay between multiple genetic susceptibility factors and environmental components. Sequencing of the human genome, cataloging of millions of single nucleotide polymorphisms (SNPs) together with the development of a map of common haplotypes, and technological innovations in genotyping are among the major milestones that are facilitating exploration of the genetic basis of common diseases (1, 7, 50). In the field of AMD genetics, these advances have led to the identification of several genetic susceptibility factors and enabled us to start dissecting the relationship between environmental risk factors and the genetic constitution of each individual (66, 118, 148). As a result, new opportunities are emerging for improved understanding of disease pathogenesis that may lead to better management and treatment of AMD. Clinical aspects of AMD are discussed only briefly (for a more in-depth discussion, see Reference 79).

STRUCTURE OF THE HUMAN EYE

To understand the pathobiology of AMD, we briefly describe the structure of the human eye (**Figure 1**). The perception of light begins in the retina, a part of the central nervous system that is easily accessible to evaluation and relatively amenable to treatment (123). The lens and cornea are optical elements in the eye that focus light on the fovea, a well-defined region of the retina responsible for capturing high-resolution visual information. The fovea is the center of the macula, which is 5–6 mm in diameter, has a dense concentration of photoreceptors, and a ratio of cone to rod photoreceptors that is approximately twice as high as that of the remaining retina. Overall, the human retina has approximately 20 times more rods than cones;
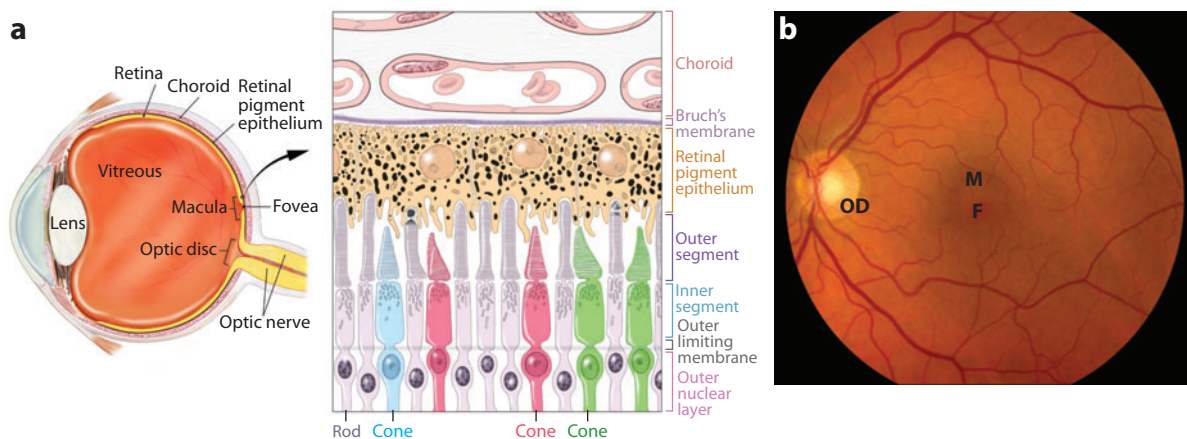
Figure 1

(*a*) Cross-section of the human eye, and schematic of the photoreceptor-retinal pigment epithelium (RPE)-choroidal layers that are affected in AMD. Cone photoreceptors are shown in red, green, or blue. RPE apical processes are intimately associated with photoreceptor outer segments. Two of the rods are shown in the active process of outer segment disc shedding. Melanosomes are shown as dark organelles in RPE cells. (*b*) Fundus photograph showing the retina of a normal individual. Retinal blood vessels are clearly visible. F, fovea; M, macula; OD, optic disc.

rod photoreceptors are highly sensitive to light and allow night vision, whereas cones are responsible for color perception and high visual acuity. Rods and cones are polarized neurons with specialized visual phototransduction proteins that are concentrated in membranous-like structures, the outer segments. These discs have high metabolic demands and undergo continuous renewal in a process controlled by light and circadian rhythms (172). As outer-segment discs are continuously shed at the distal tips of the outer segments and replaced, the retinal pigment epithelium (RPE) is responsible for clearing old shed discs by phagocytosis and supplying both cones and rods with the nutrients and oxygen they need. The RPE also serves as a barrier to the small fenestrated capillaries of the choroid or choriocapillaris, thereby playing a critical role in maintaining photoreceptor health (see **Figure 1**) (15). A thin stratified extracellular matrix, Bruch's membrane, separates the RPE from the choriocapillaris.

CLINICAL DESCRIPTION OF AMD

AMD is a late-onset, progressive degenerative disease that encompasses a broad spectrum of clinical phenotypes and pathology, primarily affecting the macular region of the human retina. AMD increases in prevalence with advancing age, with a typical onset in the sixth decade. In early stages, AMD causes minimal visual impairment; however, in severe cases it leads to the loss of high-resolution central vision in affected individuals. Numerous definitions and classifications of AMD exist in the literature (13). In this review, we use the Age-Related Eye Disease Study (AREDS) classification of AMD because beneficial preventive therapy decisions can be made based on this classification (4).

An early clinical sign of AMD is the appearance of drusen, which are yellow extracellular deposits of protein and lipid materials beneath the retina (**Figure 2**). Early AMD is defined by the presence of multiple (>5) small drusen or the presence of at least one intermediate drusen (drusen size of ≥63 μm but <125 μm).

Intermediate AMD refers to retina with many intermediate drusen or at least one large drusen (drusen size of ≥125 μm). The large drusen are often accompanied by hyper- or hypopigmentation of the RPE. Advanced AMD, the third category of AMD, includes two different types of late-stage lesions—geographic atrophy **(GA)** and neovascular (NV) AMD (**Figure 2e–b**). GA is clinically defined as a discrete area of retinal depigmentation at least 175 μm in diameter with a sharp border and visible choroidal vessels in the absence of neovascularization in the same eye. AMD is a progressive disease resulting from the development of focal loss of retinal photoreceptors and RPE as well as small blood vessels directly beneath the RPE. In NV-AMD, clinical manifestations include serous or hemorrhagic detachment of either the RPE or sensory retina, presence of subretinal fibrous tissue, and eventually widespread RPE atrophy. The onset of vision loss is usually acute with the development of neovascularization under the retina. Neovascular AMD accounts for over 90% of severe central vision loss among patients with AMD (116, 154, 161).

EPIDEMIOLOGY OF AMD

AMD is a leading cause of severe and incurable vision impairment in the United States and other developed countries (31). It accounts for more than 54% of all blindness in the USA, with approximately 1.75 million people of age 40 years or older affected with advanced AMD and another 7.3 million with intermediate AMD (51). Age is the strongest risk factor associated with the development of AMD (3, 112, 140) (**Figure 3**). The incidence of early AMD, large drusen and pigmentary changes in intermediate AMD, and choroidal neovascularization (CNV) and GA in advanced AMD increase with advancing age (51, 93). The pooled data from two population-based studies reveal an estimated prevalence of advanced AMD of ∼0.2% in ages 55 to 64, increasing to 13% in those older than 85 years (140). The number of individuals with AMD is expected to increase worldwide as the population ages and

RPE: retinal pigment epithelium

AREDS: Age-Related Eye Disease Study

GA: geographic atrophy

NV: neovascular

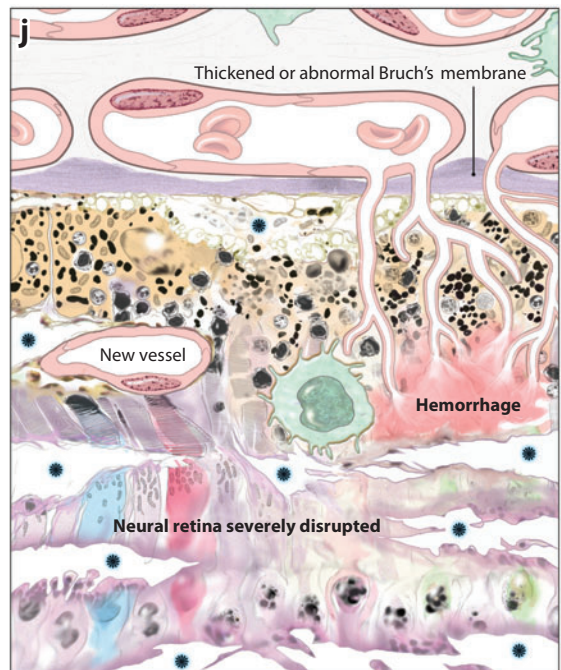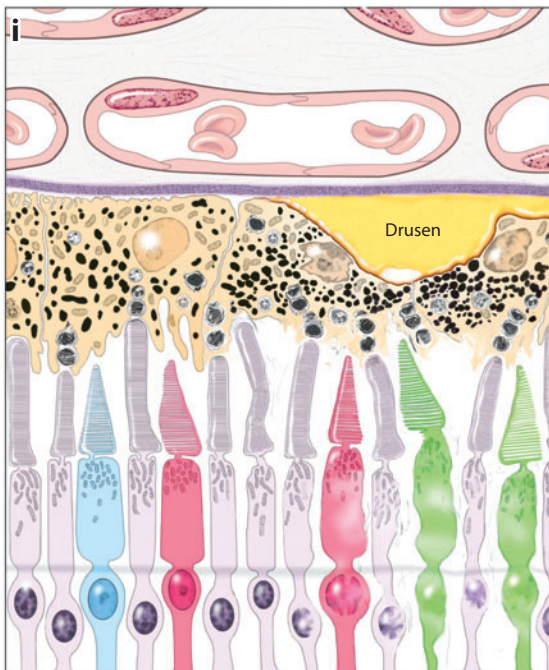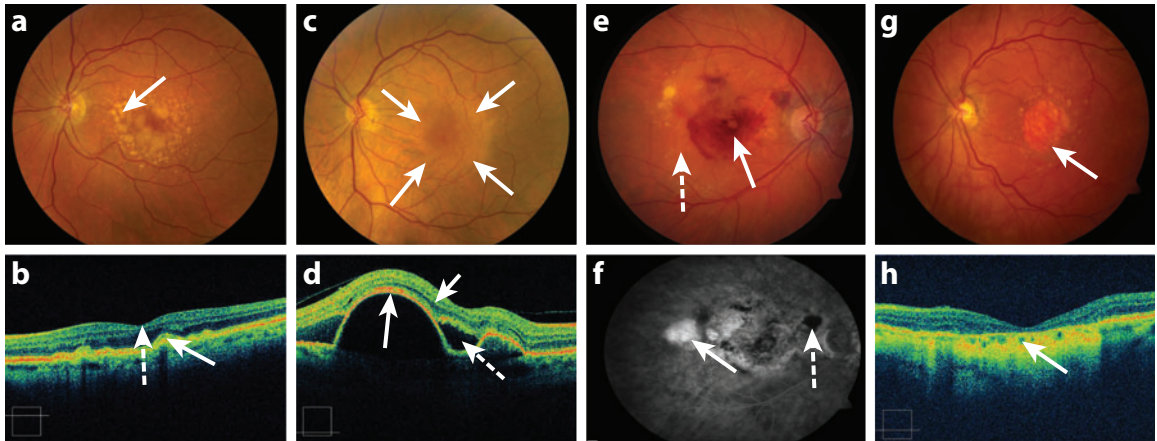**CNV:** choroidal neovascularization

treatments for preventable blindness (such as cataract) become more widely available.

Epidemiological studies have revealed differences in the prevalence of advanced AMD among different ethnic groups (51, 3, 141). An analysis of U.S. participants in the Multi-ethnic Study of Atherosclerosis (MESA) showed the prevalence of AMD in persons aged 45 to 85 years to be 2.4% in African-Americans, 4.2% in Hispanics, 4.6% in Chinese-descent individuals, and 5.4% in Caucasians (94).

PATHOLOGY OF AMD

Early pathological changes in AMD include the appearance of basal deposits in Bruch's

Drusen

New vessel

Thickened or abnormal Bruch's membrane

Hemorrhage

Neural retina severely disrupted

Gaps, tissue separating or breaking apart ✻

membrane, which is made up of the basement membranes of the RPE and the choriocapillaris. These deposits are of two distinct types (59): (*a*) basal laminar deposits between the RPE and basement membrane, and (*b*) basal linear deposits, which are composed primarily of membraneous material located external to the RPE basement membrane in Bruch's membrane and are believed to be more specific to AMD (126). Accumulation of these deposits together with secondary RPE changes may lead to the formation of drusen. Biochemical analysis of drusen has identified glycoconjugates and components found in atherosclerotic plaques including vitronectin, apolipoprotein B and E, alpha-crystallin, complement proteins, and lipids (32, 106, 115).

Drusen can present as small discrete nodules, known as hard drusen, or as large nodules with ill-defined, indistinct boundaries, known as soft drusen (19, 126). Soft drusen are associated with more extensive damage to the retina, RPE, and choroid, and predict more severe clinical outcomes, such as GA and CNV (45). GA shows abnormal RPE with cellular hypotrophy and hypertrophy, hypopigmentation and hyperpigmentation, loss of photoreceptors, Bruch's membrane thinning, and degeneration
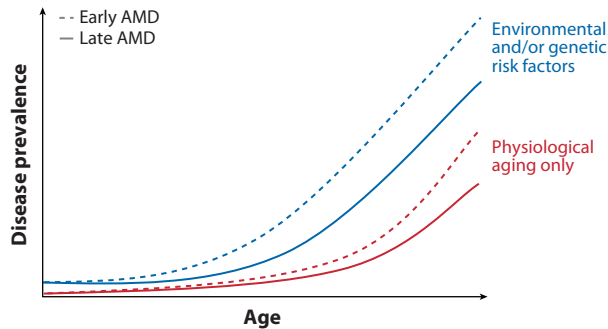
Figure 3

A representation of disease prevalence with age. The incidence of both early and late AMD greatly increases in all individuals at a late age (age 80 and above). We suggest that individuals with genetic susceptibility variants and those who have been exposed to environmental risk factors exhibit the disease at much earlier age (age 60 and above).

of the choriocapillaris (59, 103). Although RPE is the most affected cell layer, the photoreceptor layer is also severely attenuated (87). CNV refers to the growth of new blood vessels from the choroid through Bruch's membrane and under the RPE (125). These vessels eventually break through the RPE (60), resulting in severe clinical phenotypes including (*a*) neovascular AMD, where lipid-rich fluid accumulates under the RPE and/or neural retina; (*b*) hemorrhagic AMD, where blood breaks

Figure 2

(*a*) Fundus photograph of the left eye of a patient with large, "soft" drusen (*arrow*), indicative of intermediate AMD. The patient has good vision. (*b*) The optical coherence tomography (OCT) image of the patient in (*a*) with drusen. This image has the inner retina pointing toward the top with the choroid shown at the bottom of the image, a reverse of our schematic seen in panels (*i*) and (*j*). The solid arrow points to corresponding areas of drusen that have caused an elevation of the retinal pigment epithelium, giving it a "bumpy-lumpy" appearance. This section of the OCT shows the center of fovea with the retinal layers shown as a central "foveal depression" (*dashed arrow*). (*c*) Fundus photograph of a patient with an elevated RPE detachment, which is the creamy-colored round lesion centered on the macula (outlined by the arrows). The patient still has good vision because the retina has no intraretinal or subretinal fluid. This is another form of advanced AMD that may lead to NV or GA disease. (*d*) The OCT image of the patient in (*c*) with elevated RPE detachment (*long solid arrow* indicates the RPE layer). There is little intraretinal fluid (*short solid arrow*) and subretinal fluid (*dashed arrow*) detected. (*e*) Fundus photograph of the right eye with evidence of advanced AMD, the neovascular form with evidence of subretinal hemorrhage (*solid arrow*), retinal hard exudates (yellow waxy lesions, *dashed arrow*), and large drusen. The visual acuity is 20/160. (*f*) Fluorescein angiogram of patient with neovascular AMD, leakage of the fluorescein dye in the late frames through the abnormal neovascular complex, resulting in hyperfluorescence (*white area*, *solid arrow*). The subretinal hemorrhage blocked the dye, resulting in an area of hypofluorescence (*dashed arrow*). (*g*) Fundus photograph of patient with the "dry" form of advanced AMD, with GA centered on the macula, a well-defined area of atrophy with the loss of the neuroretina, RPE, and the choriocapillaris. The large vessels within the atrophic area are the remaining vessels of the choroid that can be viewed readily because of the lack of the pigmented layer of the RPE (*arrow*). Peripheral to the GA are large drusen. The visual acuity is 20/200. (*h*) The OCT image of the patient in (*g*) with thinning of the retina and the loss of the RPE and choriocapillaris in the center of the macula (*arrow*). (*i*) Schematic of the photoreceptor-RPE-choroid region from an early AMD retina. A large drusen is indicated. (*j*) Schematic of the photoreceptor-RPE-choroid region from a late AMD retina. Various abnormalities are indicated.

through the RPE into the subretinal space; and (*c*) disciform AMD, where fibrous tissue with NV and altered RPE proliferates and replaces neural retina.

Though the initial lesions in both GA and CNV involve RPE and Bruch's membrane pathology, it is the dysfunction and death of macular photoreceptors that are responsible for impairment of central vision in AMD (78).

Based on epidemiology and pathobiology, prevalence of AMD phenotypes depends on three key risk factors: environmental factors, advanced age, and genetic susceptibility variants (see **Figure 3**).

ENVIRONMENTAL FACTORS

As in other complex diseases, environmental factors play an important role in AMD development. Smoking is a modifiable and consistently associated environmental factor, typically resulting in about two-fold increased risk of developing advanced AMD (3, 112, 150, 155). A dose effect of enhanced risk of AMD with increasing number of cigarettes smoked has also been reported (86). The mechanism by which smoking affects the retina is unknown, although oxidative insults to the retina have been suggested (43).

The association of cardiovascular disease and its risk factors with AMD have not been consistently reproduced (26, 127, 136, 153). It is plausible that AMD and cardiovascular disease share common risk factors. Results from observational, case control, and population-based studies have also suggested an association of diet with AMD susceptibility. Diets high in antioxidants lutein and zeaxanthin (found mostly in green leafy vegetables) and in omega-3 fatty acids (primarily found in fish) have been linked to a decreased risk of neovascular AMD (48, 134).

Additional environmental factors that may influence AMD pathogenesis include sunlight exposure (33, 35, 85), alcohol use (23, 24, 114), and infection of bacterial pathogens (particularly *Chlamydia pneumoniae*) (11, 76, 81).

INTEGRATING THE BIOLOGY OF AGING IN STUDIES OF AMD

Aging is a genetically regulated process that is defined by progressive decline of somatic cell functions with age (46, 70). In mammals, aging-associated morphological, molecular, and functional alterations may represent an adaptive response to accumulation of damage (29, 166, 169), followed by cellular dysfunction and degeneration (16, 34, 55, 78). In humans, advanced age is accompanied by increased vulnerability to disease, with progressive and generally irreversible loss of physiological functions; in the eye, these include deficits in visual processing and degenerative changes in retinal neurons (particularly rods and ganglion cells) and the RPE (16, 78). Older individuals commonly have small drusen beneath the retina and cholesterol accumulation in Bruch's membrane (64, 74). Another sign of aging is the accumulation of lipofuscin, an undegradable fluorescent material; in the RPE, accumulation of lipofuscin may be due in large part to incomplete digestion of shed photoreceptor discs (142). A2E (a by-product of phototransduction, present in lipofuscin) and mitochondrial dysfunction are reported to impair RPE phagocytosis and activate the complement system (156, 178). The pathogenesis of Stargardt's disease, an early onset disorder whose symptoms resemble those of AMD, is thought to result from the accumulation of A2E in the RPE (110).

Aging is the single strongest risk factor for AMD; hence it is critical to integrate the biology of aging in models of AMD. Adaptive responses of the retina and RPE to aging are reflected in changes in expression of specific genes and cellular pathways (171). These changes likely contribute to the initiation of AMD; therefore, sequence variation in some of these genes may modulate individual specific risk of developing AMD. Aging-associated expression changes in genes associated with mitochondrial function, protein metabolism, and immune response have been identified in several tissues (173) and are consistent with some proposed mechanisms for late-onset neurodegenerative diseases (98, 113, 124, 166). Although an

extensive gene expression database exists for the aging mouse (173), knowledge of such changes in retina remains limited. In humans, these studies are hampered by difficulties in obtaining appropriate patient material and interindividual variability in gene expression profiles of the retina (25). Nevertheless, an initial comparison of young and aging human retinas identified differential expression of a small number of genes involved in stress and immune response, protein and energy metabolism, and inflammation (171). Retinal expression profiles of senescence-accelerated mice highlighted expression changes for a different set of genes (21). Given that gene profiles from the whole retina capture only an average of expression changes across heterogeneous cell populations and that rod photoreceptors are more vulnerable to aging (78), gene expression studies of aging in specific cell types are likely to be more informative. Initial expression analyses of GFP-tagged rod photoreceptors purified from the retinas of mice (5) demonstrate aging-associated changes in genes involved in mitochondrial function, protein degradation, and apoptosis (S. Parapuram & A. Swaroop, unpublished data). These changes may permit rod photoreceptors to adapt to aging and survive. Ultimately, it will be necessary to integrate our knowledge of aging in the human retina and in model organisms with the genetic and environmental risk factors that underlie AMD susceptibility if we are to obtain a comprehensive understanding of early steps in AMD pathogenesis.

GENETIC SUSCEPTIBILITY FACTORS

The contribution of genetics to AMD was carefully documented throughout the 1990s with reports of familial aggregation, concordant phenotypes in twins, and a higher risk of disease in first-degree relatives of affected individuals (69, 89, 91, 132). Twin studies demonstrated that genetic factors play a substantial role in the etiology of AMD, with some estimates suggesting that as much as 71% of the variation in the overall severity of AMD is genetically

determined (133). Marked progress in identifying the genetic contributors has been achieved during the past ten years (see also 148).

GENETIC LINKAGE ANALYSIS

Linkage studies of AMD using large families have been difficult because of the late onset of disease, phenotypic variability, and phenocopy effects. Klein and colleagues were the first to report linkage to chromosome 1q25–31 in an apparently autosomal dominant pedigree with dry AMD (92). As with other complex diseases, gene mapping of AMD gradually shifted in focus from studies that include a small number of large families with many affected individuals to studies of much larger numbers of smaller families with affected pairs of siblings and other close relatives (121, 148). These studies of affected relative pairs were designed specifically to facilitate gene identification in complex multifactorial traits with a late age of onset (14, 121). In AMD, several independent studies, and a meta-analysis of multiple genome scans (47), suggested susceptibility loci with large effect at chromosomes 1q31 and 10q26. In addition, these studies provided more modest evidence for linkage on many other chromosomes (2, 77, 104, 137, 160), including a region on chromosome 22 near TIMP3, the gene mutated in Sorsby fundus dystrophy (158) (**Figure 4**).

Overall, linkage studies have had only limited success in identifying complex disease susceptibility genes; nonetheless, in the case of AMD they ultimately led to the discovery of genetic variants near *CFH* and *ARMS2/HTRA1* as the strongest genetic contributors to AMD susceptibility (see below).

GENETIC ASSOCIATION ANALYSIS

Genetic linkage studies of complex traits can, at best, map disease susceptibility loci to intervals that are several megabases long and generally include hundreds of genes. Narrowing these intervals requires identification of the association between specific polymorphisms and
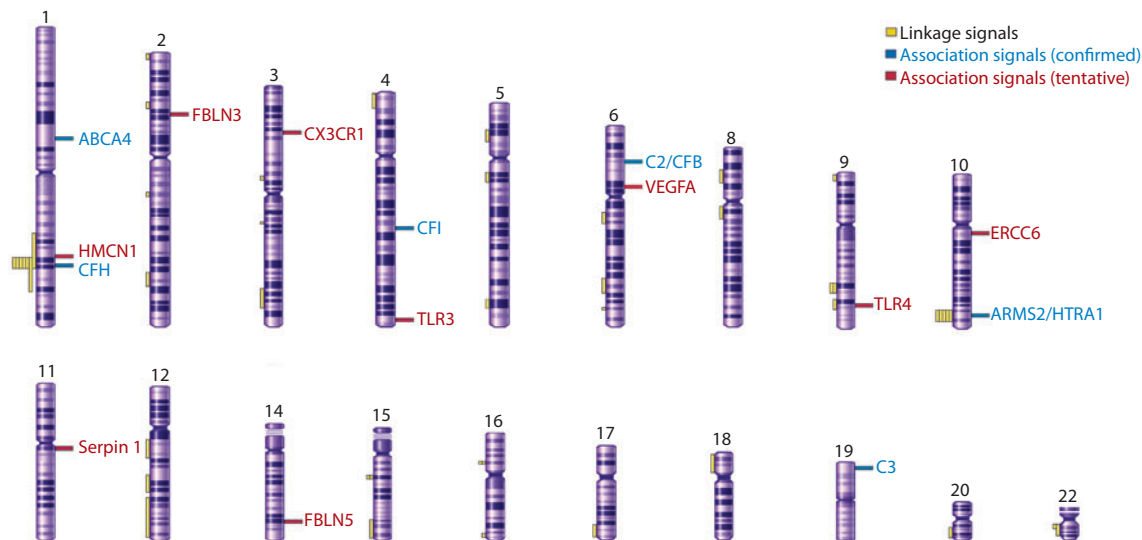
Figure 4

A schematic of human chromosomes showing regions of linkage identified in different studies and association signals (confirmed, and tentative requiring further validation) for AMD susceptibility loci/genes (modified from Reference 148).

disease. During the past few years, genetic association studies, which typically compare the frequency of specific variants between AMD cases and controls of similar ancestry, have identified several susceptibility loci. These studies can either be targeted to specific genes and candidate regions or attempt to survey common genetic variants across the genome. The latter, termed a genome-wide association study (GWAS) (71, 111), has only recently become feasible, owing to rapid advances in genotyping technology. In AMD, both focused association studies and GWAS have been extremely successful.

GENETIC ASSOCIATION STUDIES OF THE COMPLEMENT PATHWAY GENES

Perhaps the most significant breakthrough in our understanding of the genetics of AMD came in early 2005 with the identification of a strong association between disease and variants in and around the complement factor H (*CFH*) gene (**Figure 5**); this gene maps at 1q32, close to a linkage region at 1q25–31 that was identified in several independent studies (see **Figure 4**).

Initial reports of association included candidate gene approach, fine mapping of candidate loci, and the first successful whole-genome association study in humans (40, 63, 65, 95) and were immediately replicated (175). This breakthrough association has now been validated in numerous studies worldwide and has proven significant in several ways.

First, identification of a strong association between AMD and the *CFH* region prompted evaluation of genetic association between AMD and several other genes in the complement pathway. These subsequent association studies were extremely successful, and several additional complement genes have now been strongly associated with AMD. These include complement 2 (*C2*) and/or complement factor B (*CFB*) (56), complement 3 (*C3*) (170), and complement factor I (*CFI*) (44). Together, these association signals account for a large part of the genetic contribution to AMD susceptibility.

Second, identification of a strong association between AMD and *CFH* prompted finemapping studies of the region that illustrate the importance of evaluating a full set of genetic variants in each disease susceptibility locus.

GWAS: genome-wide association study
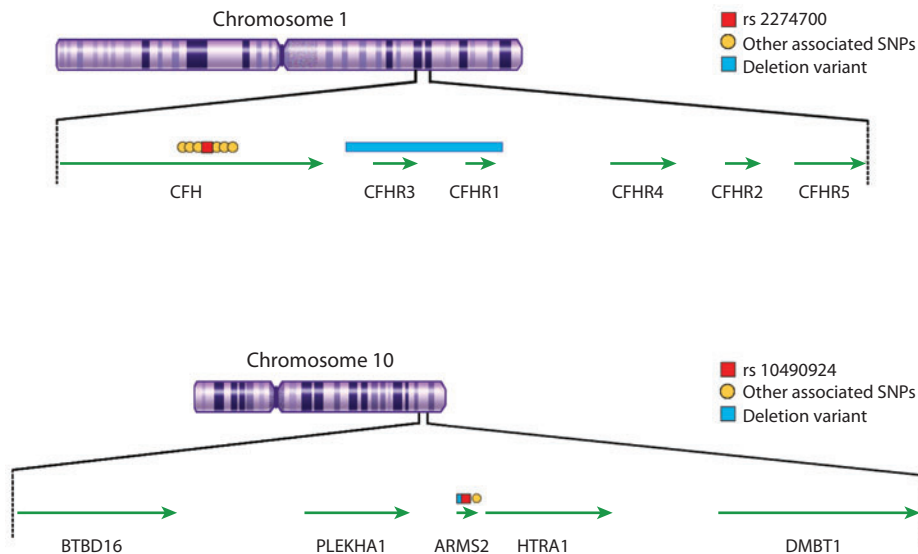
Figure 5

Genomic regions at chromosome 1q32 and 10q26 showing the genes and key associated single nucleotide polymorphisms (SNPs) and deletion variants.

These studies point to the issues and challenges that geneticists will encounter in searches for the ultimate "causal" variants at each complex disease susceptibility locus. Initial reports of association with the *CFH* gene focused on the Y402H coding variant that alters a single amino acid residue in the CFH protein. An early attempt at fine mapping the region showed that >20 variants in and around *CFH* were even more strongly associated with disease than was the Y402H coding variant, potentially suggesting that regulation of the expression of *CFH* and neighboring genes, rather than alterations in the coding sequence of *CFH*, mediates AMD susceptibility (97). This analysis also demonstrated that no single genetic variant could account for the contribution of the *CFH* locus to disease susceptibility and was soon corroborated by others (e.g., 11, 107). In addition to simple sequence changes, deletions in two genes near *CFH*, *CFHR1* and *CFHR3*, have been associated with reduced risk of AMD (75, 143) (see **Figure 5**). Therefore, detailed analyses of every disease susceptibility locus (to discover the full complement of genetic variants it contains and assess their contributions to disease susceptibility) will be required to make accurate predictions about disease risk for each individual.

Third, the association between *CFH* and AMD provided one of the first examples of how genetic association studies, and in particular GWAS, could accelerate the pace of discovery for complex traits, such as AMD, where progress using more conventional approaches had been relatively modest. In this sense, studies of AMD genetics provided the "proof-of-principle" experiment that has since been reproduced for many other traits and conditions. As geneticists and biologists evaluate the best strategies to follow up the GWAS results and further dissect genetic contributions to complex disease susceptibility, studies of AMD genetics will undoubtedly retain this pioneering role.

GENETIC ASSOCIATION STUDIES OF THE *ARMS2/HTRA1* REGION

Outside the complement pathway, the major genetic contributor to AMD susceptibility lies in the *ARMS2/HTRA1* region (see **Figure 5**).

The association signal in this region was initially identified as a result of fine-mapping efforts designed to narrow the chromosome 10q26 linkage peak (80, 122). This finding was later confirmed in several targeted studies (107) and in a GWAS (37). At least three potential candidate genes reside in the region of association; however, the identity of the gene that affects disease susceptibility is subject of controversy. Whereas two reports implicated *HTRA1* as a target of AMD susceptibility (37, 167), recent studies support the original claim (122) of *ARMS2* (previously LOC387715) as a stronger candidate (52, 82). The *ARMS2* gene sequence is detected in primates and likely encodes a mitochondrial protein (82). Equally significant, the AMD-associated deletion-insertion polymorphism in *ARMS2* is shown to mediate mRNA turnover (52). Genetic studies also suggest modification of the susceptibility effect of *ARMS2* by smoking (128), consistent with its involvement in mitochondrial function (66, 82). It is, nonetheless, possible that susceptibility variants within the *ARMS2* gene affect the activity of *HTRA1* promoter, which is only a few kilobases from *ARMS2*, so that both genes *ARMS2* and *HTRA1* influence AMD susceptibility. Biological and functional studies of *ARMS2* have been difficult because of the lack of a homologous gene in common model organisms (such as mouse or zebra fish). Additional investigations are necessary to define the role of *ARMS2* and associated causal variants in AMD pathogenesis.

CANDIDATE GENE ASSOCIATION STUDIES

A multitude of candidate gene association studies have been carried out for AMD. Their findings, however, have not been replicated as widely or as strongly as those summarized above and should, for now, be treated as tentative. Typically, these genes have been selected based on a priori knowledge of the biological pathways involved in disease or on investigator prejudices about the pathways likely to contribute to disease susceptibility (7, 149). We caution

that, in the absence of large, definitive, confirmatory studies, the possibility of publication bias cannot be discounted.

Apolipoprotein E (*APOE*) was an early target of candidate gene studies because of its presence in drusen, its key role in transport of lipids and cholesterol, and established association in Alzheimer's disease. Individually, these studies have been relatively small, and evidence for association has been modest, although together the published studies provide stronger evidence of an association between *APOE* and AMD (10, 129, 177).

Other candidate gene studies focused on genes implicated in macular dystrophies with Mendelian inheritance and phenotypic similarities to AMD (146). Genes in this category include those implicated in Stargardt's disease (caused by mutations in *ABCA*4), Sorsby fundus dystrophy (*TIMP*3), Best's disease (*VMD*2), Malattia Leventinese (*EFEMP*1 or *Fibulin-3*), Stargardt-like dominant macular dystrophy (*ELOVL*4), butterfly dystrophy and bull's eye maculopathy (*RDS*). A gene (*C1QTNF5*) mutated in patients with an autosomal dominant late-onset retinal/macular degeneration with sub-RPE deposits similar to AMD has also been examined (68). Except for two *ABCA*4 alleles (6), no variant in any other inherited macular dystrophy gene appears to show a significant association with AMD (148).

A natural progression of studies that examine genes previously implicated in Mendelian syndromes is the assessment of mutations in genes known to have similar structure or function. For example, the findings of Hemicentin 1/Fibulin 6 variant in autosomal dominant dry AMD (131) and a mutation in *EFEMP1/Fibulin-3* identified in Malattia Leventinese and Doyne honeycomb retinal dystrophy led to the screening of five fibulin genes for mutations associated with AMD; this screeening revealed a variant in fibulin-5 in AMD patients (145).

Given our relatively limited understanding of the biological pathways underlying AMD susceptibility, a wide variety of other genes have been tested for association. Consistent with the

role of mitochondria in neurodegenerative disease, a specific mitochondrial DNA polymorphism, A4917G, is reported to be associated with AMD (20). Toll-like receptor 4 (*TLR4*) was considered an attractive candidate due to its key role in innate immunity, potential function in phagocytosis, and location to a region that exhibits some evidence of linkage to AMD; an association between the D299G *TLR4* variant and AMD was initially reported (176) but has not been validated (36, 39). More recently, an association between AMD and another Toll-like receptor 3 (*TLR3*) was reported (168), but this association was not replicated in other large cohorts (41). A polymorphism in the 5′-upstream region of *ERCC6*, a gene implicated in DNA repair caused by oxidative stress and/or aging, has been associated with AMD susceptibility and was even suggested to exhibit epistatic interaction with *CFH* variants (152). Because of a potential role of retinal microglia in AMD, variants in the chemokine receptor gene *CX3CR1* were examined for association with AMD; homozygosity of the *M280* allele showed defective cell migration and appeared to be associated with higher AMD susceptibility (30). Multiple associations between AMD and HLA and cytokine genes have also been reported (57, 58). Screening of potential functional candidate genes identified association with an intronic variant within the *SERPING1* gene (42); however, other studies failed to replicate these findings (119). These are all attractive candidates based on our current understanding of pathogenic mechanisms underlying AMD, but—as with other genetic association signals–the original signals require validation in large and independent samples (71, 111).

There are now several examples where variants in loci implicated in Mendelian disorders also contribute to susceptibility to more common forms of disease; these include discoveries related to the genetics of blood lipid levels and coronary artery disease (83, 162), genetics of obesity (100, 163), and the genetics of height (96, 159). In all these instances, common variants that contribute to trait variation in loci known to harbor Mendelian variants were

missed in several earlier studies. In particular, genetic association studies apparently failed to notice positive signals in these candidate loci because they (*a*) defined the locus too narrowly (e.g., by focusing only on coding regions and a short flanking promoter sequence, although it now appears that regulatory variants can be located 10s or 100s of kb away from the gene); (*b*) relied on relatively small sample sizes (GWASs typically examine thousands of individuals and are often better powered to detect modest association signals than previous studies); or (*c*) did not comprehensively evaluate genetic variants in the candidate locus. When large and well-powered studies that examine all common variants in each of these candidate genes are carried out, we expect that some of the candidate loci implicated in disorders related to AMD may eventually be shown to also play a role in susceptibility to AMD.

PATHOGENIC MECHANISMS: CELLULAR PROCESSES AND PRIMARY AFFECTED CELL TYPES

The preceding discussion points to the complexities associated with delineating the role of genetic variants in mechanisms underlying the pathogenesis of AMD. This is not unexpected as AMD presents itself clinically in a variety of forms and affects many distinct cellular processes. To further our understanding of disease, all findings related to aging biology, genetics, and environmental risk factors must eventually be integrated into a unifying mechanistic model of AMD. A significant overlap in functional pathways highlighted by these disparate research strategies is gradually emerging.

Let us discuss the biological impact of advanced age, which is the primary risk factor for AMD. Numerous studies have demonstrated a central role of mitochondria and energy homeostasis in the pathogenesis of aging-associated neurodegenerative diseases (98, 157, 166). As photoreceptors have high oxygen consumption and energy demands, one can hypothesize increased susceptibility to DNA damage and

production of reactive oxygen species in the aging mitochondria of these cells (61, 99, 165). Photoreceptor outer-segment renewal is suggested to serve as a means to eliminate these reactive oxygen species and other damaging molecules, minimizing resulting DNA damage (164). A deficiency in antioxidant processes or increased oxidative stress could therefore make the photoreceptors and consequently RPE vulnerable to disease. Protein homeostasis is another important aspect that requires serious consideration (113). Cellular function and adaptation requires appropriate folding of proteins and elimination of damaged proteins by the combined activities of chaperone networks and protein degradation systems; aging-associated decline in these pathways can lead to protein aggregates that can compromise biochemical functions and cellular health (113, 124). Notably, protein aggregates (lipofuscin and drusen) are hallmarks of retinal aging as well as of AMD. Gene profiles of aging retina and photoreceptors (described above) provide strong support for significant dysfunction in both energy and protein homeostasis.
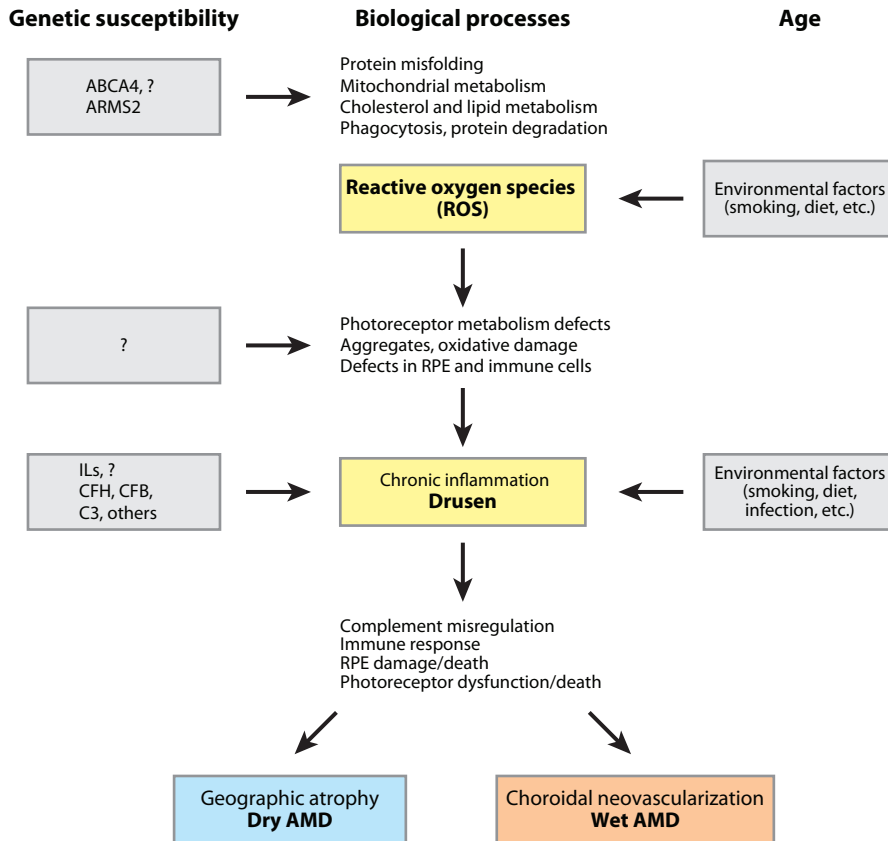
AN INTEGRATED MODEL OF AMD PATHOGENESIS

Based on extensive biochemical, pathobiological, and genetic studies implicating oxidative stress and inflammation (12, 64, 165, 174), compromised energy and protein metabolism in aging retinal photoreceptors and RPE likely constitute one of the early steps in AMD pathogenesis (**Figure 6**). The resulting oxidative stress and protein misfolding are followed by lipid peroxidation and formation of protein aggregates, which may cause damage to outer-segment membrane biogenesis and RPE phagocytosis. These initial aging-associated defects are further compounded by genetic susceptibility and environmental factors. We speculate that the ARMS2 protein plays a specific role in primate life span-dependent mitochondrial adaptation. Similarly, smoking can further exacerbate the oxidative stress and add

potentially toxic molecules. Whereas the primary triggers of AMD may originate in the photoreceptors because of their high membrane and protein turnover and exposure to light and oxygen, we believe the RPE quickly becomes involved because of its close relationship with photoreceptors. Even a slight decrease in the rate of clearing toxic protein and lipid debris could lead to their accumulation and even aggregation. This, in turn, could cause RPE damage and release of chemokines and cytokines (139). Decreased proteasome function during retinal aging (101), studies on A2E and *Abca*4-knockout mice (110, 156, 178), and changes in the RPE mitochondrial proteome (117) support this hypothesis. In addition, the CEP-mouse model demonstrates the role of oxidative damage in inducing inflammation and eventually GA-like lesions (72).

Genetic studies have established the roles of chronic inflammation and complement pathway in AMD pathology. It has been proposed that AMD is a systemic disease with specific manifestations in the macula of older individuals (118, 130). However, we believe that such macula-specific immune responses are unlikely without specific initiators of the disease process. We suggest that differential immune response to aging-associated RPE damage (as elaborated in the preceding paragraph) leads to specific clinical manifestations. Common and specific variants in complement and inflammatory response genes are therefore likely to play critical roles in generating an array of AMD phenotypes.

In attempting to integrate current knowledge on AMD, we have found a "multiple-hit" model of disease susceptibility to provide a convenient intellectual framework. Each hit represents the addition of a genetic, environmental, or aging-related risk factor that increases disease susceptibility for each individual. A clinically relevant phenotype is identified only when a threshold effect is reached after combining the susceptibility factors affecting each individual with stochastic contributions to risk (see **Figures 3** and **6**). Protective genetic haplotypes can reduce their impact on disease risk.

Figure 6

An integrated model of AMD pathogenesis. Aging is the primary driver of early cellular defects in photorecep-
tors and retinal pigment epithelium (RPE); these changes are exacerbated (or inhibited) by specific genetics
variants. Complement and immune response pathways play critical roles in the onset and severity of disease.

Our multiple-hit model can be summarized as
follows:

1. Initiation–genetic susceptibility: Variants
   in genes associated with a late-onset dis-
   ease (such as AMD) are present since
   birth, implying that these are not suf-
   ficient for disease causation. We sug-
   gest that the genetic variants alter spe-
   cific cellular function(s) or pathway(s)
   in different cell types (such as photore-
   ceptors and RPE) at early stages of life
   but that the functional consequences are
   not severe enough to produce a disease
   phenotype.
2. Modification–aging-associated changes:
   Multiple cellular pathways are concur-

rently affected during the normal ag-
ing process. These changes may initi-
ate the disease process and/or exacerbate
the impact of genetic susceptibility vari-
ants. Some of the changes (such as those
involving mitochondrial function) may
be overlapping and synergistic, whereas
others may simply be additive. Genetic
variants and aging together may lead to
milder disease phenotypes and at a rela-
tively late stage.

3. Clinical manifestation–impact of envi-
   ronment: Environmental factors, includ-
   ing smoking, high-fat and antioxidant-
   deficient diet, chronic infection and/or
   inflammation, may provide the final

trigger(s) for disease causation. The adaptive cellular mechanisms already weakened by genetic variants and aging may finally break down, leading to severe disease phenotypes and advanced stages of AMD.

In this model, cumulative genetic load (i.e., net effect of different susceptibility or protective alleles) and exposure to environmental factors unique to each individual will produce variable age of onset and a range of AMD phenotypes. We also emphasize that this model, although conceptually convenient, obviously might not reflect the full complexity of susceptibility to AMD.

AMD AND OTHER LATE-ONSET NEURODEGENERATIVE DISEASES

Alzheimer's (AD) and Parkinson's (PD) are late-onset neurodegenerative diseases (17) that share similarities with AMD. An association of impaired cognitive function with advanced AMD was reported in AREDS (27). Additionally, a prospective population-based study identified an increased risk of developing AD in individuals with advanced AMD (88). Inheritance of the *e4* allele of *APOE* is the only well-established genetic risk factor for sporadic AD (147). Mitochondrial dysfunction and oxidative stress, including mitochondrial DNA damage, have been implicated in all three diseases—AD, PD, and AMD (12, 17, 98, 165). The pathological hallmarks of AD include the presence of senile plaques, generated by accumulation of the amyloid beta (Aβ) peptide (138), whereas those of PD include Lewy bodies, which are cytoplasmic aggregates of fibrillar misfolded proteins (17). Toxic amyloid oligomers have been demonstrated in drusen, basal deposits and RPE of human donor AMD eyes but not in control age-matched eyes without drusen (102). Other components of amyloid deposits and drusen include complement and immune modulators, suggesting a potential role for inflammation in both AMD and AD (9).

ANIMAL MODELS

AMD primarily affects the macula, a specialized region identified in the retina of primates, but not in other mammals or vertebrates. Notably, the human *ARMS2* gene appears to have a highly conserved homolog only in non-human primates (49). Because of inherent difficulties associated with using monkeys, mouse models of AMD are being developed to dissect the underlying mechanisms and evaluate treatment paradigms (108). Despite their relatively short life span and absence of a fovea, mouse models offer the ability to dissect distinct characteristics of a complex human disease and investigate contributions of individual components to the complete phenotype. In our opinion, an ideal mouse model would incorporate all three aspects of AMD in humans: genetic susceptibility, advanced age, and environmental factors. No mouse model perfectly mimics AMD in all three respects; in this review we focus on a few selected mouse models that appear robust and reproducible for experimental manipulation [for more details, see other reviews (108, 120)].

The $Sod1^{-/-}$ mice lacking the Cu/Zn-superoxide dismutase 1 gene, a key regulator of oxidative stress in the cytoplasm, exhibit age-dependent development of drusen, sub-RPE deposits, and CNV (67). These deposits are immunopositive for many of the markers present in human drusen, supporting a role for oxidative stress in the pathogenesis of AMD. However, these mice also develop a progressive retinal degeneration, which is not associated with AMD.

The *APOE4* mouse carries a targeted replacement of the mouse gene with the human allele. *APOE4* mice aged over one year and fed a high-fat diet enriched with cholesterol (HFC) for 8 weeks (*APOE4*-HFC mice) develop pathological features characteristic of both dry and wet human AMD (105). Pathologies observed include diffuse lipid- and protein-rich drusen-like sub-RPE deposits that are immunopositive for complement and inflammatory markers (**Figure 7**), thickening of Bruch's membrane, patchy regions of RPE atrophy, and CNV. This phenotype presents in a temporal, incompletely

○ C3 fragments
○ apoE
○ Cell nuclei

10 μm

○ C5b-9
○ RPE
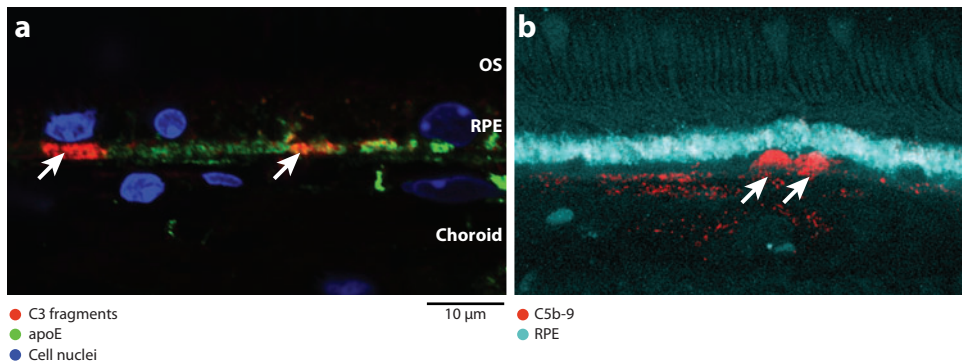
Figure 7

Activated complement components in sub-RPE (retinal pigment epithelium) deposits. (*a*) Immuno-localization of C3 fragments [C3b/iC3b/C3c (*red*)] and apoE (*green*) in basal, sub-RPE deposits (*arrowheads*) and Bruch's membrane of 75-week-old *APOE4*-HFC mouse eye. C3 fragments are found in small, discrete patches that appear to be associated with individual RPE cells (*blue* is nuclear DAPI stain). (*b*) Immuno-localization of C5b-9 (*red*) in human AMD donor eye (*cyan* is lipofuscin autofluorescence from the RPE). C5b-9 immunoreactivity is associated with similar sub-RPE deposits (*arrowheads*). OS, outer segments. Images courtesy of J-D. Ding, Duke University (*a*) and L. Johnson, University of California, Santa Barbara (*b*).

penetrant and noninvasive manner that is analogous to human AMD progression (105). Visual deficits detected in electroretinograms are also associated with the development of the AMD phenotype in these mice.

Mouse models of targeted knockout of chemokines or their receptors appear to exhibit some characteristics of AMD. Aged mice deficient in Ccl-2 (monocyte chemoattractant protein-1) or its cognate receptor Ccr2 (C-C chemokine receptor-2), which are involved in recruiting macrophages to loci of complement opsonization, sporadically present drusen accumulation and lipofuscin deposition in swollen and vacuolated RPE cells (8). In CX3C chemokine receptor (CX3CR1) knockout mouse, subretinal microglia accumulate with age, with albino background, and in response to laser injury (30). Some of these microglia are bloated with ingested lipid and account for the drusen-like appearance on fundus images. A double knockout mouse, generated by crossing $Ccl2^{-/-}$ and $Cx3cr1^{-/-}$ mice, develops a fairly severe phenotype as early as 6 weeks and includes photoreceptor degeneration, RPE damage, basal deposits, and in some animals even CNV (151).

Because of the established role of the complement pathway, attempts are under way to make transgenic mouse strains expressing the human allotypic variants of CFH associated with AMD. Low protein sequence similarity (70%) between the human and mouse CFH proteins may complicate any resulting phenotypes. The $Cfh^{-/-}$ mice exhibit an age-related decrease in visual acuity and impaired photoreceptor function with evidence of C3 deposition and ultrastructural changes in the retina, but less basal membrane thickening and less sub-RPE drusen-like material compared with age-matched wild-type controls (28). Another approach to test the effect of complement activation on AMD pathogenesis in vivo may be by dysregulating complement inhibition in a mouse model through the use of another regulator of the alternative complement pathway.

A nontransgenic mouse model deserves special mention as it is based on oxidative stress and elicitation of a systemic immune response to carboxyethylpyrrole (CEP), an ocular antigen found in eye tissue and plasma from AMD patients (73). As they age, these mice accumulate sub-RPE deposits that are immunopositive

for complement components (C3d) in Bruch's membrane. Another mouse model carrying the R345W mutation in the *EFEMP1* gene (responsible for Malattia Leventinese) closely recapitulates the early pathophysiology of human AMD patients (53, 109); this includes small, isolated sub-RPE deposits that increase in size and number, eventually becoming continuous sheets. In older mice, membranous debris is observed within the sub-RPE deposits and within Bruch's membrane.

Although we do not seem to have an ideal model that recapitulates the whole spectrum of AMD disease phenotype, many new mouse models are emerging to test molecular mechanisms and examine novel targets for therapies. For example, anti-Aβ antibodies are being evaluated to prevent retinal damage in *APOE4*-HFC mice (38). Although some might argue that the slow progression of disease in the advanced age-dependent mouse models (e.g., *EFEMP1*$^{ki/ki}$, CEP, or *APOE4*) prohibitively increases the cost of research, we believe that aging is an important risk factor in AMD and should be a key component of any model of complex multifactorial aspects of AMD pathology.

MANAGEMENT OF AMD

Dietary Supplements

Because few treatment options exist, prevention is the first approach to reduce vision loss in AMD. Control of modifiable risks factors such as smoking, hypertension, and elevated BMI may reduce the risk of developing AMD by as much as half. AREDS, a multicenter randomized and controlled clinical trial of oral supplements, demonstrated that high doses of antioxidant vitamin C, vitamin E, beta-carotene, zinc, and copper are effective in slowing the progression of advanced stages of AMD by 25% (4). This formulation, if given for the next 5 years to all those who are at risk in the United States, has the potential to prevent 300,000 individuals from developing advanced AMD. Dietary supplements are being further evaluated in a new

clinical trial, AREDS2, in which lutein, zeaxanthin, and/or omega-3 fatty acids (1 gm) will be evaluated for the prevention of advanced AMD (**http://www.areds2.org**).

Treatment of Neovascular AMD

For patients who have already developed neovascular AMD, early detection is associated with a better prognosis. The treatment of neovascular AMD has significantly improved with the development of inhibitors for vascular endothelial growth factor (VEGF), which acts as both a neuroprotective and angiogenic factor (18). In June 2006, the FDA approved ranibizumab (Lucentis), a fragment of a monoclonal antibody that binds to and inhibits all forms of VEGF-A, for the treatment of neovascular AMD (144). This drug, when injected intravitreally on a monthly basis, prevented vision loss and was the first AMD treatment associated with improvements in vision for many patients. Differences in visual acuity score in the treated and control groups were considerable, with a mean increase of 7 letters in the treated group compared with a decrease of 10 letters in the placebo, sham-injected group ($P < 0.001$) (144). A randomized trial comparing bevacizumab (a closely related antibody) with ranibizumab will directly evaluate the potential effects of reduced treatment frequency to alleviate the burden of monthly intravitreal injections (**http://www.med.upenn.edu/cpob/studies/CATT.shtml**).

THE FUTURE OF AMD TREATMENT

We expect that in the near future, genetic testing will complement clinical exams, to identify patients at risk of progressing to the advanced forms of AMD. Currently, by using a simple scale for severity of AMD, the presence of certain ocular lesions can be utilized to make useful predictions about disease progression. For example, persons with bilateral large drusen and pigmentary changes have a 50% risk of progressing to advanced AMD in one eye

VEGF: vascular endothelial growth factor

over the next 5 years. Additional evaluation of the genetic mutations may provide increased precision in these predictions. A study of AREDS participants demonstrated that the presence of *CFH* polymorphisms and *ARMS2-A69S* independently contributed to progression to advanced AMD. The presence of these polymorphisms with the AREDS-type dietary supplements, smoking, and elevated body mass index (25 or greater) increased the susceptibility to advanced AMD (135).

Therapies of the future may use genetic information to modulate and target individual therapies. A preliminary analysis of interaction between *CFH* and *ARMS2* susceptibility variants with the AREDS-type dietary supplements (90) suggests that oral supplementation with zinc was especially helpful for individuals with low-risk disease alleles at *CFH*.

ANTICIPATED PROGRESS IN GENETIC STUDIES

The pace of discovery in genetic studies of AMD has been extremely rapid, and we expect major advances within the next few years. GWAS including thousands of AMD cases and controls are now under way and will enable us to further examine the genome in an unbiased manner. We believe these studies will implicate several additional loci in disease susceptibility and clarify the role of variants examined in previous candidate gene studies. In each identified region/gene/locus, we expect that a detailed analysis of genetic variation, aided by advances in sequencing technologies, will enable us to thoroughly assess their contributions to disease susceptibility. In parallel, because of significant differences in disease prevalence and phenotypes, we believe it will be critical to study genetic contributions to AMD in different ethnic groups. Much of the AMD genetics research has focused on Caucasians; nonetheless, genetic analyses of relatively small cohorts of other ethnic groups have begun to reveal interesting insights (22, 37, 54, 84). Examination of individuals of different ethnicities (by resequencing the associated genomic regions) will be particularly helpful in attempts to identify causal alleles at each AMD susceptibility locus, since linkage disequilibrium will limit the resolution of genetic studies that focus on a single population.

We believe that changes in the management of AMD (as well as other late-onset degenerative diseases) will not occur in a vacuum. With the rapid decrease in costs of genome sequencing and genotyping and a concomitant increase in our understanding of the genetic basis of complex and common disease, we predict that within the next decade genetic information will be used to tailor individual diagnosis and treatment plans and become an integral part of the personalized medical care.

Institutes of Health, The Foundation Fighting Blindness, Macula Vision Research Foundation, and Research to Prevent Blindness.

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