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Age-related Macular Degeneration

From Clinic to Genes and Back to Patient Management

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Foreword

This is an ideal time for a textbook that summarizes the state of the art in knowledge about age-related macular degeneration (AMD), which is a leading cause of visual loss and blindness in older individuals. In the United States and higher-income countries, there have been demographic shifts toward an aging population because of increased life expectancy and decreased fertility rate. In middle- and lower-income countries with larger populations, these same trends are occurring. From a societal perspective, the impact of AMD has never been more significant.

When I finished my clinical training in 2001, little was known about the genetic foundation of AMD, and management typically involved laser photocoagulation based on findings from the Macular Photocoagulation Study trials. Since that time, genetic studies have significantly increased our understanding of AMD pathogenesis and have highlighted the role of immunological pathways. Advances in ophthalmic imaging have created unprecedented opportunities to examine the structure and function of the retina noninvasively. Clinical research and trials have evolved management so rapidly that many of today's trainees are not even aware of the Macular Photocoagulation Study trials that were the basis for my education. Advances in scientific computation and multi-omic analysis are creating potential for integrating all of these approaches like never before. New treatment frontiers include cell-based and gene-based approaches. From scientific and medical perspectives, there has never been so much excitement in studying AMD.

Drs. Anand Swaroop and Emily Y. Chew are world-renowned experts in AMD at the National Eye Institute, and their complementary areas of expertise make them ideally suited to edit this textbook together. Dr. Swaroop's laboratory has made important advances in our understanding of AMD pathophysiology using genetic, epigenetic, and systems biology approaches. Dr. Chew has led pioneering clinical trials that have provided key insights regarding the connection between AMD, nutrition, and genetics. Together, they have recruited an outstanding group of authors to cover the field of AMD comprehensively, across the spectrum from basic science to clinical management to population health: "from clinic to genes and back to patient management."

It is my hope that this book will help readers better understand the multidisciplinary contributions to our understanding of AMD today and that this knowledge will help readers develop creative new collaborative approaches toward advancing that science for the benefit of patients in the future.

National Eye Institute, National Institutes of Health, Bethesda, MD, USA

Michael F. Chiang

Introduction

Age-related macular degeneration (AMD), a leading cause of largely incurable blindness worldwide, is projected to double from 2.07 million to 5.44 million individuals by 2050 in the United States. The disease has enormous socioeconomic impact on the affected individuals, their families, and society. This monograph will bring together the state-of-the-art basic science knowledge and the results of the clinical trials and address the challenges for future research in AMD. The intersection of the different disciplines will provide potential areas for further investigations to reduce the burden of blindness from AMD.

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Age-Related Macular Degeneration: Epidemiology and Clinical Aspects

1

Tiarnán D. L. Keenan, Catherine A. Cukras, and Emily Y. Chew

Abstract

Age-related macular degeneration (AMD) is a degenerative disease of the human retina affecting individuals over the age of 55 years. This heterogeneous condition arises from a complex interplay between age, genetics, and environmental factors including smoking and diet. It is the leading cause of blindness in industrialized countries. Worldwide, the number of people with AMD is predicted to increase from 196 million in 2020 to 288 million by 2040. By this time, Asia is predicted to have the largest number of people with the disease. Distinct patterns of AMD prevalence and phenotype are seen between geographical areas that are not explained fully by disparities in population structures. AMD is classified into early, intermediate, and late stages. The early and intermediate stages, when visual symptoms are typically absent or mild, are characterized by macular deposits (drusen) and pigmentary abnormalities. Through risk prediction calculators, grading these features helps predict the risk of progression to late AMD. Late AMD is divided into neovascular and atrophic forms, though these can coexist. The defining lesions are macular neovascularization and geographic atrophy, respectively. At this stage, visual symptoms are often severe and irreversible, and can comprise profoundly decreased central vision in both eyes. For these reasons, the condition has major implications for individuals and society, as affected individuals may experience substantially decreased quality of life and independence. Recent advances in retinal imaging have led to the recognition of an expanded set of AMD phenotypes, including reticular pseudodrusen, nonexudative macular neovascularization, and subtypes of atrophy. These developments may lead to refinements in current classification systems.

Keywords

Age-related macular degeneration · Macula · Drusen · Neovascular · Exudative · Atrophic · Geographic atrophy · Reticular pseudodrusen · Subretinal drusenoid deposits

1.1 Introduction

Age-related macular degeneration (AMD) is a degenerative disease of the human retina, with pathology occurring predominantly in the *macula lutea*. It arises from a complex interplay between increased age, genetic contributions, and

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environmental risk factors. It is responsible for between 6% and 9% of global blindness [1, 2], though this proportion is likely to increase in the coming decades, given the demographic changes occurring globally. The number of people with AMD worldwide is predicted to be 196 million in 2020, increasing to 288 million by 2040 [1]. However, the proportion of visual impairment caused by AMD is much higher in industrialized countries. In the USA, it was estimated in 2004 to account for 54% of blindness and 23% of visual impairment in Caucasian people [3]. In the United Kingdom, the equivalent figures in 2013 were 50% and 53%, respectively [4].

The onset of clinically apparent AMD varies between individuals but typically begins at some point after the age of 55 years [5]. Visual symptoms are usually absent or mild at this stage. However, in advanced disease, visual symptoms may be substantial, including severely reduced central vision, often in both eyes. For these reasons, the condition has major implications for individuals and for society, as patients with advanced AMD may experience many years of decreased quality of life, reduced independence, and even depression [6–9].

1.2 Epidemiology

1.2.1 Population-Based and Prospective Cohort Studies

Several long-term population-based studies provided the basis for much of our early understanding of AMD epidemiology, and continue to generate important data on AMD natural history and disease associations. In particular, the Beaver Dam Eye Study (based in Wisconsin, USA) and the Blue Mountains Eye Study (based near Sydney, Australia) are two population-based studies that have provided rich sources of epidemiological and clinical data in recent decades.

The Beaver Dam Eye Study started in 1987. Its purpose has been to collect information on the prevalence, incidence, and potential causes of AMD (as well as cataract and diabetic retinopathy). The study involved baseline examinations on approximately 5000 of the 6000 people aged 43–84 years in Beaver Dam, Wisconsin. The ocular examinations included color fundus photography, and the fundus photographs then underwent standardized macular grading using the Wisconsin Age-Related Maculopathy grading system [10]. Follow-up examinations have taken place at 5, 10, 15, 20, and 25 years after the study baseline. The study has provided data for over 360 publications. For example, it provided some of the first prevalence and incidence estimates of AMD in white populations, and demonstrated the very strong dependence of prevalence rates on increased age [11, 12]: in 1992, the prevalence of neovascular AMD was observed at 5.2% in people aged 75 years or older, while that of geographic atrophy (GA) was 2.0%. The study also provided strong evidence of genetic involvement in AMD through sibling studies [13], which paved the way for subsequent molecular genetic analyses. Important clinical findings were published in 1997, that the risk of progression to late AMD was significantly higher in eyes with soft drusen or pigmentary abnormalities in the macula [12]. These two risk factors are now known from multiple studies to be very important predictors of disease progression, and have been incorporated into AMD classification systems and severity scales used to predict risk of disease progression (see below). Another main focus of the Beaver Dam Eye Study has been to detect and discover risk factors for AMD, particularly long-term environmental exposures. Indeed, it provided strong evidence of the association between cigarette smoking and AMD [14], and has published data on multiple other potential associations, such as cardiovascular risk factors [15] and sunlight exposure [16].

The Blue Mountains Eye Study, based in Australia, has been another rich source of important data on AMD epidemiology. This has been another long-term population-based study. Similar to the Beaver Dam Eye Study, the Blue Mountains Eye Study conducted baseline and follow-up examinations on approximately 3500 people aged 49 years and older, beginning in 1992. Again, ocular examinations included color fundus photography, with standardized macular

grading of the fundus photographs using the Wisconsin Age-Related Maculopathy grading system. Many findings were similar to those in the Beaver Dam Study. It provided important prevalence and incidence data, and again observed a very strong link between AMD prevalence and increased age [17]. Consistent with the Beaver Dam Eye Study, higher rates of progression to late AMD were observed in eyes with soft drusen or pigmentary abnormalities in the macula [18]. A strong emphasis in the study was on examination of potential risk factors for AMD; evidence demonstrated association between AMD and multiple risk factors including cigarette smoking [19], plasma fibrinogen levels [20], and family history [21].

The Age-Related Eye Disease Study (AREDS) was a long-term, multicenter, prospective study of the clinical course of AMD (and age-related cataract) [22]. Its longitudinal nature has some similarities with the Beaver Dam Eye Study and Blue Mountains Eye Study, but the AREDS was a clinical trial rather than a population-based study. In addition to the purpose of performing a randomized clinical trial of high-dose antioxidant and mineral supplements for AMD, the aim was to gather natural history data on progression rates and risk factors. In the AREDS, approximately 3600 participants underwent baseline and annual follow-up ocular examinations, including color fundus photography. The fundus photographs were graded by the Photograph Reading Center (University of Wisconsin) using the AREDS grading system [23], which was adapted from the more complex Wisconsin Age-Related Maculopathy grading system mentioned above [10]. The main outcomes were the development of neovascular AMD or central GA.

One important achievement of the AREDS was the recognition that oral supplementation with a combination of antioxidants and minerals led to decreased risk of progression to late AMD, for those in specific categories of disease at baseline [24]. Specifically, supplementation was associated with approximately 25% decreased progression risk in those with bilateral intermediate AMD or unilateral late AMD. Oral supplementation remains the mainstay of interventional treatment at the stage of intermediate AMD, and will be discussed in more detail in Chap. 12.

The AREDS also reported on risk factors of AMD, including cigarette smoking and cardiovascular factors [25, 26], consistent with previous studies, as well as associations with potential serum biomarkers such as C-reactive protein [27]. However, one important novel contribution was the generation of a Simplified Severity Scale (using data on large drusen and pigmentary abnormalities from both eyes) to calculate a score (0–4) for the 5-year probability of progression to late AMD in either eye [28] (discussed below). This system is simpler than the research-based AREDS grading system, and is therefore feasible to perform in clinic for prognostic information and stratification of potential follow-up and interventions. The Simplified Severity Scale has subsequently been externally validated in both clinical trial [29] and population-based [30] cohorts.

The Age-Related Eye Disease Study 2 (AREDS2) was another long-term, multicenter, prospective study of the clinical course of AMD [31]. The inclusion criteria (either bilateral large drusen or large drusen in one eye and neovascular AMD/central GA in the fellow eye) led to the selection of participants (approximately 4200) with a higher degree of AMD severity than in the AREDS. The study prospectively investigated the effects of modifying the formulation of antioxidants and minerals used in the AREDS, but has also provided a large body of data for additional retrospective analyses.

These population-based studies and clinical trials have provided important discovery and replication datasets for multiple hypotheses and observations, including analyses of potential genetic characteristics, environmental factors, and clinical/imaging features associated with altered incidence or progression of AMD (and other age-related eye diseases). Of course, the data are generalizable to other populations only as far as the epidemiological characteristics are shared with the population of interest, in terms of age, genetics, and environmental exposures such as smoking and dietary habits. For example, the Beaver Dam Eye Study cohort was over 99%

white, such that the applicability of findings to non-white populations is partially limited.

However, multiple other population-based studies ongoing in diverse geographical regions, as well as several large collaborative or consortium projects. These include the Rotterdam Study (Holland), the ALIENOR Study (France), the Coimbra Eye Study (Portugal), the Los Angeles Latino Eye Study (USA), the Amish Eye Study (USA), as well as the European Eye Study (seven European countries), the European Eye Epidemiology consortium (12 European countries), and the recent Macustar project (20 clinical study sites across Europe).

1.2.2 Global Prevalence and Patterns of Disease

The global prevalence of AMD has been analyzed by systematic review and meta-analysis [1]. The pooled prevalence (mapped to an age range of 45–85 years) was estimated at 8.7%; prevalence rates for early AMD and late AMD were 8.0% and 0.37%, respectively. However, considerable variation exists in AMD prevalence between different continents and populations. As demonstrated in Fig. 1.1, the prevalence of AMD was estimated at 12.3% in people of European ancestry, 10.4% in people of Hispanic ancestry, 7.5% in those of African ancestry, and 7.4% in those of Asian ancestry [1]. The equivalent rates for early AMD were 11.2%, 9.9%, 7.1%, and 6.8%, respectively, while the rates for late AMD were 0.5%, 0.3%, 0.3%, and 0.4%.

Hence, people of European ancestry have the highest prevalence of early AMD, with substantially lower prevalence rates observed in those of Asian or African ancestry. However, the difference is less substantial for rates of late AMD than of early AMD. This might suggest that those of European ancestry are partially protected (relative to those of Asian or African ancestry) from the progression of early AMD to late disease, and/or that those of Asian of African ancestry may sometimes have forms of disease characterized by more direct progression to late AMD (e.g., polypoidal choroidal vasculopathy (PCV)). In addition, people of European ancestry have a higher prevalence of the geographic atrophy subtype of late AMD than those of other ancestry; this has been estimated by meta-analysis at 1.1% (European) versus 0.2% (Hispanic), 0.2% (Asian), and 0.1% (African) [1].

These observations are relevant not just because they help inform service provision and predict future patterns of disease and service requirements, they may also provide important insights into the spectrum of AMD phenotypes, as well as clues to disease pathophysiology at different stages of disease progression. Indeed, these distinct patterns of disease prevalence and presentation between different countries are not explained fully by disparities in population structures (particularly age). Potential explanations for the residual disparities may be from differences in (i) genetic make-up (either at common loci associated with late AMD, such as *CFH* and *ARMS2/HTRA1* (discussed below), and/or in modifier genes/genetic background), and (ii) environmental factors, such as diet, smoking, sunlight, or occupational exposures. For these reasons, it is important to distinguish between ancestry and geographical area in population studies [32], since the contribution of genetics should be seen more clearly in studies stratified by participant ancestry.

For example, a recent study in a multiethnic population in the USA observed variations in the incidence of early AMD between different ethnic groups (i.e., people of different ancestries living in one geographical area) that were not fully explained by the clinical, genetic, and environmental factors included in the study [33]. Despite correction for these factors, the incidence of early AMD remained lower in African-American than white American participants; this might relate to differences in other genetic variants (e.g., *CFHR3/1* deletion or genetic background) or environmental factors that were not included in the study. In addition, in this study (unlike the results of the meta-analysis described above), the incidence of early AMD was similar in Asian-American and white American participants, despite the Asian-Americans having a lower

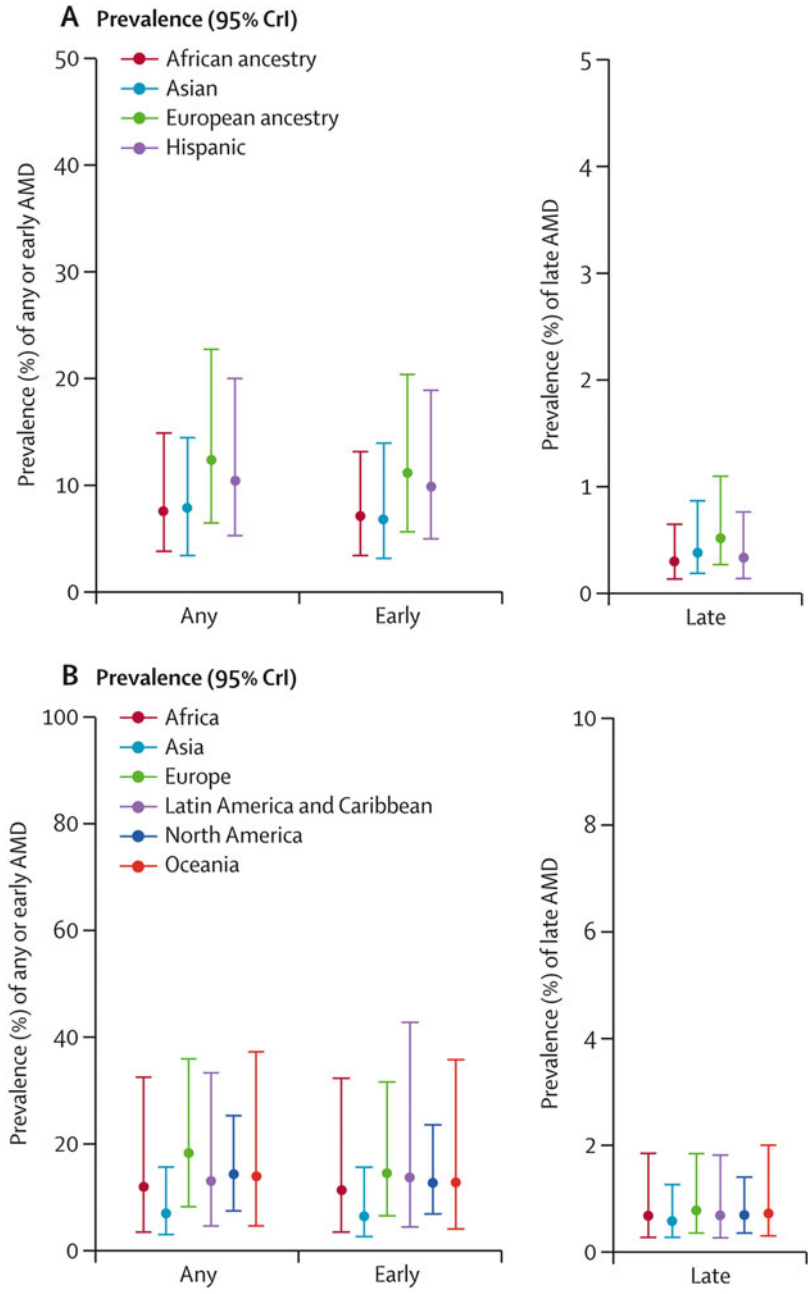

Fig. 1.1 Prevalence of age-related macular degeneration by ethnic group (**a**) and geographical region (**b**). Error bars = 95% credible intervals. Reprinted from *The Lancet Global Health*, volume 2, Wong WL et al, 'Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis', pages e106-16, copyright (2014), with permission from Elsevier

proportion of risk variants at *CFH*. Again, this might relate to other genetic or environmental factors, such as the adoption of Western lifestyle exposures by Asian-Americans. Similarly, a recent systematic review and meta-regression study analyzed population-based studies of AMD prevalence [34]. This demonstrated inverse correlations between AMD prevalence and both latitude and longitude. The most important variables for predicting prevalence appeared to be ethnicity for early AMD, but insolation (i.e., UV light exposure) for late AMD. Hence, differing combinations of genetic and environmental exposures may be important at different stages

of disease progression. An improved understanding of these factors could potentially lead to both novel therapeutic targets and refined public health recommendations.

Population studies also provide important data on the numbers and proportions of people with visual impairment caused by AMD, relative to other eye diseases. One recent study has estimated the worldwide number of people with legal blindness or moderate/severe visual impairment caused by macular disease (principally AMD) at 2.1 million [2]. In this report, AMD was the fourth most common cause of global blindness (in approximately 5.8% of blind individuals), and the third most common for visual impairment (3.9%). As mentioned above, the proportion of visual impairment caused by AMD is much higher in industrialized countries, chiefly because of increased life expectancy. Estimates from 2012 to 2013 state registrations of visual impairment in the United Kingdom showed that AMD was responsible for 50% of new registrations of blindness and 53% of visual impairment [4].

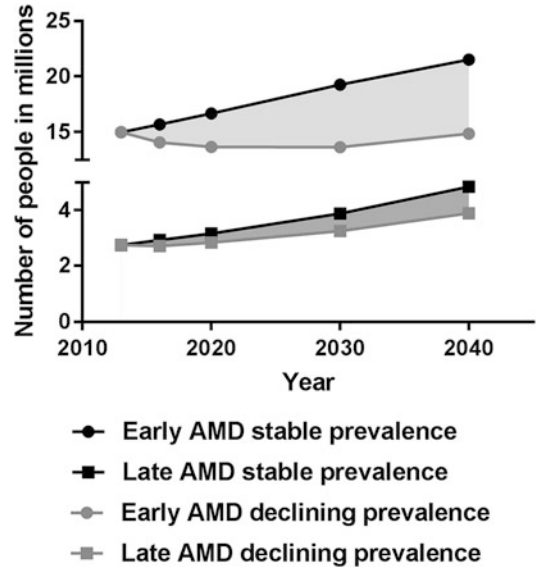

Fig. 1.2 Predicted number of persons with age-related macular degeneration in years 2013–2040 as a function of two prevalence scenarios. Reprinted from *Ophthalmology*, volume 124, Colijn JM et al, 'Prevalence of Age-Related Macular Degeneration in Europe: The Past and the Future', pages 1753–1763, copyright (2017), with permission from Elsevier

1.2.3 Changes in Disease Prevalence Over Time

Potential changes in AMD prevalence over time are important to understand, both for potential insights into AMD pathophysiology (past changes) and for planning of ophthalmic services (predicted changes) [35]. Multiple studies have demonstrated gradually increasing burdens of AMD in industrialized countries (in terms of absolute numbers with late AMD or visual loss) through increased life expectancies and aging populations. However, data from the USA (Beaver Dam Eye Study) suggest that the age-specific incidence of early AMD may have decreased over the course of the past four generations [36]; a larger dataset (gathered by meta-analysis) from Europe suggest that the age-specific prevalence of early AMD has been relatively stable between recent generations, while that of late AMD has decreased [37]. However, even if this is the case, the number of people with late AMD in Europe is still predicted to increase from 2.7 million in 2016 to 3.9 million in 2040 (as demonstrated in Fig. 1.2).

The next two decades will also observe a shift in global patterns of AMD. The number of people with AMD worldwide is predicted to be 196 million in 2020, increasing to 288 million by 2040 [1]. Asia currently has the lowest percentage prevalence but accounts for over 60% of the world population and comprises a rapidly growly and ageing population. For these reasons, by 2040, Asia is predicted to be the continent with the largest number of AMD cases, with approximately 113 million people affected (as shown in Fig. 1.3); by this time, more than half of late AMD cases worldwide will be in Asia [1]. In this respect, AMD may no longer be considered a disease primarily of European-descended populations [35].

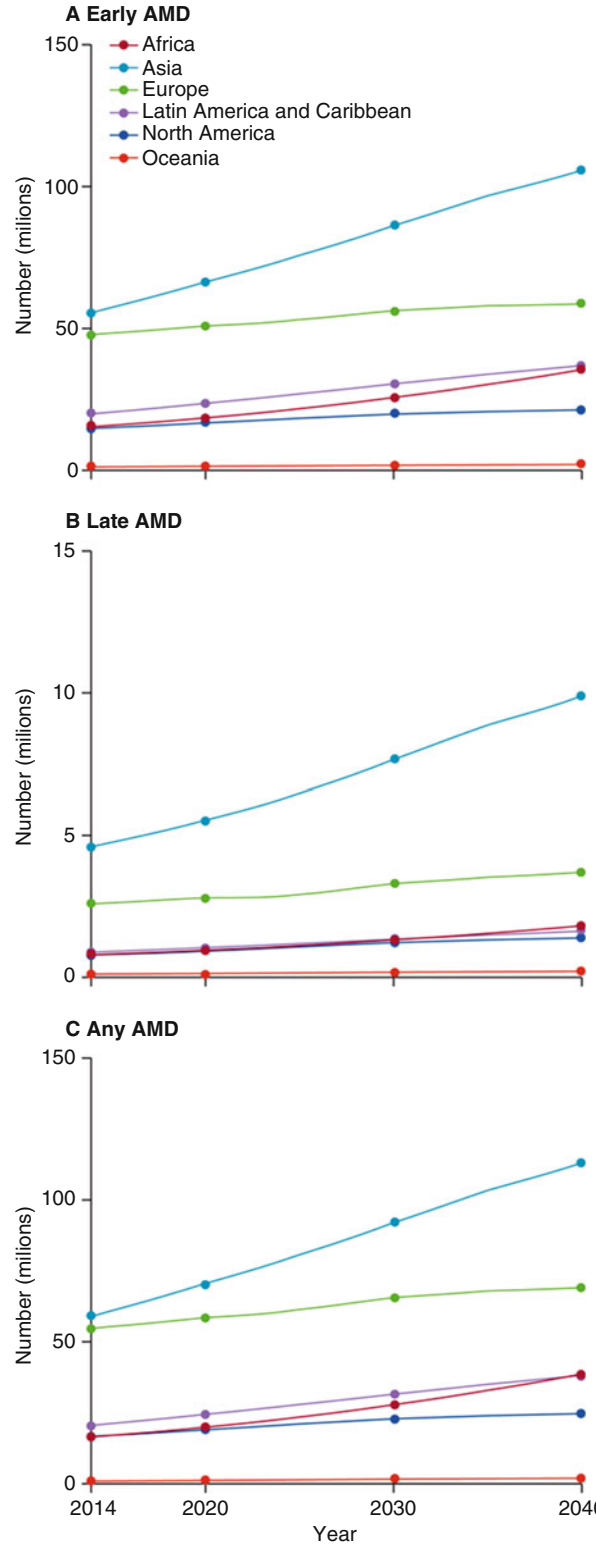

Fig. 1.3 Projection of number of people with early and late age-related macular degeneration by regions in 2014, 2020, and 2040. Reprinted from *The Lancet Global Health*, volume 2, Wong WL et al, 'Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis', pages e106-16, copyright (2014), with permission from Elsevier

Table 1.1 Clinical classification of age-related macular degeneration, as proposed by the Beckman Initiative for Macular Research Classification Committee

Classification of AMD	Definition: lesions assessed within two disc diameters of fovea
No apparent ageing changes	(i) No drusen AND (ii) No AMD pigmentary abnormalities[a]
Normal ageing changes	(i) Only small drusen[b] AND (ii) No AMD pigmentary abnormalities[a]
Early AMD	(i) Medium drusen[b] AND (ii) No AMD pigmentary abnormalities[a]
Intermediate AMD	(i) Large drusen AND/OR (ii) Any AMD pigmentary abnormalities[a]
Late AMD	(i) Neovascular AMD AND/OR (ii) Any geographic atrophy

[a]AMD pigmentary abnormalities: any definite hyper- or hypopigmentary abnormalities associated with medium or large drusen but not associated with known disease entities
[b]Small drusen are <63 μm; medium drusen ≥ 63 and <125 μm; large drusen ≥ 125 μm
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1.3 Classification of Age-Related Macular Degeneration

1.3.1 Introduction

Multiple classification systems have been used in different countries and study settings to describe the different stages and forms of AMD. Some of the most widely used systems, including the AREDS grading system [23], have been simplified versions of the more complex Wisconsin Age-Related Maculopathy Grading Scheme [10]. However, authors have recognized that harmonization between different classification systems may be important to decrease heterogeneity between study results, particularly when pooling data from multiple studies to increase power [38]. Indeed, in 2011/2012, the Beckman Initiative for Macular Research Classification Committee convened a meeting of experts to develop a unified clinical classification system by consensus using a modified Delphi process. The proposed classification system was published in 2013 [39], and is shown in Table 1.1.

Using this system, AMD is classified into three stages: early, intermediate, and late disease. The staging is based on macular characteristics (specifically drusen presence/size and AMD pigmentary abnormalities) assessed on color fundus photography or clinical examination, though other imaging modalities (particularly optical coherence tomography (OCT)) may often be used alongside. An important distinction is made between the presence of "normal aging changes" (where small drusen may be present) and early AMD (which requires the presence of medium-sized drusen), so that normal and pathological aging can be distinguished. Indeed, histological and immunological studies have helped emphasize the differences between normal aging and AMD pathology, as demonstrated in a review article entitled "aging is not a disease" [40]. In addition, the late form of AMD is divided into two forms: neovascular AMD and atrophic AMD. Neovascular AMD is caused predominantly by choroidal neovascularization (CNV), though retinal angiomatous proliferation (RAP) lesions can arise within the retina (discussed below). GA is the defining lesion of atrophic AMD; in the proposed classification system (unlike in the AREDS and AREDS2), both noncentral and central GA confer a diagnosis of late AMD. It is also important to recognize that neovascular AMD and atrophic disease can coexist in the same eye:

neovascular AMD is often accompanied or followed by macular atrophy, and GA can often be complicated during its course by neovascular activity [41].

However, recent progress in our understanding of AMD may mean that the classification system proposed in 2013 will need to be refined further. Advances in retinal imaging (including improved choroidal visualization and noncontrast angiography) and psychophysical testing (particularly dark adaptation) have led to the recognition of an expanded set of AMD phenotypes. Even the definitions of late AMD themselves may require refinement, as discussed below for the distinction between GA and isolated atrophy of the outer retina. Finally, additional data from genetic analyses (including genotype-phenotype correlations [42, 43]), ultrawide-field imaging, and serum biomarkers, may also have implications for refinements to current systems of AMD classification.

1.3.2 Early Age-Related Macular Degeneration, Drusen, and Drusenoid Deposits

Early AMD is characterized by the presence of medium-sized drusen in the macula, in the absence of accompanying AMD pigmentary abnormalities. This is defined at the level of the eye, in individuals aged 50 years and above [23, 39]. Visual symptoms are typically absent or mild at this stage of disease.

Drusen are extracellular deposits of lipid and protein that are seen clinically as yellowish spots in the posterior segment. However, multiple forms of drusen and related deposits exist, and some types are seen preferentially in normal aging, in AMD, and in other retinal conditions. For this reason, careful observation of drusen and drusenoid deposits (on clinical examination and multimodal imaging, particularly OCT) can be important in the diagnosis and staging of AMD and other retinal diseases.

Retinal imaging is discussed in Chap. 2. In addition, the importance of differentiation between the various types of drusen and drusenoid deposits has been reviewed in detail [44], as demonstrated in Table 1.2.

In brief, soft drusen (see Fig. 1.4) are traditionally considered the hallmark deposit in AMD. In contrast to hard drusen, soft drusen are usually large (often >125 μm diameter) yellow deposits that are less discrete and are found predominantly in the posterior pole. By contrast, hard drusen are small discrete deposits that occur in normal aging, and are often in both the macula and the peripheral retina. In addition, subretinal drusenoid deposits (SDD), also known as reticular pseudodrusen (RPD), are another important feature of AMD (and some other retinal diseases). The characteristics of SDD have been reviewed in detail [45, 46], and will be discussed below.

1.3.3 Intermediate Age-Related Macular Degeneration

Intermediate AMD is defined by the presence in the macula of either large drusen (diameter ≥ 125 μm) and/or RPE abnormalities accompanied by at least medium-sized drusen. This stage of AMD is an important phase in disease progression, which should be differentiated carefully from early AMD. Visual symptoms typically remain mild in intermediate AMD, ranging from no symptoms to mild blurring, metamorphosia, and/or scotomata. However, the risk of progression to late AMD is significantly higher in intermediate than in early AMD [29, 47]. The AREDS Simplified Severity Scale demonstrates this point clearly [28], where 5-year progression risk estimates range from 0.5% for the "normal aging changes" group to 50% for the highest risk category within the intermediate AMD group.

The development of the Simplified Severity Scale followed reports from the AREDS and from population-based cohorts, which demonstrated the importance of soft drusen and pigmentary abnormalities in predicting progression to late AMD. These pigmentary abnormalities arise from RPE disturbances; for intermediate AMD, they are defined as definite hyper- or hypopigmentary abnormalities

Table 1.2 Features of different drusen subtypes on retinal imaging

	Small drusen		Large drusen	Subretinal deposit
	Hard drusen	Cuticular drusen	Soft drusen	Reticular pseudodrusen
Color fundus photography	Small (<63 μm) discrete yellow-white deposits with distinct edges. Found in macula and peripheral retina	Numerous (>50) small (25–75 μm) dot-like deposits in macula and peripheral retina (may be more numerous in periphery and first visible here). Early stages not readily detectable on CFP	Larger (usually >125 μm) less discrete deposits found only in the posterior pole (more frequent in central macula, superior and temporal quadrants). Yellow appearance; central part may be whiter	Variable diameter (c. 100 μm); may be dot-like or reticular. Found preferentially in the perifovea (often more predominant superiorly). Whiter and more irregular than conventional drusen. Near infrared CSLO reflectance imaging is more sensitive than CFP
Short wavelength autofluorescence	Foci of decreased AF sometimes surrounded by increased AF	Hypo-AF dots. May appear larger than on CFP (although CFP may be more sensitive)	Moderate hyper-AF. Larger drusen may show heterogeneous AF signal	Reticular pattern, hypo-AF. A minority may be atypically hyper-AF
Optical coherence tomography	Small sub-RPE deposits, hyper-reflective. Sometimes less discernible than on color fundus photography	Sub-RPE. Prolate shape, moderate hyper-reflectivity. Saw-tooth configuration. Larger drusen erode into the RPE monolayer; apex only thinly covered by RPE	Larger sub-RPE deposits, hyper-reflective	Deposits between the RPE and inner segment ellipsoid lines (stages 1 and 2), later breaking through the ellipsoid line (stage 3) and subsequently fading (stage 4). Light stripes may be visible in the underlying choroid
Fundus fluorescein angiography	Possible mild hyperfluorescence	"Starry sky" appearance: densely distributed hyperfluorescent dots. Earlier lesions not visible (but seen on AF)	Variable, usually some later hyperfluorescence	Hypofluorescence or no change
Indocyanine green angiography	Hyperfluorescent	Early hyperfluorescence	Hypofluorescent	Hypofluorescence in mid to late-phase

AF autofluorescence; *CFP* color fundus photography; *CSLO* confocal scanning local ophthalmoscopy; *RPE* retinal pigment epithelium

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associated with medium or large drusen but not associated with other known disease entities. The Simplified Severity Scale also represented a simplification of the AREDS 9-step severity scale [47], for use in a clinical rather than a research environment. The purpose of the Simplified Severity Scale was to predict the probability of progression to late AMD at the level of the individual (i.e., in either eye) by using data on large drusen and pigmentary abnormalities from both eyes. One point is given for each of these features in each eye, and the points are summed to give a score from 0 to 4. Based on data from the AREDS, the probability of progression to late AMD over 5 years is then given: 0.5% (0), 3% (1), 12% (2), 25% (3), and 50% (4). This

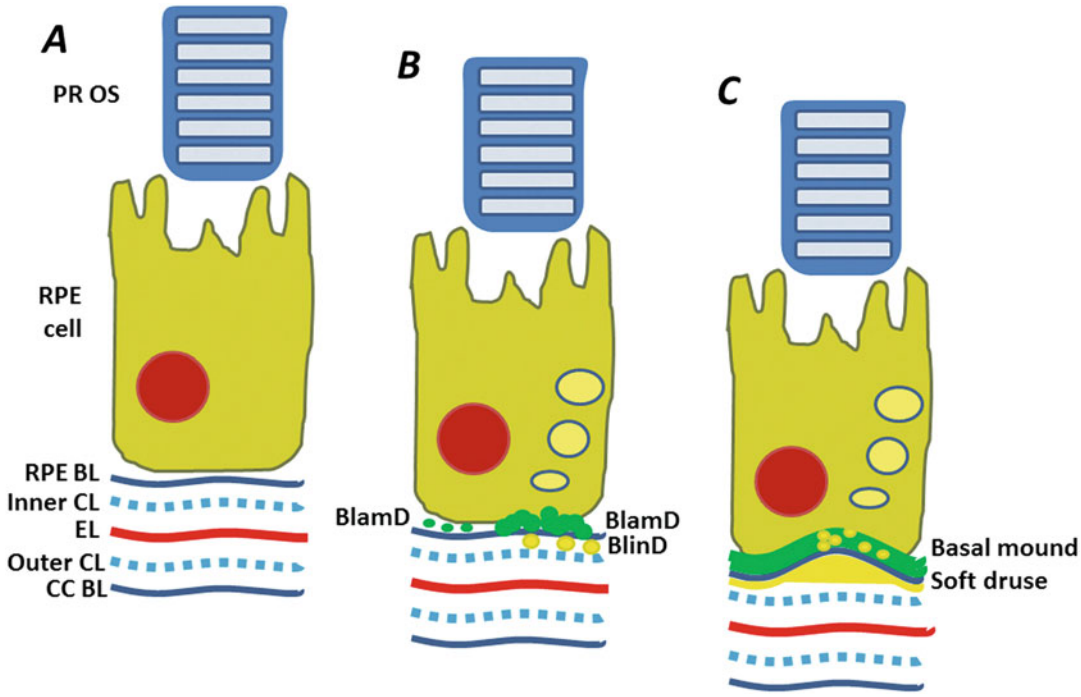

Fig. 1.4 Development of subclinical deposits and soft drusen. In this cartoon, the healthy configuration is shown on the left. With ageing (middle diagram), basal laminar deposits accumulate (internal to the RPE basement membrane) and vacuoles appear within RPE cells; early basal linear deposits (external to the RPE basement membrane) may also develop. The right-hand side shows more extensive BlinD coalescing to form soft drusen. (PR OS, photoreceptor outer segment; RPE, retinal pigment epithelium; BL, basal lamina; CL, collagenous layer; EL, elastic layer; CC, choriocapillaris; BlamD, basal laminar deposit; BlinD, basal linear deposit). Reprinted from *Progress in Retinal and Eye Research*, volume 53, Khan KN et al, 'Differentiating drusen: Drusen and drusen-like appearances associated with ageing, age-related macular degeneration, inherited eye disease and other pathological processes', pages 70–106, copyright (2016), with permission from Elsevier

information may be very useful for several reasons: as prognostic information for patients, for planning of follow-up and retinal imaging intervals, for balancing risks and benefits of potential interventions (e.g., antioxidant/mineral supplements), and for recruitment and stratification of potential clinical trial participants. The AREDS Simplified Severity Scale has subsequently been validated in the context of both clinical trial (AREDS2 [29]) and population-based (Blue Mountains Eye Study [30]) environments.

However, as mentioned above, current clinical classification systems for AMD (including the unified system [39]) do not necessarily take into account all phenotypes and features of AMD. In this context, three additional considerations are important to the discussion of intermediate AMD: (i) SDD/RPD, (ii) subclinical CNV membranes, and (iii) peripheral retinal abnormalities.

First, SDD (also known as RPD or reticular macular disease) represent an AMD phenotype whose characteristics and importance have been recognized relatively recently. This aspect of disease has been reviewed in detail [45, 46]. These deposits were first described clinically as a yellowish interlacing macular pattern seen best in blue light fundus photography [48]. Retinal imaging of SDD is discussed in more detail in Chap. 2. They have a characteristic appearance on fundus autofluorescence and on near-infrared

reflectance, while the sensitivity of detection on clinical examination or color fundus photography is relatively low. They have different cross-sectional features and localization on OCT, and appear to be located beneath the neurosensory retina (i.e., between the retinal pigment epithelium (RPE) and the retina), rather than between Bruch's membrane and the RPE (where soft drusen are located).

Although SDD often coexist with soft drusen, the genetic risk factors for their presence are partially distinct from those of late AMD: risk alleles at *ARMS2/HTRA1* are significantly associated with their presence, whereas risk alleles at *CFH* appear weakly or not associated [43]. In addition, the phenotype of SDD may be expanded to include other anatomical and psychophysical features: the constellation of SDD, thin choroid, and substantially prolonged dark adaptation [49]. Importantly, their presence is associated with a higher risk of progression to late AMD. However, they are more predictive of some forms of late AMD than others; in particular, they are associated with increased risk of GA, of RAP lesions, as well as of outer retinal atrophy (i.e., without accompanying RPE atrophy that would meet the criteria for GA). Further studies are currently underway, using data from the AREDS and AREDS2, in order to understand how to integrate this important phenotype into existing systems for the classification and prediction of disease progression. For example, it might be helpful to incorporate the presence or absence of SDD into a modified AREDS Simplified Severity Scale in order to improve the accuracy of predictions, though this alone would not necessarily differentiate risks of specific subtypes of late disease, such as outer retinal atrophy or RAP lesions.

Second, improvements in retinal imaging have led to an increased ability to detect neovascular disease. In particular, the advent of OCT angiography (see Chap. 2) has meant that angiographic examination for neovascular AMD may be performed more frequently and on an expanded set of eyes than was feasible previously with contrast dyes (i.e., fluorescein and indocyanine green (ICG)). This has led to the increased realization that some eyes assumed to have intermediate AMD from clinical examination and OCT imaging harbor occult (type 1) CNV membranes that are nonexudative or minimally exudative [50, 51]. This phenomenon potentially introduces a new class of eyes with subclinical or "silent" neovascular disease into the category of intermediate AMD (or into a new subcategory of neovascular disease). The natural history of these lesions has begun to be examined; in one series, the risk of progression from nonexudative to exudative CNV was 21% at 1 year [51]. Clearly, improvements in the detection and characterization of these lesions will lead to increased accuracy of predictions of disease progression, and may be important for recruitment and stratification of potential clinical trial participants (for both intermediate and neovascular disease categories).

Third, recent studies have revealed that eyes with at least intermediate AMD have a high proportion of peripheral retinal abnormalities (including peripheral drusen, pigmentary abnormalities, neovascularization, and GA) [52]. Together with other evidence, this suggests that AMD pathology may be more widespread than the macula alone. These data argue that AMD may be a retinal disease with abnormalities concentrated in the macula (perhaps because of the specific anatomy or metabolic properties of the retina in this location), rather than a purely macular disease without involvement of the extramacular retina. Indeed, the regional susceptibility of the retina to AMD and other retinal diseases is of great importance but currently understood only partially [53]. The parafoveal region of the retina is often the first or preferred site of involvement in multiple diseases (including AMD, Macular Telangiectasia type 2, ABCA4 and non-ABCA4 bull's-eye maculopathy, and even hydroxychloroquine toxicity), but the precise reasons for this warrant further study.

1.3.4 Late Age-Related Macular Degeneration

Late AMD is the stage of disease traditionally associated with severe central visual loss, in the form of a dense central scotoma and/or severe metamorphopsia. If present in both eyes, this can lead to severe impairment of visual function, making it impossible to read, recognize faces, or drive a car. Late AMD is divided into two classes: neovascular disease (exudative or "wet" AMD) and atrophic disease ("dry" AMD). However, as mentioned above, these can coexist in the same eye, where it can be important to understand which form was present first.

Neovascular AMD was previously responsible for over 90% of severe visual loss in AMD [54–56], since untreated neovascular disease usually leads almost inevitably to extensive macular damage and irreversible visual loss to the level of legal blindness. However, substantial improvements have been made in the treatment of neovascular AMD, most notably with the advent of anti-VEGF therapy (see Chap. 12). Following the achievement of substantially improved visual outcomes with anti-VEGF therapy, which are partially sustained in the long term, atrophic AMD represents an important disease burden and research priority, since no treatments are currently available in routine clinical use for this form of advanced disease. For example, data from meta-analysis of 14 European studies showed that the proportion of eyes with neovascular AMD that had visual impairment decreased significantly from 80% (before 2006) to 66% (after 2006, when anti-VEGF therapy was introduced); however, the proportion of eyes with GA that had visual impairment remained similar at 54% and 48% [37].

In the context of visual impairment, it is important to recognize that patients with visual loss may develop visual hallucinations, in the form of Charles–Bonnet syndrome [57]. These individuals should be counselled regarding the benign nature of these experiences.

1.3.5 Late Age-Related Macular Degeneration: Neovascular Disease

Neovascular AMD is caused predominantly by CNV. However, intraretinal neovascular disease may occur in the case of some RAP lesions, such that some authors have recommended use of the term "macular neovascularization" instead of CNV [58].

However, in the traditional view of neovascular AMD, disease is caused by the growth of CNV membranes, where new blood vessels arise from the choriocapillaris and grow into the subretinal and/or sub-RPE space. This is thought to occur under the influence of a combination of structural and biochemical factors in the local environment. Bruch's membrane normally acts as a barrier to blood vessel growth, but decreased membrane thickness and integrity permit new capillaries to breach it [59]. This occurs in association with alterations in the local balance of pro- and anti-angiogenic factors, including VEGF. The expanding CNV membrane is made up of proliferating vascular endothelial cells and RPE cells. Because its capillaries are immature, exudation from these vessels may lead to serous and/or hemorrhagic detachment of the RPE or neurosensory retina (depending on the location of the membrane). For this reason, a pigmented epithelial detachment (PED) is an important clinical and imaging finding, which raises the suspicion of active neovascular disease. However, not all PEDs are representative of neovascular activity. The nature and frequency of PEDs have been reviewed in detail [60]. They may be classified into drusenoid, serous, and fibrovascular types. This classification, together with other characteristics of the PED and accompanying clinical/imaging features, help in differentiating between neovascular and non-neovascular disease and between types of neovascularization (Table 1.3). The relative frequency of PED subtypes in one consecutive series in the USA, together with the underlying diagnoses, is demonstrated in Fig. 1.5. In addition, the natural history of drusenoid PEDs has been studied in

Table 1.3 Comparison of typical clinical and imaging features of pigment epithelial detachments associated with the various underlying etiologies

	Age-related macular degeneration				PCV	CSCR
	Nonvascularized		Vascularized			
Features	Drusenoid	Serous	Type 1	Type 3	Vascularized	Nonvascularized, serous
Demographics	Elderly, white	Elderly, white	Elderly, white	Elderly, white	Middle-aged, Asian or African	Young, male, hyperopic
Shape	Lobular	Larger, convex, steep edges	Irregular, shallow	Apical retinal vessels	Notched or peaked PED	Circular with orange ring
Location	Central macula	Central macula	Central macula	Eccentric	Central macula; eccentric	Central macula; eccentric
Size of PED (SD)						
Mean width (mm)	879 (398)	2272 (1009)	1949 (929)	1592 (1228)	1954 (1309)	1169 (385)
Mean height (mm)	155 (48)	463 (231)	245 (130)	174 (51)	537 (178)	247 (157)
Mean area (mm^2)	0.11 (0.08)	0.81 (0.57)	0.32 (0.3)	0.22 (0.2)	1.24 (0.77)	0.22 (0.2)
% of large PEDs	25	83	83	87	80	67
Choroidal features	Thin	Thin	Thin	Thin	Normal to thick, pachychoroid	Thick, pachychoroid
Nature of sub-RPE space	Homogeneous, hyper-reflective	Heterogeneous, hyporeflective	Heterogeneous, hyper-reflective	Variable appearance with stage	Focal, hyper-reflective	Homogeneous, hyporeflective
RPE tears	None	None	Common	Very rarely	Very rarely	Infrequent
Drusen	Widespread	Widespread	Common	Common, SDD	Uncommon	None
Hemorrhage	None	None	Subretinal	Small, intraretinal/ subretinal	Large, multilayer	None
Intraretinal cysts	None	None	Common	Very common	Rare	Rare
Vitelliform	Common	Very common	Uncommon	None	None	Common
Flow signal on OCTA	No flow	No flow	Flow signal, sub-RPE	Flow signal, intraretinal	Flow at polyp border, sub-RPE	No flow

CSCR central serous chorioretinopathy; *OCTA* optical coherence tomography angiography; *PCV* polypoidal choroidal vasculopathy; *PED* pigment epithelial detachment; *RPE* retinal pigment epithelium; *SD* standard deviation; *SDD* subretinal drusenoid deposit

Source: Reprinted with permission [60]. Copyright 2016, Elsevier

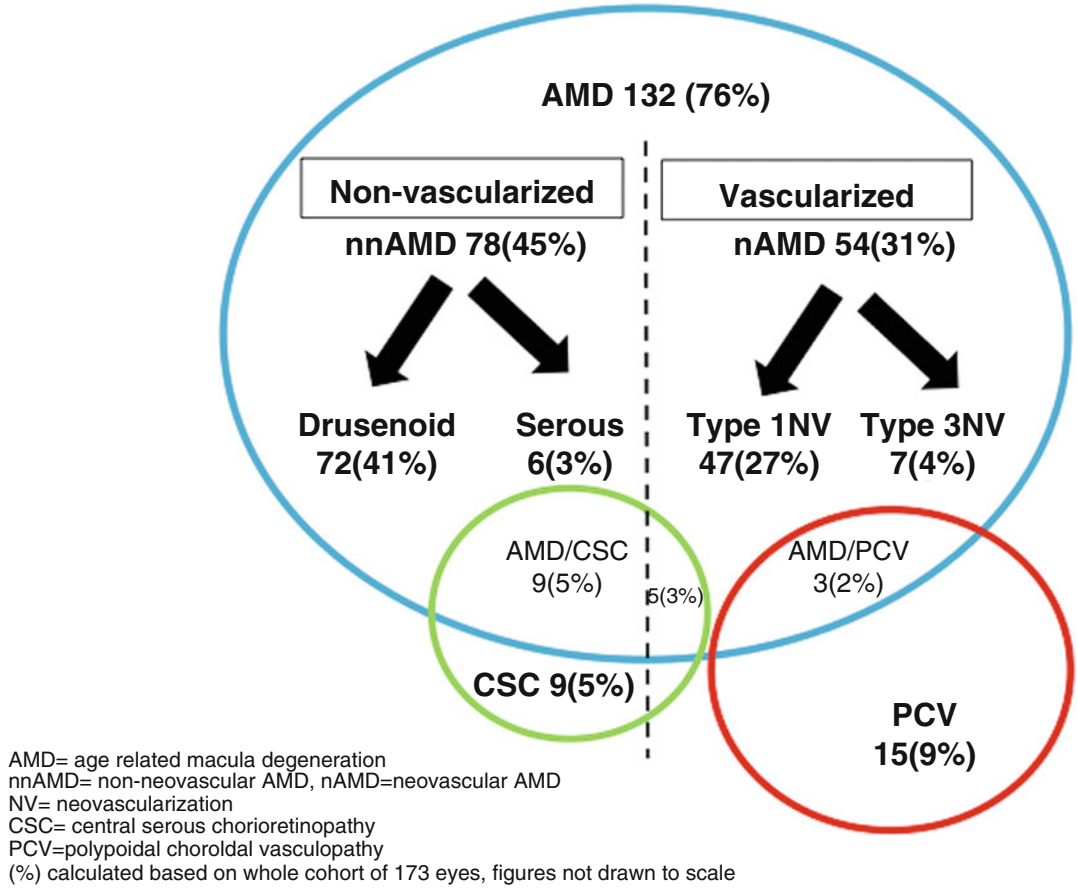

Fig. 1.5 The frequency of pigment epithelial detachment subtypes occurring in 173 eyes of 110 patients. Figures represent number of eyes and % of the total population. Reprinted from the *American Journal of Ophthalmology*, volume 172, Tan ACS et al, 'A Perspective on the Nature and Frequency of Pigment Epithelial Detachments', pages 13–27, copyright (2016), with permission from Elsevier

detail in both the AREDS and the AREDS2 cohorts; this revealed high rates of progression to late AMD and visual loss, following the occurrence of drusenoid PEDs [61, 62].

If untreated, CNV growth and activity over time is usually accompanied by hemorrhage, lipid exudation, expansion of subretinal fibrous tissue and widespread RPE, and photoreceptor atrophy in the form of a disciform scar [63]. A window of therapeutic opportunity exists after the time of incident neovascular disease and before early fibrosis and scar formation may begin. Indeed, multiple studies have demonstrated significantly superior long-term visual outcomes of anti-VEGF therapy in eyes with early neovascular disease (i.e., better baseline visual acuity and small CNV lesion size) [64, 65]. For this reason, patients are advised to perform frequent monocular monitoring of central vision (e.g., using an Amsler grid) and counselled about important warning symptoms that may herald neovascular disease activity. These include a relatively rapid increase in metamorphopsia, decrease in visual acuity, and/or central scotoma. In addition, newer devices for home monitoring of vision exist, including the ForeseeHome AMD Monitoring Program [66]. Any warning symptoms should be followed by presentation to a retinal specialist, where clinical examination and retinal imaging may be performed. Key clinical features to

suggest neovascular disease activity include retinal hemorrhage, hard exudate, subretinal and/or intraretinal fluid, observed clinically and on OCT imaging; these should prompt immediate angiographic evaluation, usually with fundus fluorescein angiography (FFA).

Retinal imaging in neovascular AMD is examined separately in Chap. 2, and has been reviewed in the literature [67]. In traditional practice, CNV membranes were evaluated and classified by FFA (with or without accompanying ICG angiography) into classic, occult, and mixed subtypes. This classification arose from the Macular Photocoagulation Study [68], where the lesion subtype was important in determining the potential benefits of photodynamic therapy. CNV membranes with occult features on FFA were assumed to be located in the sub-RPE space (i.e., type 1 histological classification), whereas classic membranes were assumed to be in the subretinal space (i.e., type 2 histological classification), though mixed sub-RPE and subretinal lesions are often found. However, the division into occult and classic membranes became less important with the advent of anti-VEGF therapy, since all lesion types are considered responsive to treatment. In addition, OCT angiography may now permit more detailed characterization of the CNV membrane location than was previously possible with FFA alone.

Two other important forms of neovascular AMD exist: (i) retinal angiomatous proliferation (RAP, or type 3) lesions, and (ii) PCV. In both cases, diagnosis is aided substantially by ICG angiography.

RAP lesions have been reviewed recently [69]. Multimodal imaging including dynamic ICG angiography helps delineate the etiology of the neovascular process; this is particularly helpful in cases of RAP that have a deep retinal vasculature component, which may or may not anastomose with choroidal vasculature [70]. The late leakage appreciated on ICG angiography can also be useful for identifying pathology that often coexists with RAP lesions, including SDD. Cystoid macular edema appreciated on OCT images constitutes an associated sign that correlates with the presence of a RAP lesion. RAP lesions are associated with the development of RPE tears and GA.

PCV is another important subtype or relative of neovascular AMD [71]. While the branching patterns of neovascular networks can be described using the same imaging modalities that are used to assess CNV in AMD, the coexisting retinal features differs from traditional AMD. In PCV, sub-RPE neovascularization (resembling type 1 neovascularization in AMD) is present but drusen and pigmentary abnormalities are often absent. The fate of eyes with PCV also differs from that of typical AMD, with few eyes developing GA. The associated finding of a thick choroid (pachychoroid) has led to hypotheses that a congested choroid plays a key and even primary role in the etiology of PCV.

These two forms of disease (i.e., RAP and PCV) have been the subject of debate and some controversy between ophthalmologists. In both cases, their partially distinct characteristics have raised questions as to whether they should properly be considered as subtypes of neovascular AMD or as forms of retinal disease distinct from AMD. Spaide has argued that RAP should be considered within the AMD spectrum because it shares phenotypic risk factors, genetic risk factors, end-stage findings, and treatments with the other forms of neovascular AMD [58]. Similar arguments apply to PCV [58], though other authors maintain that they are separate entities, and emphasize the differences between them (including choroidal features, drusen presence/characteristics, ethnic predilections, ages affected, and genetic features) [72].

However, some recent data raise the question of whether PCV may in fact represent a truly distinct entity at all (i.e., at their origin), as opposed to a secondary phenomenon. For example, Dansingani et al. [72] have observed on longitudinal multimodal imaging that many cases of PCV arise from CNV networks, such that PCV may simply represent type 1 neovascularization with aneurysms. Although these authors argue for the ongoing separation of AMD and PCV as distinct entities, they recommend shifting the focus of the question to why some patients with

type 1 neovascularization develop aneurysms while others do not [72]. In addition, Liang et al. [73] have demonstrated that the branching vascular networks and polyps of PCV can, in some eyes, coexist and communicate with type 2 neovascularization. The authors argue that aneurysm polyp formation may therefore represent a structural variant of neovascular tissue rather than a distinct pathogenic process in neovascular AMD [73]. Future studies using multimodal imaging and histological analysis may help refine our AMD taxonomy, by demonstrating whether the primary underlying disease processes are distinct or shared between these forms of disease.

1.3.6 Late Age-Related Macular Degeneration: Geographic Atrophy

1.3.6.1 Introduction

GA is the defining lesion of the atrophic form of late AMD. The term was introduced by Gass in 1973 to describe one or more circumscribed areas of RPE atrophy in the macula that may gradually enlarge and coalesce over time [74]. The atrophy was labeled geographic because confluent loss of the RPE usually occurs with a sharply demarcated border between depigmented and apparently normal retina, such that GA lesions may resemble islands. Atrophy of the RPE is typically accompanied by atrophy of the overlying photoreceptors and underlying choriocapillaris [75]. However, some heterogeneity between eyes may exist as to which tissue layer is affected first by atrophy.

GA in AMD has been estimated to affect over five million people worldwide. Data from systematic review and meta-analysis have estimated the global prevalence of GA at 0.44%, similar to that of neovascular AMD (0.46%) [1]. Interestingly, in this study, the prevalence of GA was very substantially higher in people of European ancestry (at 1.1%) than those of Hispanic (0.2%), African (0.1%), or Asian (0.2%) ancestry. In a systematic review and meta-analysis focused only on the white American population, geographic atrophy prevalence was 1.3% (representing 1.06 million people), with an annual incidence of 1.9 per 1000 adults aged 50 years or more [76].

The condition represents a substantial clinical and research priority because, unlike the situation for neovascular AMD, no drug therapies are currently available in routine clinical practice for GA, either to prevent GA development, to slow down GA enlargement, or to restore lost vision. Importantly, GA is also seen in retinal conditions other than AMD, particularly inherited retinal dystrophies such as ABCA4-retinopathy or maternally inherited diabetes and deafness. Depending on the age of the patient and associated retinal features, careful clinical examination and imaging is therefore sometimes required to distinguish between these differential diagnoses.

Visual function is severely limited in areas of the retina affected by GA, such that dense scotomata are observed in the corresponding visual field. If the fovea is affected by atrophy (so-called central GA), visual acuity is likely to be very poor. Even in individuals where GA is non-central, vision is usually highly impaired in the performance of tasks such as reading and facial recognition [77].

1.3.6.2 What is Geographic Atrophy?

Following the introduction of the term by Gass, different research groups have used definitions of GA that have varied in terms of diagnostic features and minimum size requirements; these have also been dependent on the imaging modality used to grade GA. In addition, the terminology around GA has changed over time; these semantic and historical considerations have been reviewed in detail [78].

In the AREDS, GA was defined as a sharply demarcated, usually circular zone of partial or complete depigmentation of the RPE, typically with exposure of underlying large choroidal blood vessels, that must be as large as grading circle I-1 (1/8 disk diameter in diameter) [23]. A similar definition was used in the AREDS2, except that the minimum size requirement was increased to grading circle I-2 (diameter

433 mm, i.e., 1/4 disc diameter) at its widest diameter [79]. In both the AREDS and AREDS2, a diagnosis of late atrophic AMD required the presence of central GA. However, in the unified AMD classification system proposed following the Beckman meeting, any GA (i.e., including noncentral GA) was sufficient to confer a diagnosis of late AMD. This recommendation followed the recognition that central and noncentral GA do not appear to represent substantially different entities in aspects other than location, and that noncentral GA progresses relentlessly over time to central involvement [41]. It is also worth mentioning that the term GA is usually reserved for atrophy in the absence of prior or simultaneous neovascular AMD. While some authors (including the Comparison of age-related macular degeneration Treatment Trials Research group [80]) do use the term GA to refer to atrophy following neovascular disease, others prefer to use the more general description "macular atrophy" [81].

Traditionally, color fundus photography was used as the main technique for identifying and measuring GA, as in the AREDS and AREDS2. Since then, fundus autofluorescence, near-infrared imaging, and OCT have provided additional imaging modalities to identify GA and measure progression rates. The imaging characteristics of GA on fundus autofluorescence and OCT have been reviewed [82, 83]. Fundus autofluorescence demonstrates a markedly reduced signal in the area of atrophy; patterns of hyper-autofluorescence surrounding the atrophic lesion (e.g., banded or diffuse-trickling) have also been used to characterize lesions and perhaps help predict growth rates [84].

Because of the advent of multiple imaging modalities on which to identify and measure GA, a recent consensus meeting of experts led to the proposal of a new classification system for GA. The panel recommended that OCT be used as the reference standard to identify atrophy, with the use of the other modalities as complementary [85]. The OCT-based criteria proposed for GA were: (i) a region of hypertransmission of at least 250μm in diameter, (ii) a zone of attenuation or disruption of the RPE of at least 250μm in diameter, (iii) evidence of overlying photoreceptor degeneration, and (iv) absence of scrolled RPE or other signs of an RPE tear, where all criteria must be met to define GA. In addition, the panel emphasized that photoreceptor atrophy can occur without RPE atrophy, and therefore recommended adoption of the terms outer retinal atrophy (ORA) and RPE and outer retinal atrophy (RORA), where complete RORA would correspond to GA.

Functionally, eyes with noncentral GA are not normal even if visual acuity is undisturbed [86]. Beyond microperimetric study of mesopic threshold testing, functional dark adaption has demonstrated abnormalities before GA affects central visual acuity. Supporting the observations that photoreceptor degeneration can occur without or prior to RPE atrophy, studies of dark adaptation demonstrate deficits in eyes with intermediate AMD, and particularly in eyes with SDD, even in those eyes without any overt loss of RPE [49].

1.3.6.3 Where does Geographic Atrophy Come From?

Although GA is a common form of late AMD, particularly in white populations, some heterogeneity in the origins of GA is increasingly recognized. In this respect, it seems that not all GA arises from the same precursor lesions. One recent study used longitudinal multimodal imaging in AMD and reported three major origins of central GA, defined using the OCT definition for complete RORA (i.e., three origins, aside from the spread of existing paracentral atrophy toward the fovea): (i) drusen-associated atrophy; (ii) refractile deposits; (iii) pigmentary changes without large drusen [87]. In the majority of eyes (8 out of 11), atrophy was preceded by the presence of large confluent drusen (i.e., origin (i)), with OCT features of "nascent GA" [88] noted before the features of complete RORA. Hyperpigmentary changes were present before atrophy in all cases; interestingly, RPD were present prior to GA in two of these eight eyes, but were not spatially correlated with the area of emerging GA. Indeed, throughout the study, RPD were not observed to lead directly (with

spatial association) to GA. In the other eyes (3 out of 11), GA developed from precursor lesions (ii) or (iii).

1.3.6.4 How does Geographic Atrophy Behave?

One of the largest studies of the behavior of GA was performed by analysis of the AREDS2 dataset (based on color fundus photography), which included 517 eyes with preexisting GA at baseline and 1099 eyes that developed incident GA during follow-up [41]. One striking finding of multiple studies has been that GA usually affects the parafoveal region. In the AREDS2 dataset, two thirds of incident GA was noncentral, while the remaining third of eyes had foveal involvement at the outset [41]. No apparent genetic or clinical reasons have yet been able to explain why some eyes have central involvement at the outset, while others do not [41], though the site of atrophy is usually determined by the location of preceding large drusen [87, 89].

The rate of progression to central involvement was captured in the AREDS2 dataset by Kaplan–Meier analysis [41]. For incident noncentral GA, the 4-year rate of progression to central involvement was high at 57%. This emphasizes the relentless nature of GA, even in eyes where atrophy is noncentral at the outset, since the large majority of eyes will undergo foveal involvement and visual loss in the following years. In addition, relatively high levels of progression to neovascular disease following GA were observed: in eyes with incident GA, the 4-year risk of subsequent neovascular disease was 29%.

The enlargement rate of GA was analyzed in this dataset [41], using the square root transformation to adjust for baseline lesion size [90]. Multiple clinical, imaging, and genetic characteristics were associated with altered speed of enlargement, in univariate and multivariate analyses. The factors associated with faster GA enlargement included GA presence in the fellow eye, GA characteristics (specifically intermediate size, no central involvement, and multifocal configuration), genetic variants (*ARMS2/HTRA1* risk alleles, *C3* nonrisk alleles, and *APOE* protective alleles), and possibly lower education level and positive smoking status, but not age. Other studies have provided conflicting evidence as to whether the presence of SDD is another factor associated with faster GA enlargement [91]; if this were true, it is possible that some of the association between *ARMS2/HTRA1* risk alleles and faster GA enlargement might be explained by SDD presence (since *ARMS2/HTRA1* risk alleles are associated with SDD presence [43]).

A distinction is observed between the genetic and clinical risk factors associated with incident GA and those associated with faster enlargement of GA. While some factors act in the same direction, others (such as *CFH* genotype) are not shared, and still others (e.g., *C3* genotype) appear to act in opposite directions. This suggests that the emergence of GA may be of a fundamentally different nature to the propagation of established GA, with partially distinct mechanisms and biological pathways. The important implication of this is that separate therapeutic approaches may be required to prevent the development of GA versus slow down its enlargement (see Chap. 12).

1.4 Risk Factors for Age-Related Macular Degeneration

Susceptibility to AMD, as for many other age-related chronic diseases, has traditionally been understood in terms of genetic and environmental risk factors, in addition to increased age. Of course, the situation may not as simple as this suggests. Age may represent an accumulation of multiple environmental exposures, other age-related biological changes, and perhaps even accumulated genetic and epigenetic changes. In addition, multiple potential interactions may exist between age, genetic, and environmental factors. Importantly, AMD is a heterogeneous disease; for example, the effects of environmental exposures might be different in people with *CFH*-related versus *ARMS2/HTRA1*-related AMD, and in people of different geographies and ethnic origins. It may also be informative to distinguish between genetic and environmental factors that increase

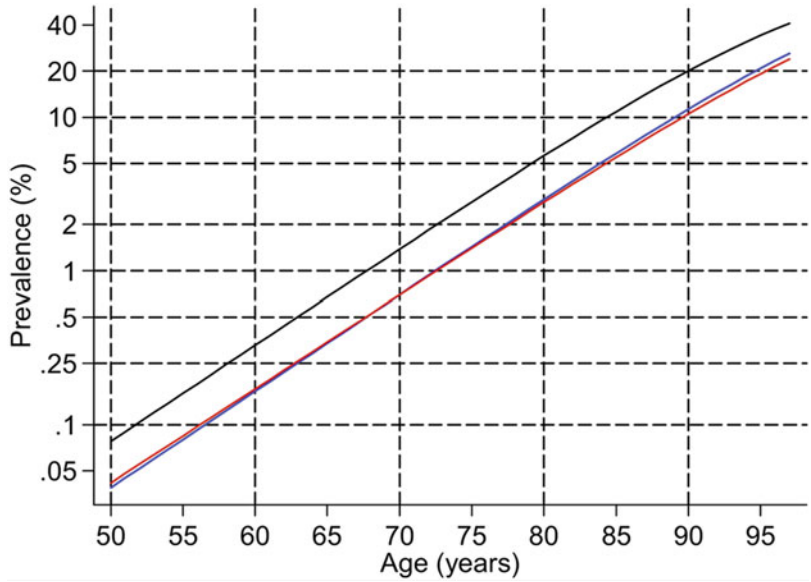

Fig. 1.6 Estimated predicted prevalence of late age-related macular degeneration (black line), geographic atrophy (blue line) and neovascular age-related macular degeneration (red line) by age from Bayesian meta-regression model with logarithmic scale on the *y*-axis. Reprinted from *Ophthalmology*, volume 119, Rudnicka AR et al, 'Age and gender variations in age-related macular degeneration prevalence in populations of European ancestry: a meta-analysis', pages 571–580, copyright (2012), with permission from Elsevier

the risk of early disease, of late disease, and of particular disease phenotypes and subtypes.

1.4.1 Age

From the earliest studies, AMD has been very strongly associated with increased age. The Beaver Dam Eye Study [11] and the Blue Mountains Eye Study [17] both reported substantially increased prevalence rates with increased age. More recently, meta-analysis of multiple studies from different countries has shown that the prevalence of AMD increases exponentially with age (see Fig. 1.6), with an odds ratio of 4.2 per decade [92]. According to this meta-analysis, the prevalence of late AMD in populations of European ancestry is 1.4% at 70 years of age, rising to 5.6% at age 80 and 20% at age 90. Considered separately, the prevalence of atrophic and neovascular AMD both increase with age: from 0.7% for each at 70 years, to 2.9% and 2.8% (80 years), to 11.3% and 10.5% (90 years), respectively.

However, the exact mechanism(s) whereby increased age leads to increased likelihood of AMD are not clear. Many of these potential mechanisms have been reviewed [40]; in this review article, the aging paradigm is considered in terms of seven damaging events that occur with increased age (see Fig. 1.7). These are discussed in Chap. 3, and can be summarized as (i) age-related changes occurring in the eye tissues, and (ii) a gradually increasing burden of para-inflammation on the retina (Fig. 1.8).

In this respect, AMD may be considered to occur as a loss of retinal homeostasis, through an interplay between (i) age-related changes and (ii) environmental and genetic risk factors. Each of these alone is necessary but not sufficient, such that the combination of the two is required for disease formation. Together, these lead to a chronic low-grade immune response, such that AMD has been considered as exacerbated aging-related changes mediated by immune activation [40].

For these reasons, epidemiological and genetic studies should ideally incorporate the possibility of interactions between age, genetics, and environmental factors, in order to advance our understanding of the complex interplay between these elements. For example, recent data from the IAMDGC conducted a large-scale GWAS of late AMD, but stratified according to age and sex [93]. This highlighted the existence of

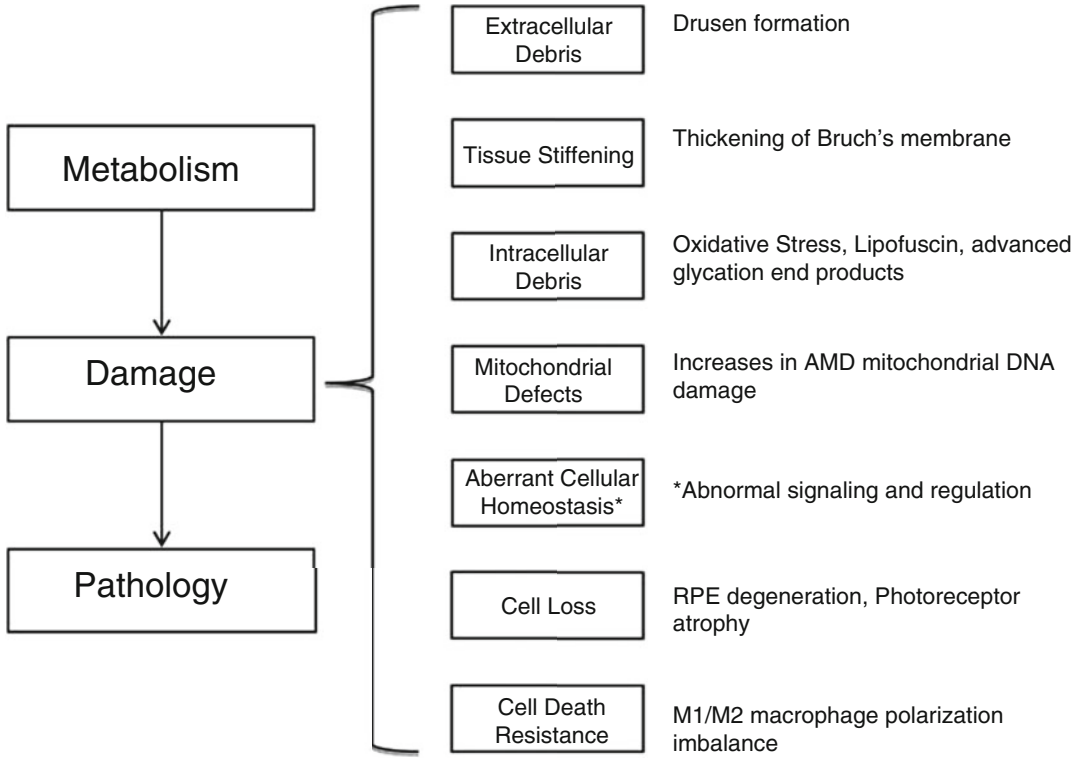

Fig. 1.7 The aging paradigm in AMD. The seven damaging events are indicated in the second column and the correlates observed in AMD in the third column. Reprinted from *Progress in Retinal and Eye Research*, volume 37, Ardeljan D et al, 'Aging is not a disease: distinguishing age-related macular degeneration from aging', pages 68–89, copyright (2013), with permission from Elsevier

age-dependent genetic effects, whereby three variants in the *CFH* and *ARMS2/HTRA1* loci had significantly stronger effects in younger individuals. Similarly, it is important to investigate and control for age, genetic, and environmental factors in studies of the molecular basis of AMD. In one study of post mortem human macular tissue, the molecular characteristics of AMD were investigated according to genotype and smoking status, while age was controlled [94]. This study demonstrated molecular signatures associated with *CFH* genotype and with smoking status, but also suggested an interaction between the *CFH* risk genotype and positive smoking status, in terms of increased levels of local tissue inflammation. In this way, links may be found between known epidemiological risk factors for AMD and molecular pathology at the anatomical site of disease formation.

1.4.2 Genetic Risk Factors

The genetics of AMD are examined separately in Chap. 7. In summary, initial evidence for strong genetic involvement in AMD was demonstrated in epidemiological studies [95, 96]. Since then, our understanding of AMD genetics has increased substantially. Two important discoveries have been (i) the report by several groups, in 2005, of a strong association between late AMD and variants at the *CFH* locus on chromosome 1q [97–100], and (ii) the report of an another strong association with variants in two tightly linked genes on chromosome 10q26 (*ARMS2/HTRA1*) [101, 102]. Haplotypes at these two loci have subsequently been consistently observed as significantly associated with late AMD, with large effect size, in multiple genetic studies of

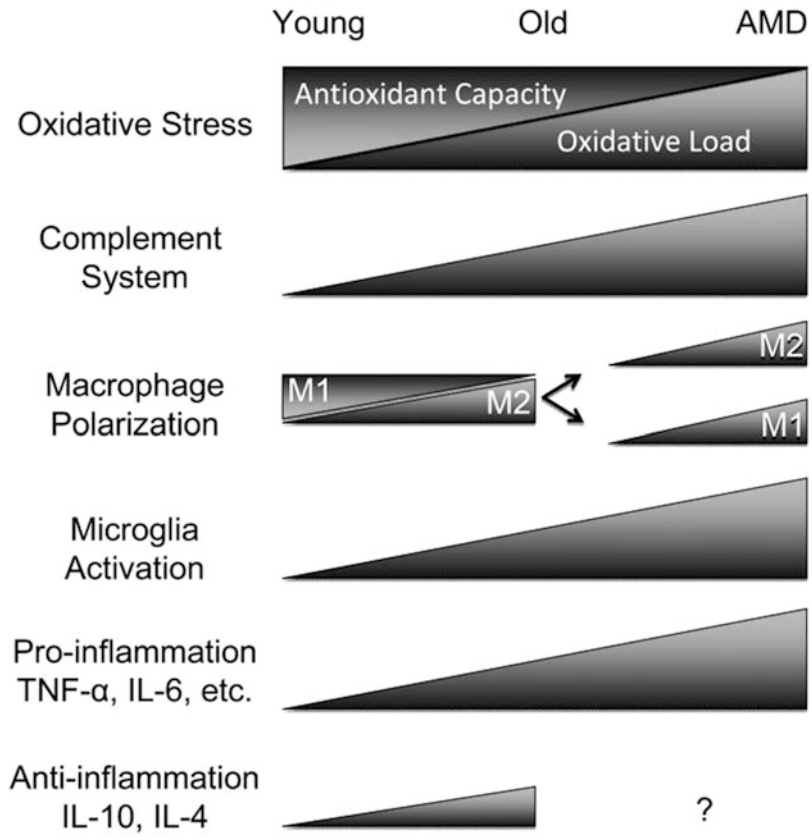

Fig. 1.8 Immune changes in aging versus age-related macular degeneration. The aging eye sees shows increased oxidative load and diminished antioxidant capacity, increased immunoreactivity toward complement factors, shifts in macrophage polarization, generally increased microglia activity, and upregulation of pro- and anti-inflammatory factors. In AMD, each of these balances is disturbed, evidenced by excessive increases in each of these categories that synergize to launch the system into a disease state. Reprinted from *Progress in Retinal and Eye Research*, volume 37, Ardeljan D et al, 'Aging is not a disease: distinguishing age-related macular degeneration from aging', pages 68–89, copyright (2013), with permission from Elsevier

European-descended (and other) populations from diverse countries and research settings.

Following this, multiple genome-wide association studies (GWAS) reported additional genetic variants associated with late AMD. Research through the International AMD Genomics Consortium (IAMDGC) led to a large GWAS of late AMD, which analyzed data from 16,000 participants with late AMD and 18,000 control individuals [103]. This led to the identification of 52 independently associated genetic variants across 34 loci. These variants and loci relate to genes involved in several distinct biological pathways: the complement system (e.g., variants in *CFI*, *C9*, *C2/CFB*, and *C3*), lipid transport (e.g., *LIPC*), extracellular matrix remodeling (e.g., *TIMP3*), angiogenesis (e.g., *VEGFA*), and cell survival (e.g., *RAD51B*) [95, 103]. These biological pathways are therefore highly implicated in AMD pathogenesis, and functional studies of these variants may provide important insights into disease mechanisms.

The results from the GWAS are thought to explain approximately 50% of the heritability of late AMD [103]. While future GWAS with even higher power might discover additional variants and loci associated with late AMD, it is likely that these variants will be relatively rare or have relatively modest effect sizes. However, some of the missing heritability might be explained in the future by discoveries from other techniques [104], such as analyses of copy number variation (e.g., *C4* [105]), quantitative trait loci, mitochondrial genetics, and/or epigenetic studies [106]. Meanwhile, a useful development from the IAMDGC GWAS has been the methodology for generation of an AMD Genetic Risk Score (GRS) for an individual [103]. This AMD GRS is calculated as a weighted risk score, based on the 52 variants. This tool has the advantage that a

single value can be used to represent the total genetic load (based on known SNPs) for AMD.

Some important genotype-phenotype associations have emerged in recent years. In a large study using data from the AREDS and AREDS2, risk alleles at the *CFH* locus were preferentially associated with macular drusen, while risk alleles at the *ARMS2/HTRA1* locus were preferentially associated with neovascular disease accompanied by subretinal/sub-RPE hemorrhage, and also with worse visual acuity [42]. Together with other evidence of genotype–phenotype associations, this has implications for studies of AMD pathophysiology; these might benefit from analyses stratified by *CFH*-related versus *ARMS2/HTRA1*-related disease, as an attempt to deal with the heterogeneity of this condition.

1.4.3 Environmental Risk Factors

As mentioned above, AMD arises through a complex interplay between increased age, genetic variants, and environmental influences. Many environmental exposures have been considered and examined as potential risk factors. This aspect was an important focus of research in the Beaver Dam Eye Study and the Blue Mountains Eye Study; both studies collected detailed information on environmental exposures and analyzed their potential influences on AMD risk. Multiple other studies in different countries and research settings have also addressed this question. From this work, some factors have been consistently and strongly associated with AMD risk, such as cigarette smoking [107]. For other factors, such as systemic hypertension, the associations have been consistent but of moderate strength. In many other cases, the evidence has been inconsistent and/or of weak effect size. Accurate knowledge in this area is important not just to identify individuals at risk and plan public health services, but also to provide insights into disease pathogenesis and potential therapeutic targets.

Potential risk factors of AMD have been considered (alongside biomarkers) in a recent review [108]. Aside from increased age and genetic variants, these may be considered as biomarkers of susceptibility (including sex, ethnicity, iris color, obesity, and systemic hypertension) and as biomarkers of exposure (including cigarette smoking, sunlight, and diet), distinct from biomarkers of disease and progression (including homocysteine, C-reactive protein, cholesterol, metabolomic signatures, and many other potential factors). Of course, other undiscovered environmental factors may increase or decrease the risk of disease incidence or progression, and some of these may have partially distinct effects in the presence of AMD associated with different genetic variants, while others might act equally on disease risk irrespective of genotype.

Because of the multitude of studies in this area with different methodologies and varying research settings, systematic review and meta-analysis has provided an efficient way to condense these data. A meta-analysis was performed in 2010, which evaluated the available evidence for 16 pre-selected potential clinical risk factors [109]. The number of studies meeting the inclusion criteria was 18 prospective or cross-sectional and 6 case–control studies, with a total of 113,780 participants (including 17,236 cases of AMD). It is important to emphasize that this research pertained only to late AMD. The results are summarized in Table 1.4. Aside from increased age and a family history of AMD, the clinical factors that showed strong and consistent associations with late AMD were current cigarette smoking and previous cataract surgery. Risk factors that had moderate and consistent associations were higher body mass index, history of cardiovascular disease, systemic hypertension and higher plasma fibrinogen. Risk factors with weaker and inconsistent associations were sex, ethnicity, diabetes mellitus, iris color, history of cerebrovascular disease, and serum total and HDL cholesterol and triglyceride levels.

However, this meta-analysis has limitations and does not provide the definitive answer to the question of environmental exposures. Only 16 factors were examined out of a possible 73 potential risk factors identified from the literature by the authors [109]. Many factors were excluded from the analysis, including ocular

Table 1.4 Summary of pooled relative risks (for prospective studies) and odds ratios (for cross-sectional and case–control studies), by systematic review and meta-analysis: environmental factors with strong or moderate association with late age-related macular degeneration

Risk factor	Prospective	Cross-sectional	Case-control
Cigarette smoking	1.86 (1.27–2.73)	3.58 (2.68–4.79)	1.78 (1.52–2.09)
Cataract surgery	3.05 (2.05–4.55)	1.59 (1.08–2.34)	1.54 (1.24–1.91)
Body mass index (≥25)	1.28 (0.98–1.67)	1.21 (0.97–1.53)	1.52 (1.15–2.00)
Cardiovascular disease	1.22 (0.92–1.63)	1.12 (0.86–1.47)	2.20 (1.49–3.26)
Hypertension	1.02 (0.77–1.35)	1.15 (0.88–1.51)	1.48 (1.22–1.78)
Plasma fibrinogen (per standard deviation increase)	1.03 (0.81–1.32)	1.45 (1.22–1.73)	–

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factors (e.g., axial length and cilioretinal artery presence), many medical conditions (e.g., obstructive sleep apnea and many autoimmune diseases), medication use, and physical exercise; dietary intake was also excluded, as this was considered to be a specialized field. In addition, the results of the meta-analysis are necessarily subject to many of the same biases that were present in the constituent studies. For example, many cross-sectional and case–control studies of cataract surgery may be subject to confounding by indication, since individuals with visual symptoms from undiagnosed AMD may be more likely to receive cataract surgery, which can subsequently be associated artifactually with increased incidence or progression of AMD. Indeed, study that use methodologies (such as randomization, matched pair analysis, or propensity score techniques) to address confounding have generally not supported the idea that cataract surgery is associated with incident AMD or disease progression [110–112].

Data from the AREDS and AREDS2 studies have also provided evidence of potential risk factors associated with AMD, including the use of longitudinal data to analyze factors linked to disease progression. A recent bivariate analysis of both datasets demonstrated that factors associated with higher risk of progression to late AMD included increased age, lower education level, positive smoking status, high baseline AMD severity, and high AMD GRS [113]. Again, however, only a limited number of potential environmental factors was considered.

Cigarette smoking has consistently been linked to increased risk of AMD in multiple studies in different countries [107], including in the meta-analysis mentioned above [109]. However, some research has examined different disease stages. For example, data from the Beaver Dam Eye Study has shown that a greater number of pack-years smoked was associated with an increased risk of progression from no AMD to minimal early AMD, and from severe early AMD to late AMD [114]; this and other studies have sought to examine potential interactions between smoking status and *CFH* and *ARMS2/HTRA1* genotype. In addition, smoking status has been weakly linked to faster enlargement of GA [41], and to worse visual outcomes with anti-VEGF therapy in neovascular AMD [64].

The role of nutrition and diet as potentially modifiable environmental factors has received considerable interest [115]. Dietary factors that may be implicated in disease risk or protective effects include carotenoids (lutein, zeaxanthin, and beta-carotene), vitamins (A–E), mineral supplements (zinc, copper and selenium), dietary fatty acids (including omega-3 fatty acids), and carbohydrates [116]. For example, dietary consumption of lutein and zeaxanthin has been linked to decreased risk of intermediate and late AMD [117]. While high total dietary fat and high glycemic index diet are known to increase risk of disease, higher consumption of omega-3 fatty acids is suggested in some studies but not others to be linked with reduced risk of AMD [116]. Data from multiple countries and research settings have demonstrated that higher levels of adherence to a Mediterranean diet are associated with lower risk of progression to late AMD (particularly GA) [118–121]. The specific dietary

components responsible for this protection appear to include higher intake of fish. Oral supplementation with different combinations of antioxidants, zinc, carotenoids, and fatty acids is discussed separately in Chap. 12.

The role of systemic medications in influencing AMD risk has been examined in multiple studies. Common drugs discussed in the literature have included aspirin, statins, and anticholinergic medications. Conflicting reports of the potential effects of aspirin have created controversy. The authors of an editorial on this topic in 2014 stated that, in general, "the cross-sectional studies reported an association with early AMD, while this was not found in the cohort studies," and that "the cohort studies found an association with only neovascular AMD ... with no associations with early AMD or GA" [122]. Many studies in this area have been limited by the potential for confounding by indication, since indications for aspirin use (e.g., history of cardiovascular disease) may also represent risk factors for AMD. Studies with methodologies designed to address this limitation (e.g., by randomization or propensity score analyses) have not supported an increased risk of late AMD with aspirin use [123–125]. Interest has existed in the potential for statin use to decrease the risk of AMD progression, but data from the AREDS2 and other studies did not support this idea [126]. Finally, one recent study has suggested that anticholinergic drugs may increase the risk of late AMD [127].

Further potential risk factors continue to be examined and reported, such as the role of obstructive sleep apnea in increasing risk of disease and of poor response to treatment [128, 129]. In addition, physical exercise may be an important protective factor in AMD progression, but was not included in the meta-analysis mentioned above [109]. However, a systemic review and meta-analysis of this particular topic (in white populations) observed that physical activity was protective against both early AMD and late disease [130]. The effect size was relatively modest for early AMD (odds ratio 0.92) but large for late disease (odds ratio 0.59).

In summary, multiple environmental factors and exposures may have risk and protective effects. It is likely that those with the most strong and consistent effects, such as smoking, have already been discovered. However, it is important to recognize that environmental factors may have differential effects according to an individual's age, ethnicity, and AMD genotype, and may have differential influences at particular disease stages (e.g., early vs late), subtypes (e.g., neovascular versus GA), and on specific disease phenotypes (e.g., soft drusen versus SDD). Studies that attempt to incorporate these parameters into their design may generate more accurate and consistent data, and provide further insights into disease pathogenesis and potential therapeutic targets.

1.5 Conclusion

AMD is a common blinding disease. It has traditionally been considered a condition of high-income countries with older populations. However, the next decades will see a large shift in global patterns of disease; by 2040, Asia is predicted to have the largest number of people with AMD, with more than half of late AMD cases. In this respect, AMD can no longer be regarded as a disease primarily of European-descended populations.

AMD is a heterogeneous disease, and arises from a complex interplay between age, genetics, and environmental factors. However, it is possible that some of this complexity has arisen from studying its epidemiology and biology as if it were a single homogeneous entity. The past decade has seen substantial improvements in our understanding of AMD genetics, phenotypes (including SDD), and forms of advanced disease (such as ORA, RORA, and subtypes of neovascularization). Genotype–phenotype analyses suggest that AMD may consist of two or more partially distinct forms of disease (related to *CFH* and *ARMS2/HTRA1*). Biological and clinical studies should ideally incorporate genetic and phenotypic information into their study designs, in order to understand similarities and differences

within this heterogeneous condition. Importantly, potential interactions with environmental factors may differ between these forms of disease, and differing combinations of genetic and environmental exposures may also be important at different stages of disease progression, as suggested for genetic factors in recent bivariate analyses [113]. Indeed, distinct patterns of AMD prevalence and presentation are seen between geographical areas that are not explained fully by disparities in population structures. Genetic variation may go some way to account for these, but some aspects remain difficult to explain, e.g., the high prevalence of PCV in African and Asian populations, and the high prevalence of GA in white populations.

Current classification systems for AMD are likely to need refinement in the near future. Following advances in imaging, genetics, and psychophysics, these may need to incorporate an expanded set of phenotypes (e.g., SDD), to distinguish between forms of advanced disease (e.g., ORA and RORA), and to establish the role of the choroid in defining disease presence and risk of progression. In particular, the latter will help in understanding precisely how to incorporate and/or differentiate between AMD and the pachychoroid spectrum conditions. For RAP and PCV, ongoing multimodal imaging and histological studies may help us understand whether these conditions share the same primary underlying disease mechanism as typical neovascular AMD, or whether the neovascularization in RAP and PCV originates via a different mechanism. Updating current classification systems may also mean refining existing risk prediction models, such as the AREDS simplified scale and published risk calculators. For example, current classification systems and risk calculators may not differentiate well between the varied courses of: (i) a younger Asian patient with scant drusen and thick choroid progressing to PCV; (ii) an Hispanic patient with large soft drusen progressing to a nonexudative type 1 neovascular lesion, which later becomes exudative; and (iii) an elderly white patient with SDD and thin choroid progressing to ORA or to RAP. Ultimately, the classification system that performs most accurately in the prediction of disease progression, visual loss, and response to treatment is likely to be most useful.

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2 Ocular Imaging for Enhancing the Understanding, Assessment, and Management of Age-Related Macular Degeneration

Marco Nassisi and Srinivas R. Sadda

Abstract

Age-related macular degeneration (AMD) is a progressive neuro-retinal disease and the leading cause of central vision loss among elderly individuals in the developed countries. Modern ocular imaging technologies constitute an essential component of the evaluation of these patients and have contributed extensively to our understanding of the disease. A challenge with any review of ocular imaging technologies is the rapid pace of progress and evolution of these instruments. Nonetheless, for proper and optimal use of these technologies, it is essential for the user to understand the technical principles underlying the imaging modality and their role in assessing the disease in various settings. Indeed, AMD, like many other retinal diseases, benefits from a multimodal imaging approach to optimally characterize the disease. In this chapter, we will review the various imaging technologies currently used in the assessment and management of AMD.

Keywords

Age-related macular degeneration · Geographic atrophy · Macular neovascularization · Optical coherence tomography · Optical coherence tomography angiography · Dye-based angiography · Fundus photography · Fundus autofluorescence · Adaptive optics · Widefield imaging · Multimodal Imaging

List of Abbreviations

AMD	Age-related macular degeneration
AOSLO	Adaptive optics scanning laser ophthalmoscopy
CC	Choriocapillaris
CFP	Color fundus photography
cORA	Complete outer retinal atrophy
cRORA	Complete retinal pigment epithelium and outer retina atrophy
EDI	Enhanced depth imaging
EFC	Emission fluorescence components
EZ	Ellipsoid zone
FA	Fluorescein angiography
FAF	Fundus autofluorescence
FLIO	Fluorescence lifetime imaging ophthalmoscopy
GA	Geographic atrophy
ICGA	Indocyanine green angiography
iORA	Incomplete outer retinal atrophy

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IRF	Intraretinal fluid
iRORA	Incomplete retinal pigment epithelium and outer retina atrophy
LED	Light-emitting diode
MFP	Multicolor fundus photography
MNV	Macular neovascularization
NIR	Near-infrared reflectance
OCT	Optical coherence tomography
OCTA	Optical coherence tomography angiography
ORT	Outer retinal tubulation
PCV	Polypoidal choroidal vasculopathy (aneurysmal type 1 neovascularization)
PED	Pigment epithelium detachment
PRPD	Peripheral reticular pigmentary degeneration
qAF	Quantitative autofluorescence
RPE	Retinal pigment epithelium
SD-OCT (A)	Spectral domain optical coherence tomography (angiography)
SLO	Scanning laser ophthalmoscopy
SRF	Subretinal fluid
SS-OCT (A)	Swept source optical coherence tomography (angiography)
TD-OCT	Time domain optical coherence tomography
SW-FAF	Short wavelength fundus autofluorescence

2.1 Background

Advances in imaging have dramatically affected our assessment of age-related macular degeneration (AMD). The high definition and high magnification of certain imaging modalities such as optical coherence tomography (OCT) or adaptive optics scanning laser ophthalmoscopy (AOSLO) have allowed the in vivo visualization of the retinal tissue, comparable to histologic samples. Thus, the opportunity to study the disease in all its phases and phenotypes has yielded new insights into the pathophysiology of AMD.

Multimodal approaches using different modalities in an integrated fashion have been a key strategy for clinical assessment. In fact, the combination of cross-sectional and en face images obtained simultaneously through OCT devices equipped with a confocal scanning laser ophthalmoscope (SLO) allows a microscopic correlation between clinical, anatomical, and pathological findings in multiple conditions [1–3].

Once diagnosed, AMD is a lifelong condition that requires accurate assessment and potentially long-term management [4]. Current treatment approaches (in clinical practice or clinical trials) largely target advanced stages of the disease like macular neovascularization (MNV) and geographic atrophy (GA). In the near future, treating AMD earlier in the disease course could increase the likelihood of the preservation of vision, while significantly improving the cost-effectiveness of drug treatments and reducing the costs of drug development.

Different mechanisms have been proposed to be relevant to the development of AMD including oxidative stress, accumulation of lipofuscin in retinal pigment epithelium (RPE), and primary damage to Bruch's membrane [5–7]. These microstructural alterations progressively lead to the early clinical findings of the disease, including features such as RPE depigmentation, drusen, and RPE hyperplasia. Eventually, complete outer retinal, RPE and choriocapillaris (CC) atrophy may develop, resulting in the late stage of dry AMD: geographic atrophy (GA). On the other hand, the sudden and often unpredictable development of neovessels, under the stimuli of vascular endothelial growth factor (VEGF) and other inflammatory factors, leads to MNV marked by exudation, bleeding and, eventually to fibrosis and atrophy with poor visual outcomes if left untreated.

Traditionally, disease grading relies on the photographic evaluation of the fundus, with the determination of the extent of drusen as well as the presence of pigmentary abnormalities in the posterior pole [8–12]. The advent of digitalization, together with new imaging technologies, provides often a higher sensitivity and has facilitated an automated or semiautomated quantitative analysis of all the features that might be considered at risk for the development and the progression of AMD [13–15].

A proper imaging assessment is fundamental in clinical therapeutic trials for advanced AMD as it can influence patient selection, and overall duration, costs, and outcomes of the studies. Given the rapid advance of new devices and post-acquisition image analysis algorithms, the selection of appropriate imaging modalities can be challenging. Currently, a multimodal approach is broadly accepted as the gold standard for imaging assessment of AMD patients [1, 2].

2.2 Color and Multicolor Fundus Photography

Color fundus photography (CFP) captures an image of the retina, with various degrees of fidelity with respect to clinical fundus ophthalmoscopy, depending on the different machines and modalities. It allows the early detection of a broad range of pathological changes associated with the early (i.e., drusen, lipids, crystalline deposits, and alterations of pigmentations) or late stages (i.e., hemorrhages, fluid, exudates, atrophy and fibrosis) of AMD. Multicolor fundus photography (MFP) is relatively newer technology and provides a "false color" image of the fundus combining three different wavelengths of light. The relative ease and tolerability of acquisition of these modalities offer important advantages to the management of AMD patients. Furthermore, digital photographs offer the possibility of post-processing analysis, paving the way to the development of automated algorithms for quantification of AMD-related features. This will be an important step forward for the early detection of disease and its monitoring.

2.2.1 Color Fundus Photography

Historically, CFP has always been considered the gold standard to demonstrate fundus alterations. Fundus cameras are designed to provide an upright, magnified view of the fundus using a white flash for illumination. A typical camera views 30–50° of retinal area, with a magnification of 2.5×.

A series of crucial improvements to fundus photography have been made over the last decades, such as nonmydriatic imaging, electronic illumination control, automated eye alignment, and high-resolution digital image capture [16]. This has contributed to advanced fundus photography as an essential tool in clinical practice to document retinal pathologies, and its use is often considered mandatory in disease definition and classification.

CFP is also considered the gold or reference standard for the evaluation of newer imaging technologies, and it is currently in use in clinical studies of AMD. During the last decades, several groups have proposed AMD classification systems based on CFP [8, 17]; the most recent of which utilize the presence of fundus pigmentary abnormalities (either hypo or hyperpigmentation) and drusen size to distinguish early, intermediate, and late AMD.

The latest classification was proposed in 2013 by the Beckman Initiative for Macular Research Classification Committee: early AMD was defined as medium drusen (i.e., between 63 and 125 μm) and no associated pigmentary abnormalities. Intermediate AMD demonstrated large drusen (i.e., >125 μm) and/or any AMD pigmentary abnormalities. Finally, the presence of MNV and/or GA was the key findings associated with the late stages of the disease [18].

This staging does not take into account the type of drusen present, with regards to features other than size. The relatively low contrast of the color photographs sometimes makes the identification of AMD-associated abnormalities challenging. Soft and cuticular drusen are easily visualized in CFP as diffuse spots with variable shades of yellow, depending on their constitution and the health of the overlaying RPE that can variably attenuate the blue light [19]. On the other hand, subretinal drusenoid deposits (SSD, aka reticular pseudodrusen (RPD) [20]), which are important risk factors for AMD progression [20–22], can be challenging to detect as they overly the RPE and are not affected by the RPE-related filtering of blue light. In fact, without

additional processing, CFP has a relatively low sensitivity for SSD detection (range: 33–42%).

The presence of MNV in CFP is also challenging to recognize, and signs of exudation and bleeding generally must be present to facilitate detection (Fig. 2.1). Previous studies report a sensitivity for the detection of MNV using CFP of about 78%, which drops to 38% when CFP alone is to be used to determine whether the MNV is active or not [23]. A multimodal approach implementing dye (i.e., FA and ICGA) and/or nondye-based (i.e., OCT and OCT angiography [OCTA]) techniques is therefore essential.

On the other hand, GA lesions are more easily identifiable on CFP and in fact the classic definition of GA is based on a qualitative and a quantitative evaluation of CFP images. The International Age-Related Maculopathy Epidemiological Study Group defined GA as any sharply delineated roughly round or oval area of hypopigmentation or depigmentation with increased visibility of the underlying choroidal vessels and of at least 175 μm in diameter on 30° or 35° CFP images [17].

Nevertheless, CFP is also limited for the purpose of measuring GA precisely owing to difficulty in delineation of the boundaries of lesions, specifically in case of relatively smaller size and multifocality.

The stereoscopic acquisition with CFP can provide higher sensitivity to detect topographic alterations at the periphery of atrophic regions and/or the presence of fluid and pigment epithelial detachment (PED). On the other hand, stereoscopic imaging is not always feasible, even in clinical trials, as it requires good cooperation from patients, and it should be performed by operators with senior experience.

2.2.2 Multicolor Fundus Photography

Multicolor images are generated from the simultaneous use of three different wavelengths: blue reflectance (wavelength: 488 nm), green reflectance (wavelength: 518 nm), and typically NIR (wavelength: 820 nm) [24]. The resulting three reflectance images are then combined into a "multicolor" image, also defined as "pseudocolor" or "false-color" image since it is not the result of the whole visible light spectrum but only of the sum of three wavelengths. Multicolor imaging systems use SLO techniques for image acquisition. In SLO systems, a confocal aperture is designed to clear backscattering light from outside the focal plane, making possible the imaging of individual layers of the retina with higher contrast and spatial resolution than with previous approaches [25].

The three different light wavelengths used in MFP can gather distinctive information from the various layers of the retina, as they penetrate them to different extents. In particular, the short wave reflectance (blue) highlights the inner retina and the vitreoretinal interface; green reflectance enhances the examination of the deep structures of the retina; and, finally, NIR facilitates the visualization of the choroid and the outer retina [24].

At present, a small number of report regarding this innovative modality have been published [22, 26, 27], and the findings in AMD are highly correlated with CFP. In the context of GA, the borders of the lesion are better visualized because of the higher contrast of the MFP (Fig. 2.2) [27].

Furthermore, fibrotic alterations and hemorrhages can be easily detected because of their optical reflection properties; however, subtle hemorrhages can sometimes be misjudged for pigmentary lesions using only MFP alone.

While CFP is more susceptible to media opacities and poor mydriasis because of the use of high-intensity and broad-spectrum light, it is possible to acquire high-quality multicolor images through nondilated pupils. However, the blue spectrum can be adversely affected by media opacities and poor mydriasis as well, influencing in an unpredictable way the signal strength of the resulting image [28].

A recent interesting addition to the available instruments for CFP is a new confocal device using white light. This machine (CentervueEidon; Centervue, Padova, Italy) offers the advantages of providing a "true color" image of the fundus, with increased contrast because of its confocality (Fig. 2.3) (using slit rather than circular confocal pinhole) [29]. However, no large studies comparing these confocal white-light color images with standard CFP have yet been performed.

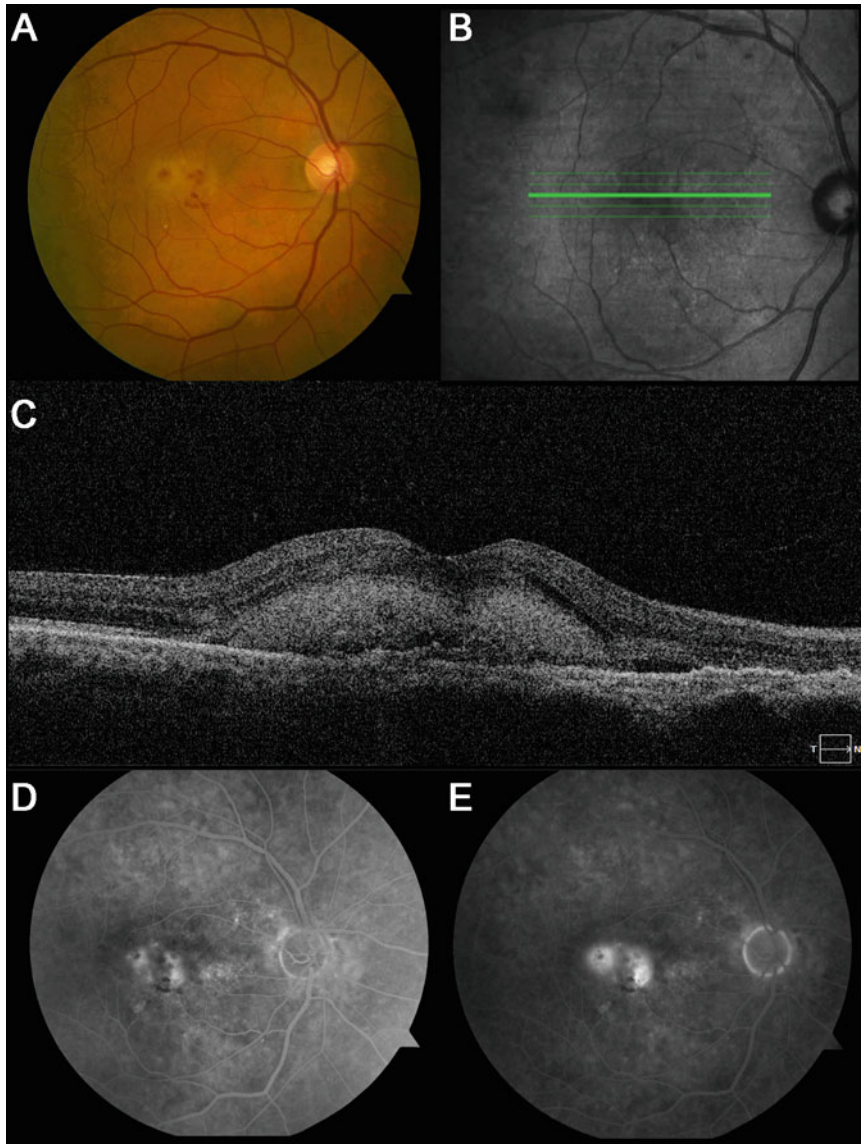

Fig. 2.1 Multimodal imaging of a patient with macular neovascularization (MNV) secondary to age-related macular degeneration in the right eye. In the color fundus photograph, (**a**) hemorrhages suggest the presence of MNV, which is confirmed by optical coherence tomography (OCT, infra-red reference (**b**) and B-Scan (**c**)). The B-Scan (**c**) shows a type 2 MNV (subretinal hyperreflective material) with subretinal fluid. The fluorescein angiogram shows a classic MNV (with some blockage from the hemorrhages) in the early phases (**d**) with evidence of dye leakage later in the exam (**e**)

2.2.3 Near-Infrared Reflectance

In AMD, most of the information needed for the evaluation of the fundus is gathered by the NIR (Fig. 2.2). Indeed, it has not been specifically investigated if using the two additional wavelengths could enhance or hide any details in the MFP image.

The NIR SLO image has little interference and absorption by media opacities and the luteal pigment in the macula providing high contrast, above all for lesions involving the fovea. Near-

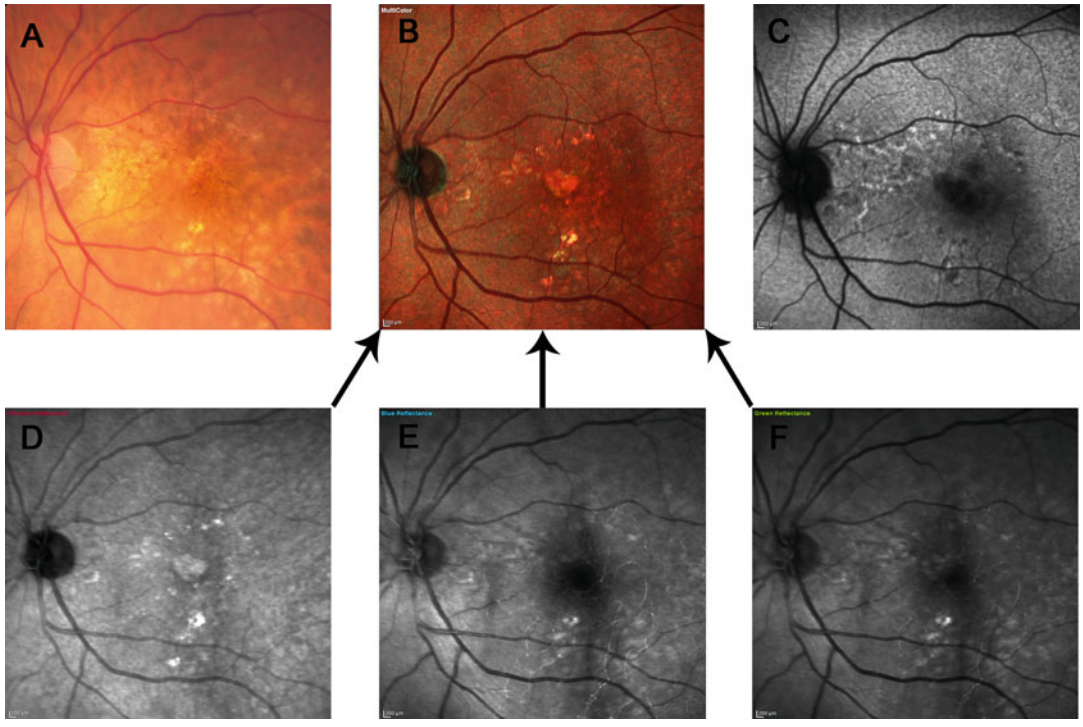

Fig. 2.2 Color fundus photograph (**a**), multicolor fundus photograph (**b**), and short-wavelength fundus autofluorescence image (**c**) of a patient with geographic atrophy and drusen secondary to age-related macular degeneration (AMD). The definition and contrast of the lesions are enhanced in the confocal image (**b**) that allows the precise identification of all atrophic foci similar to what is possible with the autofluorescence image. The confocal multicolor image is the result of the sum of three different wavelengths: near-infrared reflectance (**d**), blue reflectance (**e**), and green reflectance (**f**). Among the three, the near-infrared reflectance gathers most of the information about the (AMD) lesions as they are deep to the retina, and it seems the least affected by media opacities (floaters in this case)

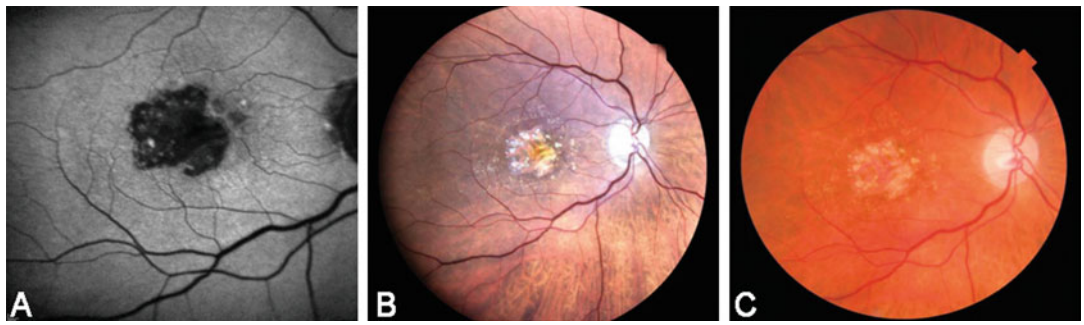

Fig. 2.3 Age-related macular degeneration patient with geographic atrophy, imaged by three different modalities: short wave-length fundus autofluorescence (**a**), confocal white-light fundus photography (**b**), and traditional color fundus photography (**c**). The confocality appears to enhance the contrast and details of the fundus image, allowing a better demarcation of the atrophic lesion

infrared light can better capture subretinal characteristics [30]; hence, NIR is the modality with the highest sensitivity for the detection of reticular pseudodrusen [20].

Three subtypes of SDD have been recently described by a recent study using multimodal imaging analysis: (1) dot pseudodrusen are usually located in the superior perifovea and appear

as small whitish deposits regularly arranged that correspond to small hyporeflective spots in NIR; (2) ribbon pseudodrusen (reticular pattern); (3) peripheral pseudodrusen are more uncommon and usually appear as yellowish aggregates that correspond to hyper-reflective spots in NIR images and are just outside the perifoveal region. Each subtype may have a specific composition, which may result in different hazards for the progression toward later stages of the disease [31].

NIR was first designed as a tool to guide the OCT acquisition; but later evolved to an independent output imaging technique (with a range of wavelength between 750 and 840 nm).

Accurate drusen assessment benefits from the use of infrared imaging as infrared light has deeper penetration in tissue and is less absorbed by media opacities, which are common in elderly patients [30]. Furthermore, the fraction of the infrared light that is scattered from the fundus may be detected using specific capture systems in "retromode" [32]. These systems are based on the different positions of the aperture which in "retromode" is not central (as in the standard systems), but it is lateral (right or left) and ring-shaped. "Retromode" imaging better highlights the deep retinal structures and the RPE [33], giving to elevated structures (e.g., drusen) a characteristic pseudo-3D "surface relief" pattern that makes their edges more visible.

2.3 Fundus Autofluorescence

Since its first description in the late 80s by Delori, fundus autofluorescence (FAF) has rapidly spread and become an invaluable tool for retinal evaluation [34]. This imaging modality allows the detection of fluorophores, which are natural molecules that interact with lights, absorbing and emitting it at specific wavelengths. The use of these specific wavelengths excites specific fluorophores that thereby become detectable. Specifically, melanin and lipofuscin can be detected using near-infrared (NIR-FAF) and short-wavelength (SW-FAF) light, respectively. Two systems are currently used to acquire FAF images: SLO systems and flash fundus camera-based systems. The former provides FAF images with enhanced contrast, resolution, and quality as they are able to suppress autofluorescence from anterior structures such as the crystalline lens. Given the unique information provided by FAF, its scope and clinical applications have expanded, and it is a particularly valuable tool for assessment of atrophic AMD.

2.3.1 Short-Wavelength Fundus Autofluorescence

Short-wavelength fundus autofluorescence has been the most well-studied FAF technique, and applications for many retinal diseases have been proposed. SW-FAF utilizes blue-light excitation (500–750 nm range), which is mostly adsorbed and emitted by lipofucsin, a dominant fluorophore located in the RPE. Pupil dilation and opacity of the media may affect the signal, influencing SW-FAF imaging quality. Furthermore, macular pigments intercept blue light, resulting in a drop in the intensity of the signal at the level of the fovea.

In SW-FAF, the intensity of the signal is mainly influenced by the quantity of lipofuscin. The alterations may be hypo-, iso-, or hyper-autofluorescent [35]. In the early stages of dry AMD, SW-FAF can demonstrate a larger affected area in comparison to color photography or fundoscopy. Pigment abnormalities on ophthalmoscopic exam may correspond to either hypo or hyper-autofluorescence based on their lipofuscin content [36]. Depigmented, hypoautofluorescent spots correlate with RPE atrophy, an early finding of GA [36, 37]. Recently, the International FAF Classification Group described eight different SW-FAF phenotypes that could be associated with early dry AMD: normal, minimal change, focal increase, patchy, linear, lace-like, reticular, and speckled [38]. These patterns may have clinical relevance as they may predict the development of late AMD stages and in particular choroidal neovascularization. Previous studies have suggested that the patchy, linear, and reticular patterns have the strongest correlation with progression to neovascular AMD [39, 40].

Similar to fundus photographs, drusens' features may widely vary in FAF imaging, according to size, composition, or condition of the overlying RPE and ellipsoid zone (EZ) [19, 41]. Large drusen may produce FAF alterations, whereas smaller drusen can appear iso-autofluorescent and may not be detected [42]. Intermediate drusen (diameter ranging between 63 and 125 μm) show a typical central hypo-autofluorescence surrounded by a ring of hyper-autofluorescence corresponding to the condition of the overlying RPE [43]. Cuticular and crystalline drusen appear hypo-autofluorescent with FAF [19] while soft confluent drusen tend to appear as hyper-autofluorescent lesions [42]. It should be highlighted that although these manifestations are the most common, drusen can show a plethora of FAF patterns.

Fundus autofluorescence may aid in the identification of two important features of high risk progression that could be problematic for detection by CFP or MFP: (1) drusenoid PED show a typical appearance of hyper-autofluorescent spots with a hypo-autofluorescent halo, but may be characterized by intermediate to decreased signal in case of overlying RPE atrophy or fibrovascular scarring [44]; (2) subretinal drusenoid deposits [20] were first reported as dot-like lesions by blue light photography and may be better visualized by SW-FAF, NIR-FAF, or OCT [44, 45]. These usually show the appearance of small and round, elongated foci of hypo-autofluorescence connected by interspersed reticular pattern of hyper-autofluorescence [46].

In general, early choroidal neovascularization may be not visualized on SW-FA, as RPE and photoreceptor layers are often relatively intact [47]. Subsequently, both types 1 and 2 MNVs may appear hypo-autofluorescent for different reasons: in type 2 MNV, the hypo-autofluorescence may be the effect of light blockage by the fibrovascular complex overlying the RPE in the subretinal space (Figs. 2.4 and 2.6); in type 1 MNV, the hypo-autofluorescence may be owed to the overlying RPE atrophy (Fig. 2.4) [48].

Macular neovascularization shows a hyper-autofluorescent halo in 38% of cases that may be due to a window defect secondary to the photoreceptor loss or to an RPE proliferation [39]. Hemorrhages and exudates may vary their appearances according to their age: at first they absorb light, appearing hypo-autofluorescent, but then when they become organized, they may appear hyper-autofluorescent.

Advanced lesions with RPE atrophy (GA), with the local loss of lipofuscin, produce areas with a low to extinguished SW-FAF signal. Given the high contrast between the areas with intact and atrophic RPE, the border of these lesions are typically sharply demarcated (Figs. 2.2 and 2.3) allowing semiautomated or even automated measurements of their area [49]. Nevertheless, there are some important limitations: (1) when the borders of the lesion are close and/or involve the fovea, they may be difficult to visualize due to the interference by macular pigment; (2) inside the atrophy, there may be some residual SW-FAF signal, preserved by retained RPE cells or debris and basal laminar deposits [50, 51]; (3) a halo of hyper-autofluorescence may surround the GA lesions, indicating the presence of ongoing RPE cell dysfunction or vertically superimposed RPE cells. This halo was associated with variable levels of atrophy expansion [52, 53] and has been reported with various appearances: none, focal, diffuse, banded, and patchy. The diffuse and banded phenotypes are associated with a faster rate of atrophy enlargement.

Another challenge is to distinguish GA and MNV when both coexist, and a multimodal approach is often required to discriminate atrophy, fibrosis, hemorrhages, or hard exudates [54]. Indeed, a combined analysis of SW-FAF and NIR images and FA may help achieving a precise identification and assessment of the atrophy [54, 55].

The strong interest in SW-FAF imaging in the clinical and research environments is clearly justified by its ability to precisely assess atrophy in AMD and potentially predict its progression [56]. In fact, current clinical trials on AMD use SW-FAF to measure GA not only for the demonstrated reliability of the measurements [57], but also for its correlation with the visual

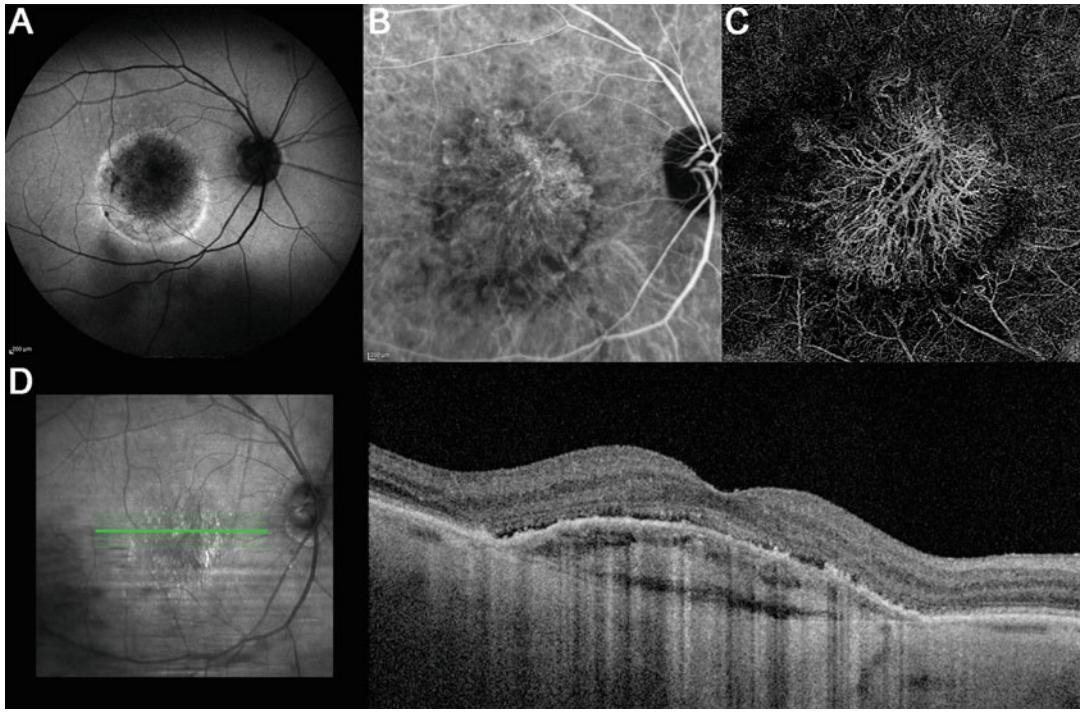

Fig. 2.4 Multimodal imaging of a patient with a type 1 macular neovascularization (MNV) secondary to age-related macular degeneration. The short wavelength autofluorescence (**a**) shows a round hypo-autofluorescent lesion (probably due to the subretinal fluid and overlying RPE atrophy) surrounded by a hyper-autofluorescent ring. Indocyanine green angiography (**b**) shows the neovascular network that perfectly corresponds to the enface optical coherence tomography (OCT) angiography image (**c**). OCT B-scan shows the location of the neovascular plexus between the retinal pigment epithelium and the Bruch's membrane

function; that is, when the autofluorescence signal is absent, it is correlated with a loss of retinal sensitivity [58, 59]. It should be noted, however, that in case of MNV, this assumption may not be completely accurate. Nevertheless, confluent patterns of autofluorescence loss in central macula are strongly related to visual acuity, contrast sensitivity, and reading speed in eyes treated with anti-VEGF agents for neovascular AMD [60, 61].

2.3.2 Near-Infrared Fundus Autofluorescence

Confocal SLO instruments can integrate multiple sources of light with different wavelengths, with a lower sensitivity to optical media opacities and macular pigment than SW-FAF. Confocal NIR-FAF (830 nm) is able to detect the melanin of the RPE and also, to some extent, melanin in the choroidal layers [62]. This has been confirmed by multiple animal and donor human eye studies [63]. In normal subjects, NIR-FAF is characterized a central area of high signal in correspondence of the foveal region, where the RPE are taller and thus have a higher concentration of melanin. This area of higher NIR-FAF corresponds to the physiologically reduced central SW-FAF [63, 64].

In early AMD, the correspondence between NIR-FAF and SW-FAF is not absolute, depending on the relative amount of lipofuscin and melanin in each lesion [65]. In eyes with neovascular AMD, the predominant finding is the blocking of NIR-FAF and SW-FAF by subretinal blood or MNV. The main difference is in areas of exudation activity where SW-FAF is

typically increased, whereas NIR-FAF is usually decreased [65].

For GA, the application of NIR-FAF can be complimentary to the SW-FAF due to some important differences: (1) the area of hypo-autofluorescence corresponding to GA atrophy is usually significantly larger in NIR-AF compared to SW-AF [65, 66]; (2) when the lesion is close to the fovea, SW-FAF might not have enough contrast for a distinct delineation of the borders, due to the masking effect of the macular pigment; (3) visualization of the borders of the lesion in NIR-FAF may also be difficult in pigmented individuals where the higher melanin signal from the choroid can reduce contrast [62]; (4) the fluorescence of the borders might be different between the two modalities: an iso-SW-FAF border may correspond to a hyper-NIR-FAF border—these areas are thought to have a persistent photoreceptor layer over an already damaged RPE, suggesting that NIR-FAF may detect areas of damaged RPE/photoreceptors cells earlier than SW-FAF [66].

Thus, optimally, a combination of both NIR-FAF and SW-FAF imaging should be implemented to detect and monitor morphological and functional RPE in AMD, especially in eyes with GA.

Several SLO and fundus camera systems implemented other wavelengths to investigate the retina; however, very few of them have been properly validated (i.e., 532 nm fundus cameras or 514 nm SLO systems) [67, 68]. A potential advantage of green FAF over blue FAF is that it is of lower energy and generally more comfortable for patients. The lower energy may also offer theoretical safety benefits, particularly in diseases eyes that may be more susceptible to light toxicity—however, this hypothesis requires careful study. A recent study compared green FAF versus blue FAF in measuring GA lesions, finding a slightly higher reproducibility and accuracy of measurements for green FAF. A post-hoc analysis related the inter-reader differences to the opacification of the media, which has a higher impact on the quality of the blue FAF compared to green FAF, hence affecting the precise grading of the lesions [69].

2.3.3 Color Autofluorescence

Recently, a confocal blue-light FAF device (CentervueEidon) using a 450 nm wavelength and a light-emitting diode (LED) light source has been introduced. The 450 nm wavelength is thought to excite different fluorophores from the ones excited with the classical 488 nm [70]. As this device is equipped with a color sensor, the full-spectrum of the emitted light can be detected, resulting in a "color FAF" image. This complete emission spectrum can be divided into long-wave and short-wave emission fluorescence components (EFC): "red" (560–700 nm) and "green' (510–560 nm). The evident advantage is that minor fluorophores, whose emission is usually overwhelmed by major ones (e.g., lipofuscin), could be isolated and studied as they emit in the shorter wavelength end of the spectrum (green EFC) [71, 72]. For example, in GA, while there is an absence (or major reduction) of the high red EFC component coming from lipofuscin, the green EFC signal is still present, even if diminished, and it seems to originate from subretinal hyper-reflective material. It is possible that drusen-like metabolites with highly glycated products have fluorescent capability and could be the source of this signal (Fig. 2.5) [72].

Thus, it is evident that 450 nm FAF imaging may yield further insights into the pathologic processes behind AMD pathogenesis and progression. However, more work is necessary to define the role of color FAF on the assessment and prognosis of AMD patients.

2.3.4 Quantitative Fundus Autofluorescence

While SW-FAF allows qualitative evaluation and quantitative measurements of areas of definite hypo- or hyper-autofluorescent alteration, traditional SW-FAF approaches do not allow the absolute FAF intensity to be quantified. A methodology that overcomes this limitation is quantitative autofluorescence (qAF), which employs an internal fluorescent reference to calculate the intensity of the autofluorescence of the

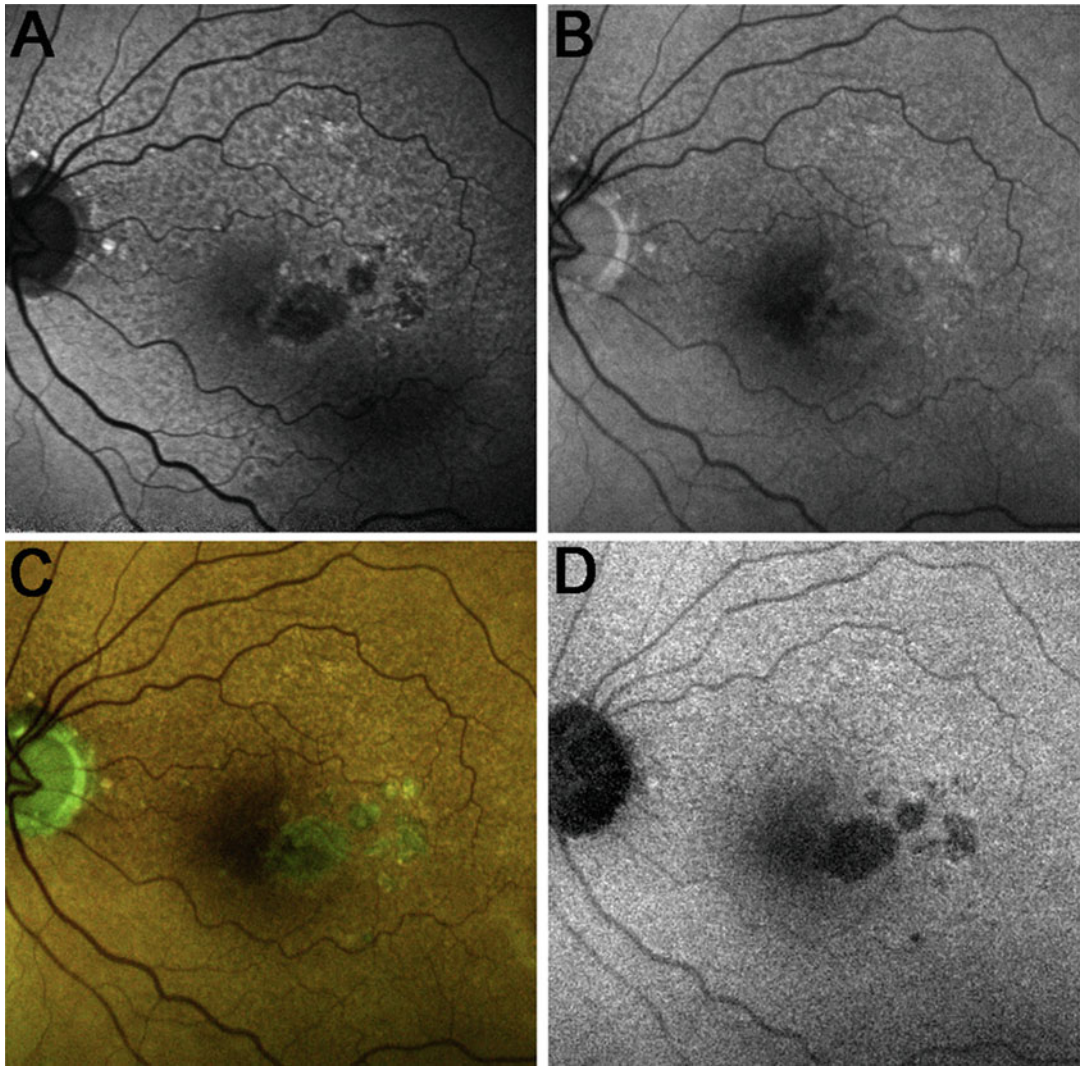

Fig. 2.5 Fundus autofluorescence images from an eye with geographic atrophy secondary to age-related macular degeneration. The 488 nm fundus autofluorescence (FAF) image (**a**) shows well demarcated areas of atrophy that are not visible in the 450 nm FAF (**b**). The color FAF (**c**) shows that in these areas the green emission component (probably due to subretinal deposits) is still present while the red emission component (isolated in **d**) is absent in atrophic regions (due to reduction/absence of lipofucsin)

fundus [73]. Normative studies validated this modality and reported that qAF intensity increases with age, and also vary with sex (higher in women) and ethnicity (higher in whites and Hispanic) [71, 72]. Of note, foveal and perifoveal qAF values are inversely correlated with macular pigment measurements [71, 72]. Obtaining high quality, reliable qAF images does require careful attention to technical details such as uniform illumination, adequate bleaching, optimal focus, and central alignment of the camera with the pupil [73–75].

qAF techniques may have important techniques in inherited retinal diseases, many of which (Stargardt's pattern dystrophies, Best disease) feature accumulations of lipofuscin, and in some cases may be confused with AMD [76–78]. In addition, qAF is being studied in the

setting of AMD, and qAF findings are being correlated to functional measurements (e.g., from electrophysiology and microperimetry) [79]. Large, longitudinal studies are still needed.

2.3.5 Fluorescence Lifetime Imaging Ophthalmoscopy

Fluorescence lifetime imaging ophthalmoscopy (FLIO) uses a blue laser light impulse to excite retinal fluorophores and measure their time span of emission, which is independent from the signal intensity. This parameter is specific for each molecule, depending on its structure and interactions with the local metabolic environment [70]. The latter can change very early during degenerative processes, revealing information about the integrity of RPE and photoreceptors before these changes can be seen by standard imaging modalities [80, 81]. In AMD, fluorescence lifetimes are prolonged compared to healthy subjects [82, 83]. In GA, areas of complete outer retinal and RPE atrophy (cRORA) have longer lifetimes compared to areas where there are surviving photoreceptors segments. This is probably related to fluorophore emission from the connective tissue components and the underlying choroid.

This is a relatively innovative field and more studies assessing metabolic alterations in the pathological retina are required and FLIO can help in supporting this analysis, including in dry AMD.

2.4 Dye-Based Angiography

Dye-based angiography of the retinal fundus consists of two main methodologies according to the type of exogenous fluorphore injected intravenously in the patient for the examination: fluorescein and/or indocyanine green. These fluorophores absorb and emit light at specific wavelengths that can be detected by the camera systems using distinct filters. Several clinical studies have validated the use of SLO systems and flash fundus camera-based systems in both neovascular and dry AMD. Fluorescein angiography and indocyanine green angiography may be performed separately or in combination depending on the diagnostic concern [46].

2.4.1 Fluorescein Angiography

Over the past decades, the diagnosis and grading of MNV were based on fluorescein angiography [84, 85]. It requires the intravenous injection of fluorescein, an organic molecule that shows emits fluorescence when exposed to short-wavelength light (465–490 nm). FA aids in the characterization of numerous retinal abnormalities, such as drusen, vascular alterations, and neovessels. FA is better used to visualize retinal vascularization as the melanin in the RPE absorbs both the exciting and the emitted light on FA. However, wherever the RPE is absent or shows less pigmentation, the CC and choroidal vessels may be seen.

Drusen can be easily visualized by FA but often require the complementary acquisition of other imaging modalities (such as OCT) to distinguish the subtype.

In general, staining properties of soft drusen on FA vary depending on the status of the overlying RPE and the quality of their content [19]. For this reason, they could range from hyper- to hypofluorescent, particularly in the early phases of the angiogram. Given their cross-sectional triangular shape, cuticular drusen have a significant RPE attenuation at the apex and compacting of RPE at the base. This leads to an inverse pattern of presentation between FA and FAF: in FA cuticular drusen show a pinpoint hyperfluorescence centrally ("starry sky" or "milky way" pattern), while in FAF images the apices are hypo-autofluorescent. SDD are hard to visualize on fluorescein angiography, showing absent or minimal fluorescence [86].

The guidelines for the acquisition of FA and the criteria for the identification of MNV secondary to AMD on FA were systematically defined in 1991 with the Macular Photocoagulation Study [84] that distinguished between classic and occult neovascularization. Shortly thereafter another type of neovascularization was identified as a

neovascular process starting from the retinal vasculature and characterized by retinal–retinal or retinal–choroidal anastomosis. The characteristics of each subtypes became more clear with the advent of depth-resolved imaging (such as OCT), leading to a more "modern" classification: type 1 (former "occult") MNV, type 2 ("former" classic) MNV, and type 3 (former "retinal angiomatous proliferation") MNV [3, 87, 88].

Type 2MNVs occupy the subretinal space and generally correspond to classic MNV on FA. Classic MNV is characterized by an area of bright, well-demarcated hyper-fluorescence evident in the early phases of the angiogram. In later phases, progressive leakage and pooling of dye in the overlying subsensory retinal space leads to obscuration of the boundaries of the MNV (Figs. 2.1 and 2.6) [84, 89].

Type 1 MNVs occupy the sub-RPE space and generally correspond to occult CNV on FA. Occult CNV is characterized by areas of irregular elevation of the RPE (fibrovascular PEDs) that may not be as well-demarcated or as bright as areas of classic MNV in the transit phase of the angiogram. Within 1–2 min after fluorescein injection, an area of stippled hyperfluorescence is usually apparent. By 10 min after injection, there is persistent fluorescein staining or leakage within a sensory retinal detachment overlying this area [84, 89]. The exact boundaries of fibrovascular PEDs can be determined only when fluorescence sharply outlines the elevated RPE, although frequently,

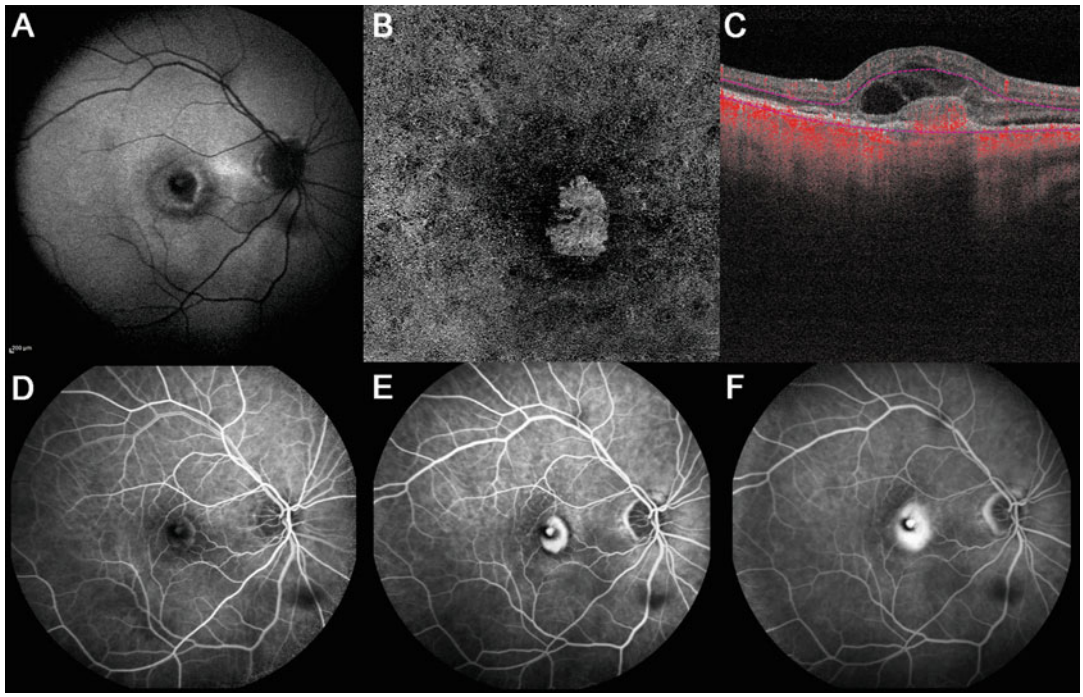

Fig. 2.6 Multimodal imaging of a patient with type 2 macular neovascularization secondary to age-related macular degeneration. Short-wavelength autofluorescence (**a**) shows a hypoautofluorescent area due to blockage by hemorrhage and the neovascular lesion which is positioned above the retinal pigment epithelium (as seen on the optical coherence tomography [OCT] B-scan (**c**)) On *en face* OCT angiography (**b**), the network of vessels is clearly visible and surrounded by a ring of choriocapillaris flow attenuation. The same MNV network is visible by fluorescein angiography, especially during the early (**d**) and late transit phases (**e**). The leakage of dye in the late phase (**f**) makes it more difficult to distinguish the borders of the lesion

the intensity of fluorescence at the boundary of the elevated RPE is quite irregular, with some areas fading relative to the fluorescence of the remaining areas of elevated RPE, making it difficult to distinguish the boundaries of fading fluorescence of the occult MNV from the fading fluorescence of the surrounding RPE. Furthermore, the borders of elevated RPE often slope gradually downward to surrounding flat RPE so that the demarcation between elevated RPE and flat RPE cannot be determined with certainty. Fibrovascular PEDs should not be confused with typical, classic serous detachments of the RPE [90, 91] In the latter, there is usually a uniform, smooth elevation of the RPE with early, sharply demarcated, fairly uniform hyperfluorescence that persists in the late phase of the angiogram [90, 91].

Type 3 MNV may be more difficult to distinguish by FA alone. In the early phases of the angiogram, it shows a leakage often in close proximity to retinal vessels that then becomes intense in late phases, often with cystoid macular edema. Sometimes, in the very early phases it is possible to visualize the retinal–retinal anastomoses. Nevertheless, to reach a sufficient level of confidence for the diagnosis of this type of MNV, ICGA, and/or OCT are often required [3, 89, 92, 93].

The distinction of the specific forms of neovascular AMD is important because some of them show a more severe progression of the disease, critically influencing patient prognostication and the decision-making process for treatment. For instance, retinal angiomatous proliferation (RAP) can present with intraretinal hemorrhage, lipid exudates, and edema in the retinal layers [89, 94]. Furthermore, it is the one with the highest rate of progression to atrophy, with poorer visual outcomes over time [95].

It should be noted that many MNV lesions can feature mixtures of these MNV subtypes that can result in peculiar phenotypeson FA [89].

Other angiographic findings associated with MNV may interfere with the visualization of the lesion boundaries: (1) hemorrhages, hyperplastic pigment, or fibrous tissue contiguous with the MNV may obscure the normal choroidal fluorescence; (2) a serous PED cause an early bright uniform hyperfluorescence that may obscure the fluorescence from MNV. The presence of any of these features can make it impossible to accurately determine the full extent of the MNV [84].

FA can be used for the imaging of GA lesions and is often included in clinical trials in order to rule out the presence of MNV [96, 97]. The presence of leakage associated with MNV may blur the edges of the GA lesion [98, 99] (Fig. 2.7).

Drawbacks of the procedure are its invasiveness, the relatively long time required for capturing the late-phases and the discomfort of patients. Other less invasive methodologies may be preferable alternatives for the differential diagnosis of GA. Furthermore, the risk of severe allergic reaction to intravenous injection of the dye should always be considered.

2.4.2 Indocyanine Green Angiography

Indocyanine green angiography (ICGA) utilizes near-infrared fundus illumination that allows better visualization of deeper structures (e.g., choroid). As this molecule has a higher affinity with plasma proteins than fluorescein, it does not leak from the tiny capillaries in the normal CC; hence, it improves the visualization of the deeper vessels of the choroid [100]. ICGA can be useful for identification of type 3 MNV, revealing a hyperfluorescent spot corresponding to the early angiomatous lesion, which over time may extend from the deep capillary plexus, toward the choroid [100].

ICGA allowed the initial understanding of another form of neovascularization otherwise difficult to recognize by FA alone: the aneurysmal type 1 neovascularization (or polypoidal choroidal vasculopathy) (PCV) [92]. These terms describe the occurrence of aneurysmal lesions that develops from type 1 neovascular networks.

Taking advantage of this better visualization of the choroidal vessels, in 1992, Spaide et al. described PCV as a hyperfluorescent vascular network that forms a “plaque,” masking the

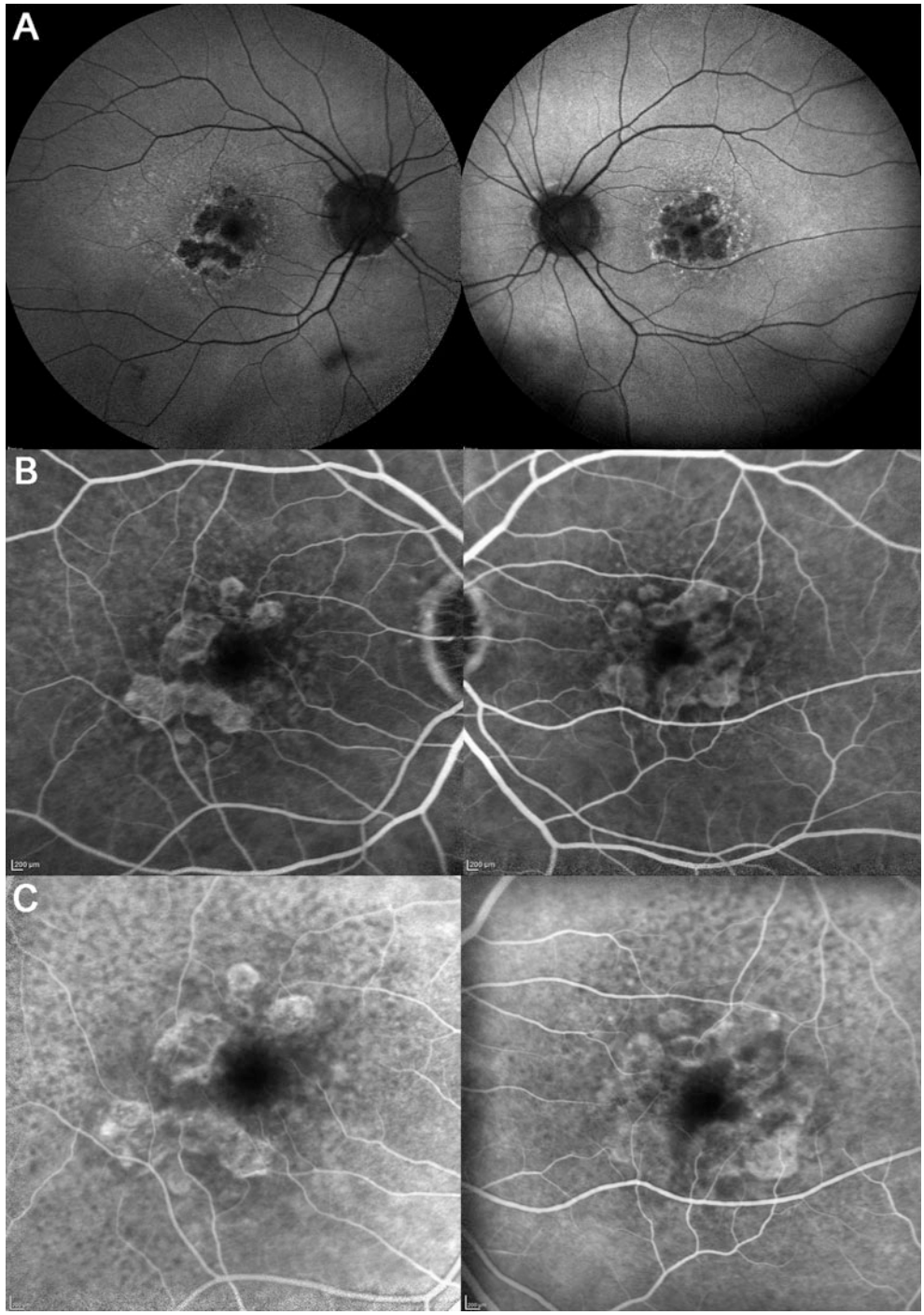

Fig. 2.7 Short-wavelength fundus autofluorescence (**a**) and fluorescein angiography (**b**, **c**) of a patient with bilateral geographic atrophy secondary to age-related macular degeneration. The borders of the lesions are clearly visible during the early phases of the angiogram (**b**) but become less well-demarcated during the late phases (**c**) because of dye leakage and staining

underlying CC and polyps themselves. The latter are evident as hyperfluorescent structures in the early phases of the ICGA, since they subsequently stain and leak from their walls [101].

Only after the introduction of OCT, was the actual depth of these lesions definitely determined [60, 88, 102]. Of note, it is still controversial as to whether PCV is a form of AMD or not [92, 103]. Indeed, there are several differences between PCV and AMD. Peculiarities of PCV are: (1) a thicker subfoveal choroid; (2) the relative absence of classical, and when drusen are present, have an unusual shape [104]; (3) it is more prevalent in African populations (in whom other forms of AMD-related MNVs are uncommon); (4) patients are usually younger; and (5) the development of polypoidal lesions.

ICGA can also aid in the discrimination of atrophic lesions of different origins. For instance, in late-onset Stargardt disease, atrophic areas are not stained by the dye ("dark atrophy"), whereas in AMD, a late staining of the lesions usually occurs [105]. The interest toward the choroid; involvement in the pathogenesis and progression of AMD is particularly high, hence its imaging can be a valuable tool to study and assess it accurately [105–107]. ICGA is an invasive method with the same limitations as FA with regards to imaging time and the risk of allergic reactions.

2.5 Widefield Imaging

Compared to other conventional imaging modalities, widefield imaging can visualize a larger retinal field. The field of view may be extended to >100° using internal or external lenses. Nevertheless, a wider view corresponds to lower resolution and contrast. The SLO modality drives a big proportion of widefield imaging machines, allowing the capture of reflectance images, FAF, or FA and ICGA on the same device. These devices do not use white light, but a mix of discrete laser sources, which are then combined, giving a "false color" image.

A system equipped with an internal ellipsoid mirror (Optos devices; Optos, Dunfermline, UK) can achieve up to a 200° field of view, which covers >80% of the ocular fundus. However, the use of the ellipsoid mirror creates distortion in the periphery of the acquisition; furthermore, the view may be vertically limited by lid and eyelash artifacts [108].

Alternatively, a contact lens may be implemented on an SLO machine in order to achieve a field of view of up to 150° [109]. The use of a contact lens requires a trained operator and can be particularly influenced by the presence of lens opacities [109]. Recently, an ultra-widefield confocal system providing a 105° of view with a noncontact lens attached to the camera has been introduced (Heidelber Engineering, Heidelberg, Germany) [110]. The smaller field of view allows higher contrast without lid and eyelash artifact [110].

Finally, a new color fundus camera with widefield acquisition has become available. This machine (Zeiss Clarus 500; Carl Zeiss Meditec, Dublin, CA) offers the advantages of providing a "true color" image of the fundus with a field of view of 133° in one single image and up to 267° by montage of six pictures. Features graded on standard color photos and widefield image can yield similar findings [111]. Widefield imaging introduced the unprecedented possibility to study, document, and follow the peripheral anomalies associated with AMD [112]. These abnormalities have been noted in >70% of eyes with AMD and include a variety of features such as peripheral drusen, RPE depigmentation, peripheral reticular pigmentary degeneration (PRPD), and/or atrophic patches (Fig. 2.8). It has been suggested that some of these peripheral features may be associated with a worse prognosis (such as PRPD [113, 114]). The natural history of these peripheral lesions and the influence they have on the natural course of the disease require further investigation [115, 116].

2.6 Optical Coherence Tomography

Over the last two decades, the availability of OCT has dramatically transformed ophthalmology,

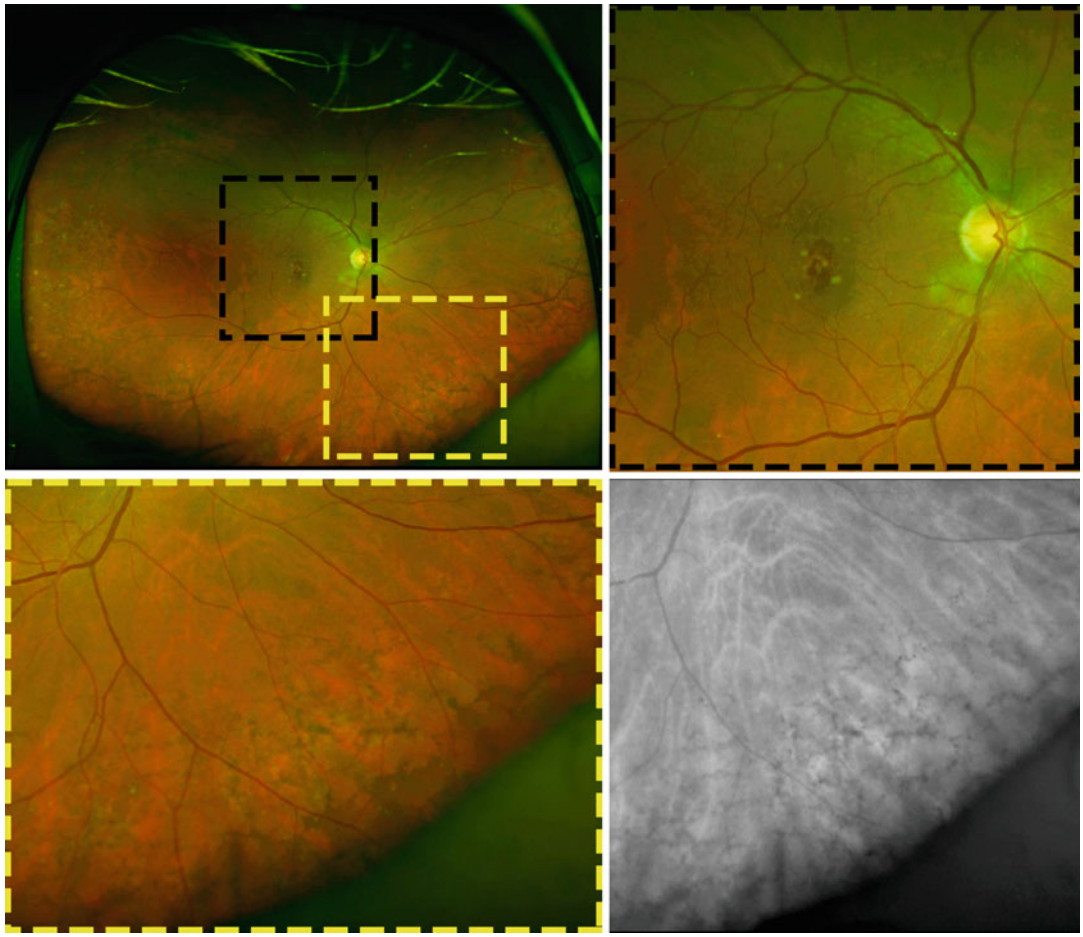

Fig. 2.8 Widefield fundus image (top left) of a patient with dry age-related macular degeneration. The presence of lid artifacts limits the view of the superior field. A magnification of the central field (black box inset on top left → top right) allows better visualization of the drusen in the macular area. Peripheral alterations such as drusen, retinal pigment epithelium depigmentation, and peripheral reticular pigmentary degeneration (PRPD) can be easily seen. The magnification of the infero-nasal field (yellow box, bottom left) highlights the presence of the PRPD, particularly evident in the infrared reflectance image (bottom right)

especially for retinal disease diagnosis and assessment. OCT uses infrared light (with high penetration properties) to measure different backscatter from biological tissues, yielding micrometer-resolution images [117]. OCT imaging can provide depth resolution inside tissues and characterize its structure based on their degree of reflection/scattering of light. Similar to ultrasound imaging, OCT captures multiple images on an axial plane (A-scan), which, when summed up on the transverse plane, provide cross-sections of the tissue (B-scan). Volumetric information is generated by sequentially acquiring multiple B-scans that are displaced perpendicular to the B-scan image, covering an entire region of the retina [117].

Unlike other imaging modalities, OCT produces in vivo cross-sections and en face images of the retinal and subretinal structures [118].

Three different generations of OCT machines have been brought to the market [119–121]. The first OCT device was based on time-domain (TD) technology, which used a light beam

calibrated on a 840 nm wavelength. This device could acquire 400 A-scans every second, and provide images with an axial resolution up to 10–15 μm. Spectral domain (SD)-OCT was the next generation of devices which were introduced and achieved faster scanning speed (25,000–85,000 A-scans per second) and improved resolution (4–7 μm). Enhanced-depth imaging (EDI) SD-OCT yields a higher penetration power and resolution for the imaging of the choroid [122]. Finally, the introduction of swept source (SS)-OCT provided a greater penetration of the choroid, exploiting a light source with wavelength around ~1050 nm. SS-OCT available devices can capture 100,000 A-scans per second, with an axial resolution of 6–8 μm, and can provide B-scans of >20 mm in length. *En face* OCT imaging is commonly used to examine the macula, providing retinal and choroidal sections on the coronal plane (C-scan), which is aligned with the RPE profile as reference.

Eye-tracking systems use anatomic features to align acquisitions from different sessions (useful for follow-up) and facilitate the acquisition of scans in eyes with poor stability of fixation. A single B-scan may be acquired quickly while a dense raster pattern of line-scans ("volume scan") often requires more time. Nevertheless, volume scans with sufficiently small spacing between consecutive B scans are necessary to elaborate the C-scans, which allow the mapping of the macula and the localization of specific features, such as drusen, reticular pseudodrusen, and pigment migration into the inner retina. In early and intermediate stages of AMD, the high-resolution of the OCT scans provides an excellent visualization of the morphology of drusen and the overlying RPE and neurosensory retina [123].

Small (hard), medium, and large (soft) drusen appear as deposits of hyper-reflective material between the RPE basal lamina and Bruch's membrane (Fig. 2.9) [19].

The coalescence of large drusen may lead to a drusenoid PED. OCT imaging can identify the presence of PED, showing a dissociation between the RPE and the Bruch's membrane [44]. The inner contour of the PED (equivalent to the RPE band) usually appears smooth and undulating. OCT can supply useful information on the dimension of drusen (i.e., height, area, and volume), but also regarding their shape and internal reflectivity, as well as the integrity of the overlying RPE. Several studies have reported that drusen show a dynamic appearance, with their volume increasing and decreasing cyclically [123, 124]. Several prognostic factors associated with drusen could be easily evaluated by OCT. A greater risk of progression to focal atrophy has been associated with (1) greater heights of drusenoid lesions or PED [123]; (2) drusen regression [125]; (3) internal heterogeneous reflectivity or hyporeflective drusen cores (Fig. 2.9) [123, 126]; (4) finally, mineralized drusen with refractile deposits (supposedly calcified or mineralized lipid material), which may be a form of drusen in regression [127]. Cuticular drusen are small drusen that demonstrate a characteristic "sawtooth" pattern on OCT (small, triangle-shaped, hyporeflective inside) [19]. Finally, subretinal drusenoid deposits (which correlate with reticular pseudodrusen on FAF) have been classified using OCT in three different stages: stage 1 shows a wavy or ribbon-like EZ, in stage 2 the EZ deflects inward as a result of the deposit, while stage 3 features the interruption of the EZ and the inward deviation of the external limiting membrane [20].

Several studies have shown that reticular pseudodrusen are associated with a higher risk of developing late AMD [128, 129] and atrophic degeneration of the outer retina. Nevertheless, it is still debated if reticular pseudodrusen may forecast the rate of future growth of the lesions, as their presence usually anticipate where GA would develop and the risk of multifocal lesions [122].

Other AMD-related features that OCT may help to identify in the earlier stages are pigmentary alterations: choroidal hypertransmission (i.e., increased light transmission through the choroid because of the overlaying retinal atrophy) may help identify focal areas of RPE loss or depigmentation [130]; pigment clumping and migration are visualized in OCT as outer retinal hyper-reflective foci. The latter have been related

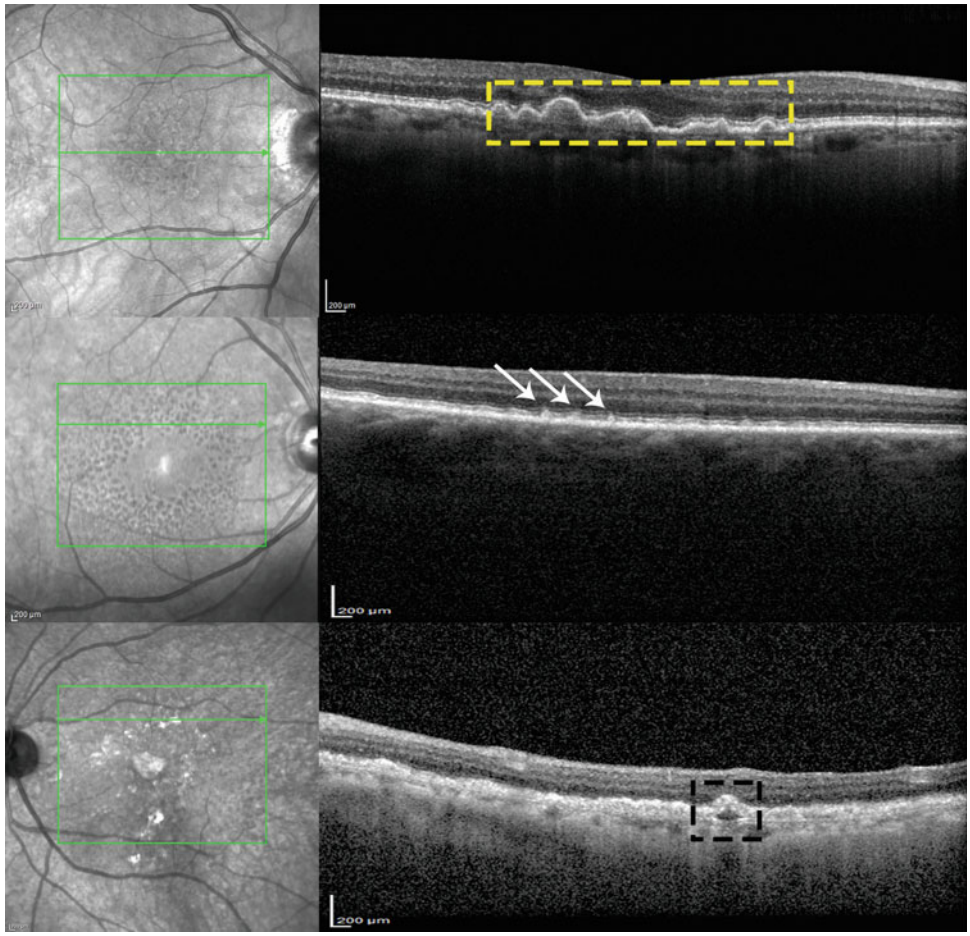

Fig. 2.9 Infrared reflectance (IR) and optical coherence tomography B-scans of three patients with dry age-related macular degeneration. The first subject (first row) shows soft confluent drusen (yellow box) close to the foveal center; the second patient (second row) shows a ring-like pattern of reticular pseudodrusen on the IR image with corresponding subretinal drusenoid deposits (white arrows) on the OCT B-scan. The third patient (bottom row) has focal areas of geographic atrophy associated with hyporeflective drusen (black box)

to RPE atrophic degeneration and associated with a higher probability for focal atrophic lesions to develop and progress [123, 129, 131, 132].

The clinical relevance of OCT is also important in the later stages of AMD (both GA and MNV), and its structural findings are often included in clinical trials as primary and/or secondary endpoints for treatment success.

OCT can identify exudative AMD at an early stage. Type 1 MNV is localized under the RPE, constituting a vascularized fibrovascular or serous PED. Serous PEDs are usually smooth regular dome-shaped hyporeflective RPE elevations. Fibrovascular PEDs are filled with a medium-to-high reflective material organized in multiple layers that are separated by hyporeflective spaces (Fig. 2.4).

Active type 1 MNV is often accompanied by subretinal fluid (SRF) that accumulates between the retina and the RPE and appears hyporeflective. Intraretinal fluid (IRF) is less common for type 1 MNV (generally present with older, more chronic lesions) and appears as round, hyporeflective, cystoid spaces within the retinal layers. Not all these spaces are indicative of exudation, and persistent cystoid spaces

despite therapy, particularly with less associated retinal thickening, may be a feature of retinal degeneration [3, 89].

Type 2 MNV is localized in the subretinal space, directly above the RPE (Figs. 2.1 and 2.6). It is frequently associated with retinal thickening, SRF, IRF, and PED [133].

Type 3 MNV is typically preceded by the migration of RPE cells (intraretinal hyperreflective foci) into the retina. These RPE cells may locally secrete VEGF and promote the development of the NV lesion at the level of the deep capillary plexus. The NV lesion may then grow downward and reach the sub-RPE space through a gap in the RPE monolayer that is commonly present. OCT features that aid in the identification of Type 3 NV include: (1) a gently sloping dome-shaped or trapezoid-shaped PED without an obvious peak and with a "flap" sign; (2) a focal funnel-shaped defect in the RPE, called the "kissing sign"; (3) presence of IRF and often (but not always) absence of SRF [93, 134] (Fig. 2.10).

In aneurysmal type 1 neovascularization (or PCV), the branching vascular network appears as a fibrovascular PED, while the aneurysmal lesions themselves appear as a PED with sharper bumps, often correlating with an internal rounded hyporeflective area (representing the polyp lumen) and exudative findings [92, 135].

To date, OCT is the gold-standard imaging technique for assessing exudative AMD over the long term and has largely replaced CFP and FA for monitoring the activity of the disease thanks to its higher sensitivity. Serial consecutive assessments of macular thickness and morphology allow the evaluation of the response to treatment. It is particularly valuable in case of individualized or evaluation-based, as-needed therapy (Pro Re Nata, PRN), which is one of the two treatment regimens commonly employed in practice. The presence of any fluid generally guides the decision to retreat patients [96, 136]. Structure–function correlation has identified OCT biomarkers that are commonly associated with reduced vision: IRF at baseline and persistent cystoid spaces at the end of the loading dose (independently from the agent and regimen chosen) [137]. Whenever IRF is present initially, best corrected visual acuity (BCVA) and the gain in BCVA may be reduced compared to eyes with only SRF [137].

OCT is also currently used for a precise assessment of GA. Its depth resolution characteristics yield precise measurements and evaluation of the single layers of both the retina and choroid [138], and may facilitate the detection of early or nascent atrophy before it may be detected by CFP or FAF.

Recently, an OCT-based classification of macular atrophy was proposed by a consensus of retinal specialists and image reading center experts through the Classification of Atrophy Meetings (CAM) program [130]. Four terms

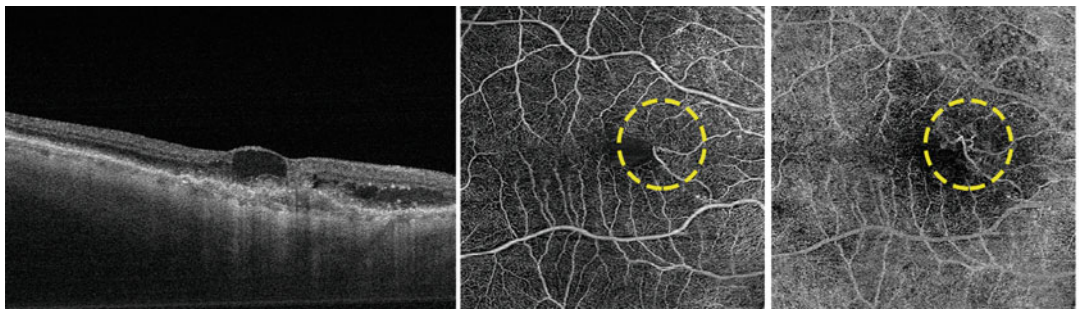

Fig. 2.10 Optical coherence tomography (OCT) and OCT angiography of type 3 macular neovascularization. The B-scan (left) shows a trapezoid-shaped pigment epithelial detachment with the typical "kissing sign" and the presence of intraretinal fluid. On OCT angiography, this type of neovascularization can be visualized as a discrete high flow linear structure (yellow dotted circle) extending from the middle retinal layers (middle) into the deep retina (right)

were proposed to describe atrophy in the setting of AMD: "complete RPE and outer retina atrophy (cRORA)", "incomplete RPE and outer retinal atrophy (iRORA)", "complete outer retinal atrophy (cORA)", and "incomplete outer retinal atrophy (iORA)".

GA was defined to be a subcategory of cRORA with no MNV, whereas cRORA is identified as a condition of macular atrophy with or without MNV. Nascent GA [139] is considered as a subcategory of iRORA with no MNV. The cRORA is defined by the concomitant presence of three specific OCT criteria: (1) an area of light hypertransmission into the choroid with a minimum diameter of 250 μm; (2) a region of attenuated or absent RPE, 250 μm in minimum diameter; and (3) signs of degeneration of photoreceptors (absence of the interdigitation zone, external limiting membrane, and EZ and reduction of the thickness of the external nuclear layer), without a scrolled RPE or other evidence of an RPE rip. In ambiguous cases, it has been advised that OCT evaluation should be assisted by other imaging technologies.

OCT is valuable not only for the diagnosis, but also for assessing the progression of GA: when the lesion borders present with irregular RPE elevations [140], an evident separation between the RPE and the Bruch's membrane [140], and/or a thickening of the inner nuclear layer thickness [141], the GA is more likely to grow faster than lesions with smooth edges. Furthermore, an outer retinal thinning often appears at the border of the lesions before the atrophy progresses [142].

Another OCT finding that may be observed in eyes with RPE loss (including eyed with GA) is outer retinal tubulation (ORT). OCT B-scans detect ORTs as round-shaped structures in the outer nuclear layer. Using the *en face* OCT, it is often evident that these structures are connected in a multiple branching morphology [142–144]. ORTs show a hyporeflective lumen surrounded by a hyper-reflective ring that is the outer limiting membrane. It is still questioned if ORTs have clinically important prognostic value [122, 145, 146].

The rising availability of EDISD-OCT and SS-OCT has facilitated evaluation of the choroid in various disorders including AMD. It has been speculated that a drop in the perfusion of the choroid can result in ischemia of the external retina, which is considered an important pathogenic trigger in the pathogenesis of both non-neovascular and neovascular AMD [146–148]. Some studies have associated the thickness of the choroid with the AMD condition: the choroid tends to be thinner as the disease progresses, especially in dry AMD [149, 150, 151]. However, this is still debated as other reports did not confirm these findings [152, 153].

2.7 Optical Coherence Tomography Angiography

Optical coherence tomography angiography (OCTA) is another promising and rapidly evolving technology that can provide visualization of flow in the retinal microcirculation in a depth-resolved fashion [154–156]. The relatively quick acquisition time, lack of need of intravenous dye, and the high-resolution and contrast of the resultant images are major advantages of OCTA.

This technology is based on the principle that the flow in the retinal blood vessels is the main source of motion in the posterior segment of the eye. Thus, by acquiring repeated B-scans at the same position, differences in phase and amplitude of the reflected light signals can be used to identify regions in B-scans where presumed flow is present.

The image resulting from the pixel-by-pixel comparison of two or more repeated B-scan (automatically done by different algorithms in different machines) at the same position is displayed as a motion contrast image. OCTA represents the volumetric reconstruction of a dense raster of repeated consecutive B-scans, which allows the depth-resolved *en face* visualization of the retinal and choroidal microvasculature. Unlike FA, OCTA allows the capillaries in different retinal layers to be isolated and visualized. In this way, a precise correlation between vascular, structural, and functional

changes can be performed in vivo in both cross-sectional and longitudinal fashions.

The first OCTA system was implemented on an SD-OCT platform. Recently, the implementation of this system on SS-OCT with longer (1050 nm) wavelengths has facilitated better assessment of the CC and choroid (Fig. 2.11).

Choriocapillaris alterations have been observed throughout all phases of non-neovascular AMD. In early and intermediate AMD, OCTA has identified reduced CC flow signal under and around drusen, confirming previous histological studies [157–159]. These findings could be indicative of true nonperfusion due to CC impairment or may simply reflect a reduction of blood flow velocity below the detectable threshold of current OCTA technology. Regardless, both scenarios may be associated with hypoxia of the RPE and photoreceptors, with consequent derangement of the local metabolic environment. Eyes with reticular pseudodrusen seem to have even more extensive impairment of the CC as well as a reduced choroidal thickness, particularly in the extrafoveal quadrants [160, 161].

This CC impairment in the earlier stages of AMD seems also to correlate with photoreceptor function, since a significant association has been found between the absence of flow signal and electroretinogram implicit times [162]. In intermediate AMD, but not in early AMD, OCTA has also shown alterations in the superficial and deep

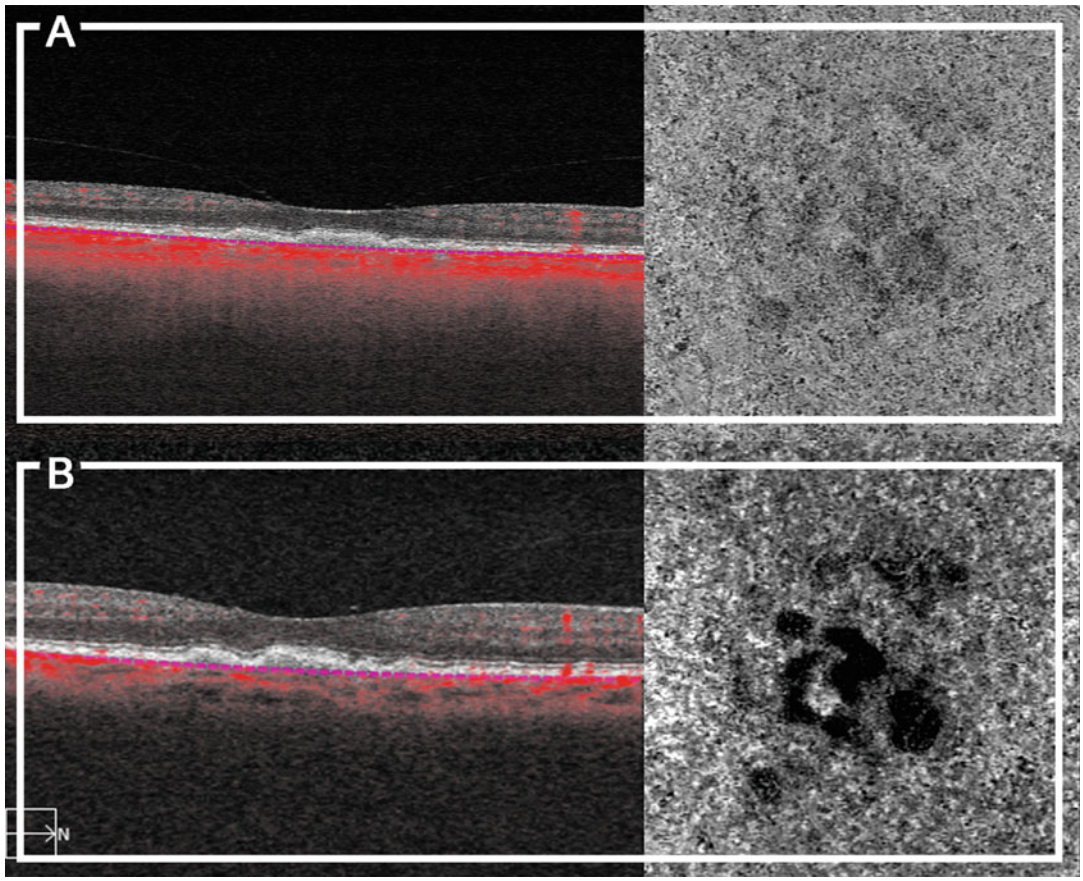

Fig. 2.11 Swept-source (**a**) and spectral domain (**b**) optical coherence tomography angiography of a patient with drusen. The higher penetration of the longer wavelength used by the swept-source systems allows a clearer visualization of the choriocapillaris (right) under the drusen

retinal plexuses, which also seem to correlate with choroidal thickness reduction [163].

In GA, the CC is highly impaired in within these advanced atrophic lesions, even though some residue of flow can be still detected near the border of these lesions. However, the CC immediately surrounding the GA lesion (under apparently intact RPE) can show substantial impairment (Fig. 2.12) [164, 165].

Recently, a report on OCTA showed a significant relationship between areas of nascent GA and CC alterations [166].

It is still debated whether the RPE or the CC disruption occurs first in AMD with evidence

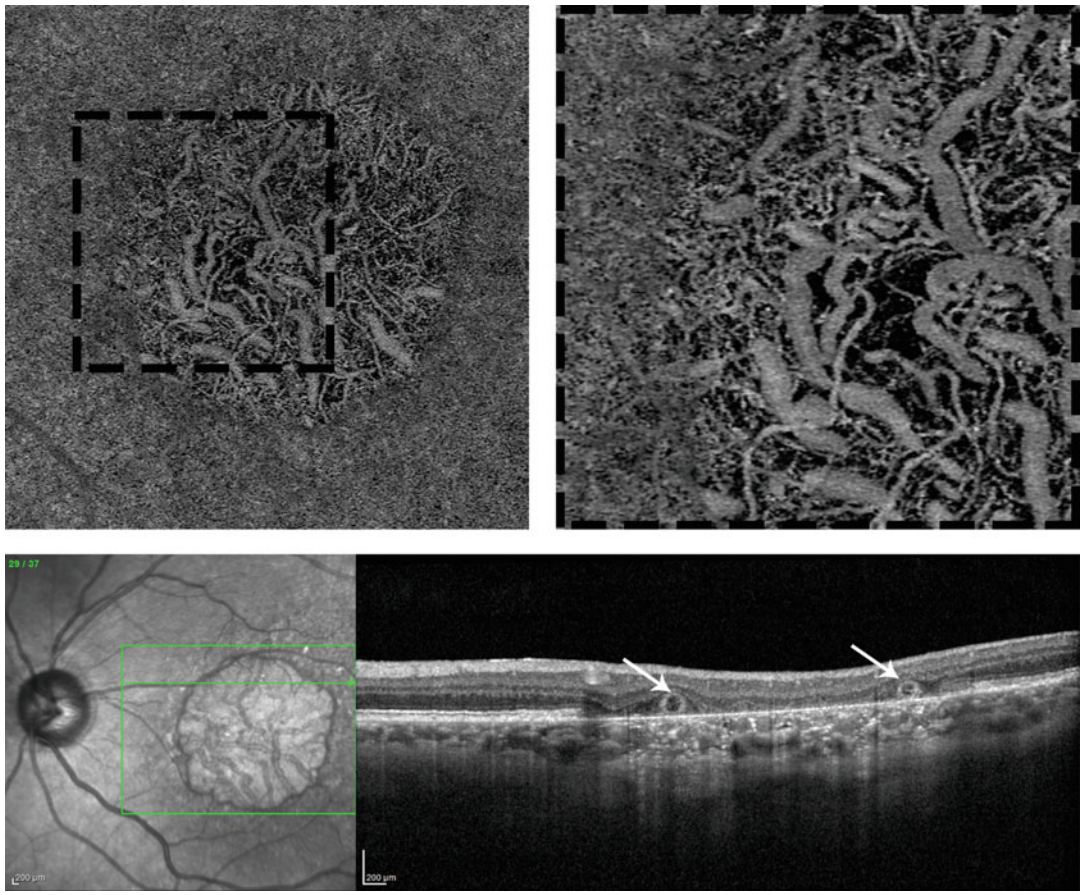

Fig. 2.12 Multimodal imaging of a patient with geographic atrophy secondary to age-related macular degeneration. Optical coherence tomography (OCT) angiography (first row) shows evidence of flow in large choroidal vessels within the atrophic region where the choriocapillaris (CC) is significantly impaired/atrophic and the retinal pigment epithelium (RPE) is absent. The CC also appears to be impaired near the margin of the atrophy (black dotted box). The near infrared image (second row, left) shows the exact boundaries of the lesion. In the OCT B-scan (second row, right), evidence of complete RPE and photoreceptor atrophy with choroidal hypertransmission, attenuation of the RPE band, and thinning of the overlying outer retina can be seen. Areas of outer retinal tubulations (white arrows) can also be observed

pointing in both directions [158, 166–169]. Recent in vivo studies using OCTA seemed to support the hypothesis that CC loss may precede RPE degeneration as microvascular changes occur even under areas of intact RPE. Nevertheless, it is still possible that current imaging methodologies are not sensitive enough to detect the earliest dysfunction of the RPE. Furthermore, the reduced production of trophic factors from the damaged or absent adjacent RPE may also contribute to CC impairment in regions surrounding GA lesions [158, 167].

The use of OCTA has also been investigated for detecting "silent" type 1 MNV in otherwise asymptomatic intermediate AMD patients. A case series has estimated this occurrence in around 30% of all eyes with intermediate AMD [170]. OCTA may be used for diagnosing different subtypes of MNV, allowing a direct correlation with structural OCT and thereby aiding in the diagnosis and classification of the neovascular lesions [171]. Type 1 MNV is usually displayed as a network of vessels between the RPE and the Bruch's membrane, often in the setting of a fibrovascular PED as visualized on the OCT (Fig. 2.4) [172]. Conversely, Type 2 MNV is displayed as a vascular network in the avascular subretinal space (Fig. 2.6) [173]. In both these MNV types, it is often possible to see on OCTA the feeding trunk vessels: generally large vessel from which smaller vessels derive, forming anastomotic connections inside and at the borders of the lesions [174–176]. Furthermore, there may be drop out of the CC surrounding areas of MNV [168] confirming previous histological studies [177].

Type 3 MNV, on the other hand, can be visualized as a discrete high flow linear structure extending from the middle retinal layers into the deep retina and occasionally through the RPE (Fig. 2.9). OCTA findings may be difficult to see in the earliest stages of Type 3 MNV, and thus structural OCT remains important for diagnosis. When type 3 MNV progresses, vessel branches form anastomotic connections within the deep capillary plexus, extending downward to the external retina and subretinal and sub-RPE spaces [175].

Current studies using OCTA in neovascular AMD are aimed at defining imaging features that could aid clinicians to distinguish between active and inactive MNV [178]. Different authors advocate that some OCTA findings (e.g., dark haloes surrounding the lesions and tiny vessels at the MNV edges) may correlate with activity, whereas bigger "dead tree"-shaped vessels and a lack of fine vessels branches may correlate with quiescent lesions [171]. The reliability of identifying these features remains to be established. Nevertheless, a lesion defined as "active" may not always implicate poor visual acuity while an "inactive" fibrovascular scar may be associated with poor visual acuity. These observations may eventually assist in defining the optimal endpoint or outcome following anti-VEGF therapy MNV, though prospective longitudinal studies will be essential [156].

A challenge for evaluating the OCTA of eyes with suspected MNV is to avoid misinterpretation due to various artifacts, some of which include: (1) areas of atrophy that may reveal flow in deeper choroidal vessels that could be confused for neovascularization; (2) projection artifacts from retinal vessels projecting onto elevated hyperreflective structures such as serous PED, drusen, drusenoid PED, and pigmented scars thereby simulating neovascularization [177]; (3) particularly for type 3 MNV, superficial vessels may be projected on the highly reflective PED, simulating a MNV [179, 180]; and finally (4) hemorrhages or other features of exudation may obscure the flow signal, preventing visualization of the MNV [181]. Use of projection removal software as well as a systematic review of the OCTA with simultaneous viewing of the corresponding structural OCT *en face* image as well as the B-scans with flow overlay can prevent misinterpretation of these artifacts.

Various studies have evaluated the sensitivity and specificity of OCTA in identifying MNVs [174, 182], resulting in an overall sensitivity of ~50% and a specificity above 80% [182]. The sensitivity seems to be higher when considering only Type 1 MNV (66.7%), with further increase in detection rate when OCTA is accompanied by structural OCT data (87%) [172, 183].

Current commercial OCTA devices present a small dynamic range for flow velocity, with the

output saturating at low flow rates. The ability to quantifying flow rates could potentially prove to be useful for an accurate identification of MNV and the evaluation of the efficacy of treatments, but such an analysis is not currently available in clinical devices.

OCTA has several limitations that must be taken into consideration when evaluating the images: (1) the absence of signal may not indicate an absence of flow, but only a flow below the threshold of detection; (2) low OCT signal, but above the threshold level may give rise to a decorrelation signal due to noise; and (3) the presence of RPE alterations may preclude a clear visualization of the underlying CC. These issues must be considered when evaluating OCTA images for the presence of CC dropout [158]. Thus, swept-source devices, which use longer wavelength, are less prone to attenuation artifacts and may be more suitable for CC visualization [120, 184–187].

Finally, motion, projection, and segmentation artifacts are further drawback of this imaging methodology. However, more efficient tracking systems and projection removal algorithms as well as deep-learning algorithms for segmentation could potentially overcome these limitations in future [176].

2.8 Adaptive Optics Scanning Laser Ophthalmoscopy

Adaptive optics scanning laser ophthalmoscopy can produce in vivo images of individual cone photoreceptors and images of the RPE mosaic. This technology has a lateral image resolution of 2 μm achieved by compensating for ocular wavefront aberrations. This resolution allows the reproducible visualization of individual cone photoreceptors that may be reliably tracked over time [188–190]. Usually, AOSLO methodology employs along wavelength (e.g., 840 nm) [191]. Media opacities and poor fixation are major obstacles to obtain good quality images as they may cause light scattering and image distortion, respectively [192].

Increasing degrees of severity of AMD lead to increasing photoreceptors loss as demonstrated by the use of AOSLO [193]. There is a slight disruption of the photoreceptor mosaic over small drusen while in presence of soft drusen and/or drusenoid PEDs and/or areas of GA, photoreceptor density is significantly decreased [193]. In patients with GA and drusen, the correlation between AOSLO and other modalities (SD-OCT, SW-FAF, NIR-FAF, and CFP) has shown that the cone mosaic is continuous, with normal intercellular spacing over drusen up to the edge of GA [194]. Nevertheless, the reflectivity of the cones over drusen is often reduced and colocalizes with overlying hyporeflective EZ on OCT [194].

No correlation was found between the AOSLO dark signal and the presence of hyperautofluorescent GA borders in FAF images [195]. The spacing of the cones has a high specificity but not the same sensitivity in measuring the integrity of the mosaic-like structure of photoreceptors and thus cannot be a reliable index of the progression of the disease [194]. Of note, in the presence of RPE or retinal profile alterations (e.g., drusen or GA), the alignment of the cones might change making them appear artificially hyporeflective [194, 196]. These findings do not consistently correlate with histology and OCT studies that report a decrease in the density of photoreceptors over drusen [197, 198]. On the other hand, they correlate well with histology and OCT studies reporting photoreceptor alterations at the edge of GA [51, 195, 199]. Recently, photoreceptor abnormalities were detected in AMD patients in areas of normal SW-FAF [192], which seem to correspond with histologic changes of AMD [52]. Therefore, AOSLO could anticipate the identification of abnormal findings in RPE cells and/or in the overlying photoreceptors before they become visible with SW-FAF, NIR-FAF, or even SD-OCT. These findings may represent features of AMD progression that can be tracked in a quantitative and reproducible fashion.

2.9 Summary

In summary, advances in ocular imaging have significantly enhanced our ability to comprehensively evaluate the eyes of patients with AMD. These imaging approaches can allow early diagnosis of AMD and precise monitoring of its

progression. The various imaging modalities provide complementary information and are optimally used in multimodal approach.

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Histopathology of Age-Related Macular Degeneration and Implications for Pathogenesis and Therapy

3

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Abstract

Aging is associated with a number of histological changes in the choroid, Bruch's membrane, RPE, and neuroretina. Outside of the normal physiologic aging spectrum of changes, abnormal deposits such as basal laminar deposits, basal linear deposits, and soft drusen are known to be associated with AMD. Progression of AMD to advanced stages involving geographic atrophy, choroidal neovascularization, and/or disciform scars can result in debilitating vision loss. Knowledge of the angiogenic pathway and its components that stimulate neovascularization has led to the development of a new paradigm of intravitreal anti-VEGF pharmacotherapy in the management of neovascular AMD. Currently however, there are no available treatments for the modification of disease progression in non-neovascular AMD, or for the treatment of geographic atrophy. Further understanding of the histopathology of AMD and the molecular mechanisms that contribute to pathogenesis of the disease may reveal additional therapeutic targets.

Keywords

Age-related macular degeneration · Histopathology · Basal laminar deposits · Basal linear deposits · Drusen · Geographic atrophy · Choroidal neovascularization · Disciform scar

3.1 Introduction

Age-related macular degeneration (AMD) is an ocular disease characterized by pathologic changes in the posterior pole of the eye, in structures such as the outer neurosensory retina, retinal pigment epithelium (RPE), Bruch's membrane, and the choriocapillaris in elderly patients. These pathological abnormalities differ from a number of age-related histological changes considered to be part of the physiologic spectrum.

AMD is broadly classified into "dry" and "wet" subtypes. Histologically, accumulation of waste material resulting in basal deposits (i.e., basal laminar deposits (BlamD) and basal linear

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deposits (BlinD)) beneath the RPE and into Bruch's membrane characterizes early dry, non-exudative, or non-neovascular AMD (Fig. 3.1). Drusen are the clinical hallmark of AMD that can be noted on fundus ophthalmoscopic examination and represent deposits within Bruch's membrane. Wet, exudative, or neovascular AMD is mainly distinguished from dry AMD by the presence of choroidal neovascularization (CNV), which represents abnormal new blood vessel formation.

Loss of vision occurs through a variety of mechanisms, which in turn have important resulting clinical implications. At the level of the RPE and photoreceptors, alterations such as RPE hypopigmentation, depigmentation, atrophy, hypertrophy, and photoreceptor attenuation may manifest. In wet AMD, fluid or blood in the intraretinal, subretinal, or sub-RPE spaces may additionally result in decreased visual acuity, metamorphopsia, or other visual symptoms. Less commonly, massive subretinal hemorrhage or breakthrough vitreous hemorrhage may further reduce vision. Progression to geographic atrophy and disciform scarring occur in late stages of dry and wet AMD, respectively.

Examining the histopathology of AMD provides us with a better understanding of the pathogenic mechanisms that drive vision loss, which may ultimately aid in better determination of visual prognosis, patient counseling, and development of new treatments.

3.2 Histopathology and Anatomy of the Normal Retina, RPE, Bruch's Membrane, and Choroid

The retina is a thin, multi-layered sheet of tissue derived from the neuroectoderm. It represents the beginning of the visual pathway, responsible for transmitting visual stimuli from the external environment to the visual cortex, by way of the optic nerve [1, 2]. This approximately 0.2-mm-thick structure is comprised of the neuroretina, made of 9 separate layers, and the retinal pigment epithelium (RPE) [3]. As light enters the eye, it traverses the neuroretinal layers in the following order: (1) internal limiting membrane, (2) nerve fiber layer, (3) ganglion cell layer, (4) inner plexiform layer, (5) inner nuclear layer, (6) outer plexiform layer, (7) outer nuclear layer (the photoreceptor nuclei), (8) external limiting membrane, and (9) photoreceptor (the photoreceptor inner and outer segments) layer [1, 4]. Each layer includes neurons specialized in visual information processing, totaling more than 50 distinct types [2].

In the seventh and ninth layer**,** photoreceptors (i.e., rods and cones) convert photons of light into electrical impulses, thereby initiating signal transduction to the brain [2]. The photoreceptor layer comprises an outer and an inner segment: the outer segment includes the photoreceptor discs, which contain photopigment; the inner segment includes the structures responsible for intracellular metabolism and transport, like mitochondria (found in the outer ellipsoid), Golgi bodies, and ribosomes (found in the inner myoid) [5]. Photoreceptors extend from this layer to the outer nuclear layer, which houses the photoreceptor nuclei. In between lies the external limiting membrane, which is formed by tight junctions between photoreceptors and Müller cells and is not considered a true membrane. Axons extend from the photoreceptor cell bodies, synapsing with bipolar cells in the outer plexiform layer. The inner nuclear layer (INL) is comprised of a diverse set of neurons (their nuclei) primarily belonging to three classes—horizontal, bipolar, and amacrine cells—as well as Müller cells, a type of macroglial cell. Bipolar cell nuclei predominate in the INL, and are known to demonstrate different functional responses to light. Some bipolar cells respond at light onset, some at light offset, some transiently, and some in a sustained manner. Such ON bipolar cells terminate at the inner part, and OFF bipolar cells at the outer part, of the inner plexiform layer [1, 6]. Axons extend from the INL synapsing with ganglion cells in the inner plexiform layer.

The ganglion cell layer contains the nuclei of more than 10 types of ganglion cells [7]. Ganglion cell axons, which form the nerve fiber layer, are responsible for transmitting visual information to

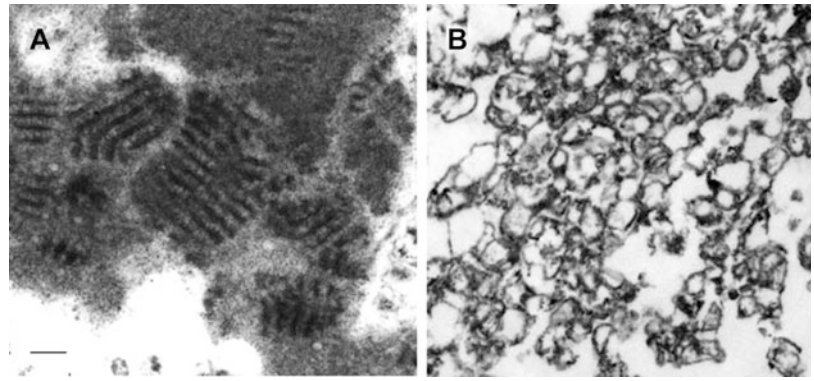

Fig. 3.1 Transmission electron micrography illustrating (**a**) basal laminar deposits (BlamD) and (**b**) basal linear deposits (BlinD) (bar = 0.5 μm)

higher visual centers by way of the optic nerve. Finally, the internal limiting membrane is a basement membrane formed by Müller cell footpads [1].

The RPE is a single layer of neuroectoderm-derived hexagonal epithelial cells primarily responsible for nourishing the overlying neurosensory retina. Its apical surface, which is covered in microvilli, faces the photoreceptors, helping in the continual turnover of their outer segments [4, 8]. The RPE basal lamina is the inner layer of Bruch's membrane, a 5-layered membrane overlying the choriocapillaris. Specifically, functions of the RPE include phagocytosis, recycling photoreceptor outer segments, converting all-trans retinal to 11-cis retinal as part of the visual cycle, secreting growth factors to maintain photoreceptor vasculature, transporting nutrients and waste between the photoreceptors and the choriocapillaris, and forming the outer blood-retinal barrier, which prevents molecules >300 kDa from passing into or out of the retina [4, 8].

Bruch's membrane, an acellular pentalaminar structure, separates the RPE and choroid. From the RPE to the choroid, the following layers are distinguished: (1) RPE basement membrane, (2) inner collagenous layer, (3) elastin layer, (4) outer collagenous layer, and (5) basement membrane of the choriocapillaris. Bruch's membrane serves as a semi-permeable filter to regulate the transport of molecules such as carbon dioxide, water, ions, waste products cleared by the RPE, photoreceptor outer segments and other substances between the retina and choroid [8].

The choroid is a vascular bed between Bruch's membrane and the sclera. Its innermost layer is the choriocapillaris, a single layer of fenestrated capillaries that supplies blood to the RPE and outer neurosensory retina. Sattler's Layer is comprised of intermediate arterioles and venules, connecting the choriocapillaris to the outer Haller's Layer. The choroidal vasculature is primarily supplied by the long and short ciliary arteries, which are branches of the ophthalmic artery. Of note, loss of endothelial cells of the choriocapillaris is an important contributor to the development of AMD [8].

3.3 Histological Changes During Normal Aging in the Retina

With non-pathological aging, the retina undergoes a number of changes such as drusen formation, Bruch's membrane thickening, photoreceptor loss, choroidal thinning, lipofuscin accumulation, and other RPE changes [9].

3.3.1 Drusen

Drusen, which are comprised of cellular debris and lipids, among others, accumulate within the Bruch's membrane with age. Drusen may be categorized by traits including their size, shape, and location, and are variably linked to disease states. Specifically, hard drusen accumulate as part of the normal retinal aging process. Typically, hard drusen are <63 micrometers with sharp borders, are

found at both the macula and periphery, and have not been causally linked to AMD development. By contrast, soft drusen are typically >125 micrometers, may have either sharp or indistinct borders, are only found at the macula, and significantly increase the risk of AMD [4, 9] (Fig. 3.2).

3.3.2 Thickening of Bruch's Membrane

Beyond drusen formation, Bruch's membrane thickening also occurs with age. In general, the five layers of Bruch's membrane become less sharply demarcated and the overall structure thickens [4, 10]. Previous work has demonstrated a linear relationship between Bruch's membrane thickness and age (from 2 mm in those <10 years old to 4.7 mm in those >80 years old) [8]. Further Bruch's membrane changes include lipid accumulation, reduced amino acid diffusion, diminished elasticity, and an increase in the expression of TIMP3, a regulatory protein that inhibits metalloproteases [9]. The implications of thickened Bruch's membranes in aging retina are a reduction in efficiency of exchange of nutrients and waste between the retina and choroid, potentially increasing susceptibility for development of retinal pathology.

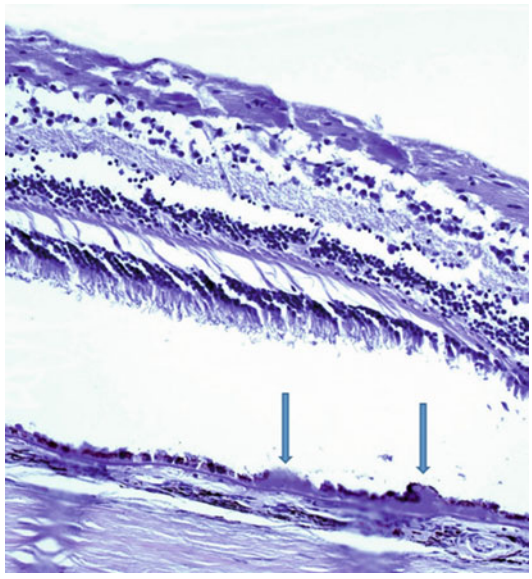

Fig. 3.2 Photomicrograph of an eye showing drusen (arrows) in the macular region (Periodic acid-Schiff (PAS), original magnification, ×100)

3.3.3 Loss of Photoreceptors

Another consequence of aging is photoreceptor loss, predominantly affecting rods more so than cones. It is estimated that 2 rods/mm^2 are lost per year in healthy retina; examination of donor retina tissue of patients over 90 has revealed minor cone loss in the fovea and 30% rod loss in the parafovea. Given their extremely high metabolic activity, photoreceptors are particularly susceptible to hypoxia. As a result, any degeneration in the choriocapillaris over time is expected to increase photoreceptors' vulnerability. Compared to older, healthy retina, more significant rod loss is observed in eyes with retinal disease, e.g., AMD (both geographic atrophy and CNV) [4, 9]. Given their role as mediators of vision in low-light conditions, loss of rods with age results in decreased scotopic sensitivity [11].

3.3.4 Choroidal Thinning

Choroidal thickness and choriocapillaris density decrease with age. Sub-foveal choroidal thickness has been observed to decrease by ~3 micrometers per year. Reduction in choriocapillaris blood flow has also been reported, which may increase risk for AMD and CNV [4, 9].

3.3.5 Lipofuscin Accumulation

Moreover, lipofuscin accumulation occurs as individuals' age. Lipofuscin is composed of granules that are byproducts of photoreceptor outer segment turnover. Accumulation in the RPE is observed in normal aging, reaching a detectable level in those over 40 years of age. Past work has not identified a significant difference in the degree of lipofuscin accumulation in patients with AMD vs. health- and age-matched controls—though this may be due to increased

cell death in AMD eyes and therefore loss of lipofuscin [8, 9].

3.3.6 RPE Changes

Finally, a number of RPE changes occur with age. Specifically, the following have been reported: reduced number of mitochondria, loss of cristae and matrix density, accumulation of lipofuscin (as previously noted), decrease in melanosomes, and increase in toxic visual cycle byproducts. Minor RPE cell loss has also been observed with age [4, 9].

3.4 Histopathology in Early Dry AMD

Clinically, a diagnosis of AMD requires the presence of extensive small drusen (Fig. 3.3), or the presence of any medium or large drusen (Fig. 3.4) in the posterior pole. Histologically, several other changes can be observed.

3.4.1 Changes in Bruch's Membrane

In AMD, thickening of the Bruch's membrane is greater than with aging alone, partly due to accumulation of inner collagenous material also known as BlamD [12]. In early AMD, further thickening and loss of normal architecture is seen within Bruch's membrane. Bruch's membrane is considered the structural analog of the vascular intima as it lies under the RPE and forms the inner margin of the choriocapillaris [13]. Analogous aging changes in the vascular intima of atherosclerosis were thought to relate to the pathogenesis of AMD in the Bruch's membrane [14].

3.4.2 Changes in the RPE

In the early stages of AMD, the RPE mosaic, a normally uniform hexagonal array, begins exhibiting pleomorphism [15]. There is also an exaggeration in RPE cell density decline, compared to the normal age-related decline [16]. As AMD progresses, RPE cells may die. In a study of the inflammatory roles of the RPE in AMD, Nussenblatt and Ferris discuss the importance of the downregulatory immune environment in the eye [17]. The hypothesis suggests that the natural environment of the eye is designed to downregulate inflammation while maintaining an equilibrium in the eye. The RPE cells downregulate the immune response in the eye, therefore in AMD it becomes a cycle of inflammatory response damaging the RPE, which subsequently decreases the downregulatory effect of the RPE leading to worsening RPE degeneration.

3.4.3 Various Deposits

Among the earliest pathological changes in early AMD are the appearance of BlamD and BlinD (Fig. 3.1). BlamD and BlinD, but not drusen, were found to have a positive association with CNV, disciform scarring, and visual loss [18].

3.4.3.1 Basal Laminar Deposits (BlamD)

BlamD are extracellular debris accumulating between the RPE and its basal lamina. They consist of membrano-granular material and foci of wide spaced collagen. Localized detachments of the BlamD result in the formation of soft drusen. BlamD stain light red with Mallory staining, and light blue with Masson's trichrome staining. Studies showed that BlamD are composed of type IV collagen, laminin, glycoproteins, glycosaminoglycans (chondroitin and heparin sulfate), N-acetylgalactosamine, esterified and unesterified cholesterol, and apolipoproteins B and E [19–21].

3.4.3.2 Basal Linear Deposits (BlinD)

BlinD are present in the superficial and deeper layers of Bruch's membrane, external to the RPE basement membrane. They are usually present in the inner collagenous layer, but can extend into the outer collagenous layer and even into the choriocapillaris pillars. They consist of lipid-rich vesicular material located in the inner collagenous

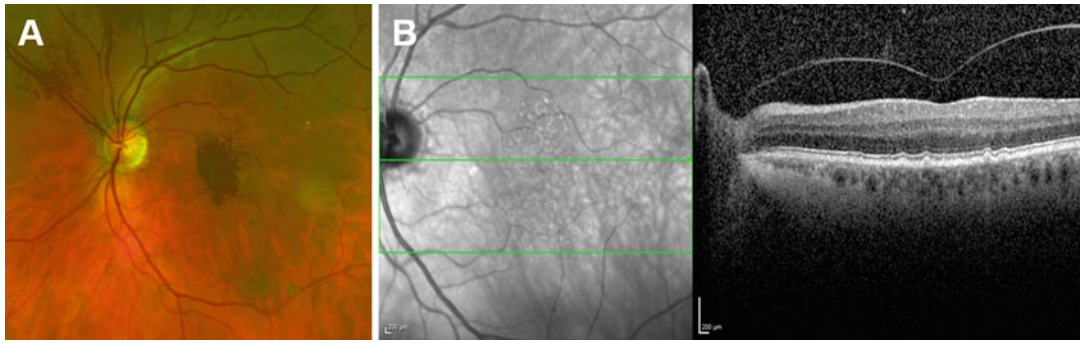

Fig. 3.3 Fundus photograph and optical coherence tomography (OCT) of a patient with early dry age-related macular degeneration. (**a**) Fundus photograph using Optos (Optos PLC, Dunfermline, Scotland) of the left eye reveals small drusen in the posterior pole. (**b**) OCT of the left eye using Heidelberg SPECTRALIS (Heidelberg Engineering, Heidelberg, Germany) reveals small drusen between retinal pigment epithelium and Bruch's membrane

zone of Bruch's membrane, which contributes to Bruch's membrane thickening. BlinD may represent a possible extension or progression of the abovementioned BlamD. They are found in association with soft drusen and RPE detachments. These deposits may not be evident on clinical examination in early AMD. They can sometimes be detected by very faint and late fluorescein staining, and inferred retinal function. They become clinically evident by secondary changes such as thinning of RPE, development of soft drusen or eventual choroidal neovascularization and disciform scarring. They are more specific for AMD than BlamD [12], and their amount is a more reliable indicator of the degree of RPE and photoreceptor degeneration [15, 22].

3.4.3.3 Drusen

Drusen are localized deposits between the RPE basement membrane and Bruch's membrane. In early AMD, they are frequently found as clusters within the macular region (Figs. 3.2 and 3.3). They vary in size and are split to small (<63 μm diameter) (Fig. 3.3), medium (63–125 μm diameter) (Fig. 3.4), and large (>125 μm diameter) drusen. The typical diameter of a retinal vein at the optic nerve head (125 μm) can be used as an estimate for classifying large drusen. Drusen also vary in shape, consistency, color, and distribution. Drusen usually increase in number with advancing age. Drusen are associated with thinning of the overlying RPE and become visible as yellow-white deposits on fundus examination.

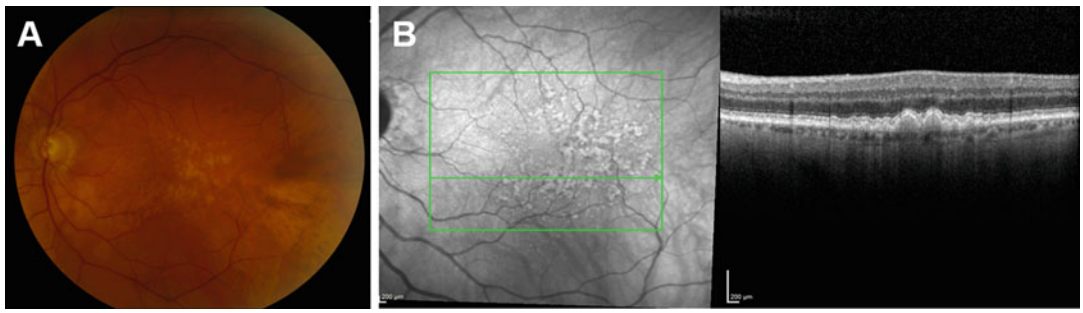

Fig. 3.4 Fundus photograph and optical coherence tomography (OCT) of a patient with intermediate dry age-related macular degeneration. (**a**) Fundus photograph using Optos (Optos PLC, Dunfermline, Scotland) of the left eye reveals large drusen in the posterior pole. (**b**) OCT of the left eye using Heidelberg SPECTRALIS (Heidelberg Engineering, Heidelberg, Germany) reveals large drusenoid pigment epithelium detachments

They are clinically classified into either hard drusen, soft drusen, or reticular pseudo-drusen. In early AMD, they are frequently found as clusters within the macular region (Fig. 3.3). Intermediate stage AMD is defined as the presence of extensive intermediate size (63–125 μm) drusen in the macula, or one large drusen (>125 μm) within 3000 μm of the foveal center (Fig. 3.4).

Types of Drusen

Hard drusen are small (less than 63 μm), yellowish punctuated deposits. They are globular in shape and stain with periodic acid-Schiff. They are not specific to AMD and are common in elderly patients even without AMD. The presence of a few small hard drusen is not an important risk factor for developing AMD.

Soft drusen are larger, paler, and more diffuse with blurry margins, and signify early AMD. They often represent localized accumulations of BlinD in the presence or absence of diffuse thickening of the inner aspect of Bruch's membrane [23]. Soft drusen are focal manifestations of a diffuse process; when there is diffuse BlamD, they may form focal accumulations that are represented as soft drusen. Through activated complement deposition in Bruch's membrane, soft drusen play a role in increased vascular endothelial growth factor (VEGF) production by RPE cells [24]. Large, soft, bilateral, and numerous drusen are significant risk factors for developing advanced AMD. The larger drusen with more RPE pigmentary changes seen in the macula confer a higher risk of progression to late AMD [25].

Reticular pseudo-drusen consist of an accumulation of extracellular debris between the apical processes of the RPE and the inner and outer segments of the photoreceptors [26]. They have been associated with a 4–8 greater risk of 5-year progression to late AMD compared to eyes with only drusen [27]. They were first described in 1990 as an outer macular yellow interlacing pattern with a 100 μm diameter that did not fluoresce on fluorescein angiogram, but instead had enhanced visibility in blue light [28]. The Wisconsin Age-Related Maculopathy grading scheme later described them as "ill-defined networks of broad interlacing ribbons" on color fundus photographs [29]. The term reticular pseudo-drusen was later introduced, described as a "yellow interlacing network 125–250 μm wide, appearing first in the superior outer macula and extending circumferentially and beyond" [30]. Curcio later named these deposits as subretinal drusenoid deposits, as spectral domain optical coherence tomography scans showed hyper-reflective deposits internal to the RPE [31]. Reticular pseudo-drusen share several common components with drusen, such as membranous debris, vitronectin, CFH, and apolipoprotein [32], but have a higher concentration of unesterified cholesterol compared to drusen. However, they do not contain opsins, glial fibrillary acid protein, or RPE marker proteins which are found in soft drusen [31]. Subretinal drusenoid deposits demonstrate the presence of complement and complement regulators [33]. Reticular pseudo-drusen are also present in other diseases such as pseudoxanthoma elasticum and Sorsby fundus dystrophy. Thickening of Bruch's membrane and pathologic changes to the Bruch's-RPE interface have been thought to play an important role in the pathogenesis of reticular pseudo-drusen [34, 35]. Reticular pseudo-drusen are dynamic structures that expand, enlarge, and/or regress over time. In early stages of AMD, they are usually located in the superior part of the macula between the superior temporal arcade and the fovea. The growth of these deposits becomes more rapid in advanced AMD, specifically in geographic atrophy [36].

Pathogenesis of Drusen

Several theories for the pathogenesis of drusen in early AMD have been proposed. Accumulation of BlinD may form a continuous layer of soft drusen which is sometimes referred to as diffuse drusen [23]. Autophagy, the process by which dysfunctional cellular components are degraded, may also play a role in early AMD pathogenesis. RPE cells exhibit reduced capacity for autophagy in AMD [37]. As the RPE is nondividing tissue, it may lead to accumulation of lipofuscin within RPE cells. Macrophage scavenging may also be impaired in AMD, resulting in decreased removal

of membranous debris. [38]. AMD donor eyes were found to contain markers for autophagy and exosomes, which suggests an increase in autophagy and intracellular protein release via exosomes by RPE as a contributor to drusen formation [39]. Apoptosis may also play a role in AMD-associated RPE and photoreceptor cell death [40]. The RPE-Bruch's membrane-choriocapillaris complex is predisposed to continuous oxidative stress, especially in the macular region [41]. Nuclear factor erythroid-2 related factor 2 (Nrf2), for example, is known to protect the RPE cells from oxidative injury [41], and smoking has been shown to suppress its upregulation [42]. Oxidative damage is thought to incite an inflammatory process, termed para-inflammation [43], which is in part mediated by the complement activation pathway at the level of RPE-Bruch's membrane-choriocapillaris [44].

Constituents of Drusen

Protein

AMD pathogenesis and drusen formation are likely multifactorial. Tissue metalloproteinase inhibitor 3, clusterin, vitronectin, and serum albumin are common drusen proteins, detectable in up to 80% of tissues in normal drusen and 60% of drusen in AMD eyes [45]. Clusterin and vitrenectin are complement pathway regulators. The identification of these and other complement system proteins (e.g., complement component 5 (C5), the membrane attack complex (MAC) containing C5b-9, and others) in drusen, coupled with genetic association studies, suggest a potential role of the complement pathway in AMD pathogenesis (please see Sect. 9.3: The Complement System) [24, 45–48]. Amyloid beta has also been identified in drusen and studies suggest that amyloid beta reduces complement factor I function, leading to chronic low-grade inflammation [49]. Moreover, protein modifications from lipoxidation and glyoxidation have been identified in drusen of AMD patients, suggesting a role for oxidative stress in AMD pathogenesis. [45]

Lipid

A variant of the hepatic lipase gene, Lipase C hepatic type (LIPC), has been found to have a significant genetic association with AMD [50]. Above, we briefly mentioned the Bruch's membrane changes in AMD that were thought to relate to changes in the vascular intima leading to the pathogenesis of atherosclerotic disease [14]. These associations were strengthened with similarities found in the protein molecular composition of drusen and atherioclerotic deposits [46]. Both conditions were found to have apolipoprotein B and cholesterol accumulation, with subsequent modification, oxidation, and aggregation. Given these associations, it was initially hypothesized that statins may affect AMD status and progression [51]. Statins are inhibitors of HMG-CoA reductase (the enzyme catalyzing the rate limiting step in cholesterol biosynthesis) and therefore suppress cholesterol synthesis. They also increase levels of liver LDL receptors, reduce apolipoprotein B synthesis, and suppress prenylation. Studies with AMD and statin use varied, and a 2015 Cochrane report concluded that there was insufficient evidence to conclude if statins have a role in the onset or progression of AMD [52]. A randomized, placebo-controlled study suggested that a daily 40 mg of simvastatin may slow the progression of early and intermediate AMD, especially with the CC genotype of the Y402H genotype of CFH. In an open-label prospective multicenter pilot study, 23 patients with large, soft drusenoid deposits were given 80 mg of atorvastatin daily for a year. Ten of those patients showed regression of drusen deposits on imaging with an associated vision gain of 3.3 letters [53].

Another study found an increased risk of neovascular AMD in patients with elevated plasma lipid levels with statin use for more than a year [54]. The authors however postulated that the risk was due to the patients' resistance to the statin treatment rather than the statins themselves causing the neovascular AMD.

RPE Alterations in AMD

Two of the common pigmentary changes seen in early AMD are RPE mottling and clumping. Pigment mottling consists of RPE attenuation, depigmentation, hypertrophy, hyperplasia, and eventual atrophy [18], while RPE clumping refers to the accumulation of pigmented cells in the subretinal space. In a study of three-dimensional OCT scans, AMD patients showed intraretinal RPE migration on OCT imaging. These areas of RPE migration corresponded with RPE pigment clumping on fundus photography. These areas of RPE clumping also showed a high incidence of underlying drusen, suggesting drusen might play a physical and catalytic role in facilitating RPE migration and the appearance of clumping [55].

3.5 Histopathology in Advanced Dry AMD with Geographic Atrophy

Advanced dry AMD is characterized by geographic atrophy, which are well-demarcated areas of confluent RPE atrophy through which underlying choroidal vessels are visible (Fig. 3.5). Geographic atrophy is characterized histologically by loss of overlying outer layers of neurosensory retina and underlying choriocapillaris (Fig. 3.6). Bruch's membrane may exhibit changes such as erosion of intercapillary pillars, but does not typically have breaks, which are the precursor to neovascular AMD [22, 56–58]. Macrophages have been reported to be the most prominent inflammatory cell type present in AMD, and have been found to be associated with drusen and areas of geographic atrophy [58]. Other cell types like melanocytes, fibroblasts, and multinucleated giant cells have likewise been reported to be associated with AMD [58].

3.5.1 Drusen and RPE Abnormalities

Drusen size and extent, along with the presence of RPE abnormalities, determine the risk of progression to geographic atrophy. However, although considered to be central to the initiation of RPE cell loss, drusen may disappear over time as AMD progresses, especially when geographic atrophy occurs [59–61].

RPE abnormalities that may precede more advanced geographic atrophy include hypopigmented areas of atrophy and areas of focal hyperpigmentation [59]. RPE abnormalities are often found on the anterior surface of drusen, but may also occur independently of drusen. As areas of discrete drusen and RPE abnormalities develop and increase in extent, adjacent areas may coalesce and form larger confluent patches. Retrospective analysis of fundus images prior to

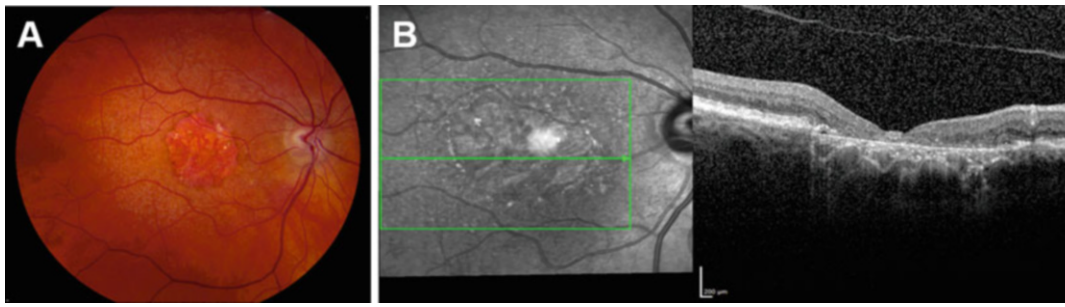

Fig. 3.5 Fundus photograph and optical coherence tomography (OCT) of a patient with advanced dry age-related macular degeneration with geographic atrophy. (**a**) Fundus photograph using Optos (Optos PLC, Dunfermline, Scotland) of the right eye reveals a geographic patch of hypopigmentation with visible underlying choroidal vessels. (**b**) OCT of the right eye using Heidelberg SPECTRALIS (Heidelberg Engineering, Heidelberg, Germany) reveals outer retinal and retinal pigment epithelium atrophy with adjacent drusen

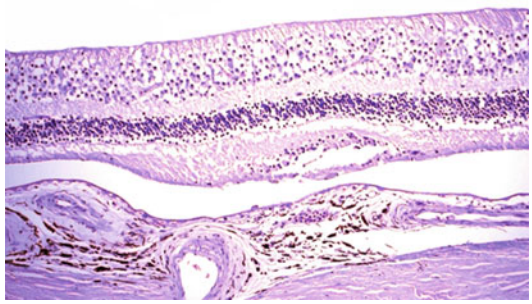

Fig. 3.6 Photomicrograph of a geographic AMD eye showing the macular lesion with total loss of RPE and mostly photoreceptor cells with a few remaining photoreceptor nuclei (asterisk) in the ONL (hematoxylin & eosin (H&E), original magnification, ×100)

the development of initial geographic atrophy revealed several retinal precursor lesions and a clinical sequence of events leading to the development of geographic atrophy [62]. This clinical sequence of events has been reported to begin with the development of large drusen (Fig. 3.4) and RPE hyperpigmentation, followed by regression of drusen, appearance of RPE hypopigmentation, loss of RPE, and ultimately by the development of geographic atrophy (Fig. 3.5). Geographic atrophy that develops from this evolution of RPE abnormalities often begins in the parafoveal region. Additionally, geographic atrophy may develop in association with drusenoid pigment epithelial detachments (PEDs), and independently from drusen in areas of RPE abnormalities. Progression and enlargement of geographic atrophy to involve the fovea typically results in precipitous decline of visual acuity.

3.5.2 Pathogenesis of Geographic Atrophy

The underlying mechanisms that lead to the eventual loss of the RPE and photoreceptors have not been fully elucidated. An exacerbated oxidative stress response is believed to occur, and treatment with antioxidants and omega-3 fatty acids has been touted as possible methods of maintaining RPE function. Healthy RPE function is known to maintain photoreceptor homeostasis, and when RPE function is compromised, photoreceptor damage and atrophy characteristic of AMD may occur (Fig. 3.6). Nevertheless, loss of photoreceptors, particularly rods, have been observed to occur prior to the development of RPE dysfunction [63]. The hypothesis that rod cell death occurs prior to RPE dysfunction is supported by the observation that greatest RPE and photoreceptor cell loss occurs in the parafoveal region, where rod density predominates [22]. It has been proposed that subclinical loss of rods without overt evidence of RPE disease may be the earliest manifestation of AMD, resulting in atrophy that encircles the fovea. Eventually, in susceptible individuals, RPE dysfunction exacerbates rod loss and cone loss begins.

3.5.3 Lipofuscin Accumulation

The role of lipofuscin accumulation in RPE cells further illustrates the close relationship between photoreceptor and RPE dysfunction. Lipofuscin is generated as a byproduct from the oxidation of unsaturated fatty acids, and enters the RPE through phagocytosis. Lipofuscin-containing phagosomes combine with lysosomes and are digested within the RPE. Abnormal molecular degradation of lipofuscin, as well as autophagy within non-dividing RPE cells, eventually leads to its accumulation over time, which may reduce RPE function and result in cell death [64]. A2E, a lipofuscin fluorophore internalized by RPE cells, has been shown to mediate membrane damage [65], and have the potential to induce activation of the complement system [66–68].

3.6 Histopathology in Choroidal Neovascularization

Choroidal neovascularization is the hallmark of neovascular AMD. It occurs as a growth of new choroidal blood vessels, and may extend through the RPE via breaks in Bruch's membrane (Fig. 3.7). These fragile new vessels are prone to

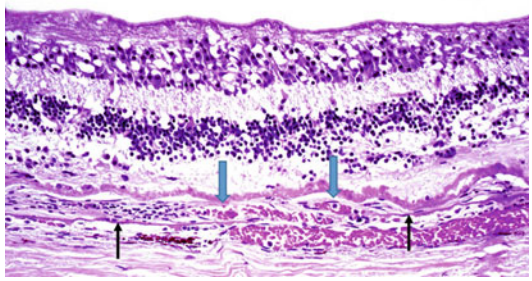

Fig. 3.7 Photomicrograph of a neovascular AMD eye showing the macular lesion with subretinal fibrovascular tissue containing many small vessels (thick blue arrows) through the broken Bruch's membrane (thin black arrows), most photoreceptor cells and RPE cells are missing (hematoxylin & eosin (H&E), original magnification, ×200)

leakage of intravascular contents due to the lack of barrier function present in mature vascular endothelial cells. Retinal edema from fluid, exudates from the deposition of proteins and lipids, and hemorrhage from erythrocytes may occur secondary to extravasation of intravascular contents. Less commonly, massive subretinal hemorrhage or breakthrough vitreous hemorrhage may occur, severely impairing visual function.

Decreased thickness and disruption of the elastic lamina of the Bruch's membrane may precede choroidal neovascularization of the sub-RPE space [69]. Calcification and breaks of the Bruch's membrane correlate with the presence of neovascular AMD [70]. Breaks in Bruch's membrane provide conduits that allow choroidal vessels to traverse the membrane into the sub-RPE space. Besides vascular endothelium, choroidal neovascular tissue consists of both cellular and extracellular components such as RPE, macrophages, lymphocytes, erythrocytes, fibrocytes, myofibroblasts, collagen, fibrin, and BlamD [71, 72].

3.6.1 Pro-Angiogenic Factors

A variety of components of choroidal neovascular tissue suggest a multifactorial etiology in its pathogenesis, with inflammatory and pro-angiogenic components contributing to its formation. Macrophages and lymphocytes have been reported to potentially have a role in the promotion of breaks in Bruch's membrane, induction of choroidal neovascularization, and increasing exudation of intravascular contents in new vessels [73]. Angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) contribute to the neovascular process, and are pharmaceutical targets for the treatment of neovascular AMD.

3.6.2 Pigment Epithelial Detachments and Types 1/2/3 Neovascularization

Pigment epithelial detachments (PEDs) occur when the RPE separates from the underlying Bruch's membrane due to the presence of drusen, serous exudate, blood, or neovascular membrane [74]. On OCT imaging, drusenoid PEDs exhibit moderate hyper-reflectivity, serous PEDs appear hypo-reflective, and a mixed pattern of reflectivity may be seen in fibrovascular PEDs. Coalescence of soft drusen results in the formation of drusenoid PEDs (Fig. 3.4). Soft drusen may indirectly promote angiogenesis via macrophages [75], and are additionally implicated in increased VEGF production by RPE cells [24]. Serous PEDs can occur in the context of a non-neovascular process, but are often associated with an area of CNV underlying an intact Bruch's membrane in AMD. Active leakage of fluid has been postulated to increase hydrostatic pressure, leading to RPE separation from the inner collagenous layer of Bruch's membrane.

Fibrovascular PED, or type 1 neovascularization, occurs when neovascular tufts break through Bruch's membrane and extend laterally under the RPE (Fig. 3.7). Type 1 neovascularization is believed to be the predominant process corresponding to "occult" CNV as observed on fluorescein angiography. The horizontal growth of neovascular tissues is facilitated by a cleavage plane between BlamD and Bruch's membrane that contains accumulated lipids. Intravascular leakage from the type 1 choroidal neovascularization can result in serous or

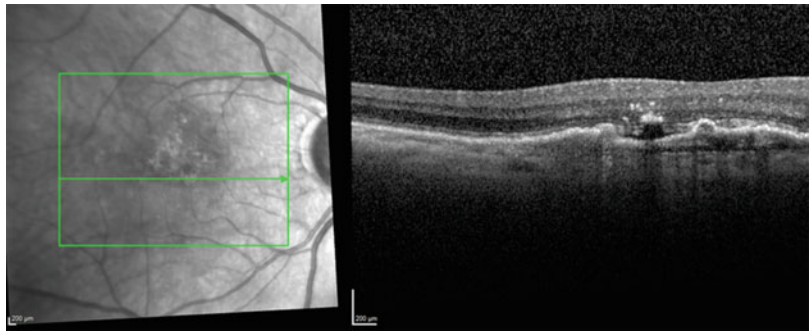

Fig. 3.8 Optical coherence tomography (OCT) of the right eye using Heidelberg SPECTRALIS (Heidelberg Engineering, Heidelberg, Germany) of a patient with mild wet age-related macular degeneration reveals a fibrovascular pigment epithelial detachment consistent with type 1 choroidal neovascular membrane with associated subretinal fluid

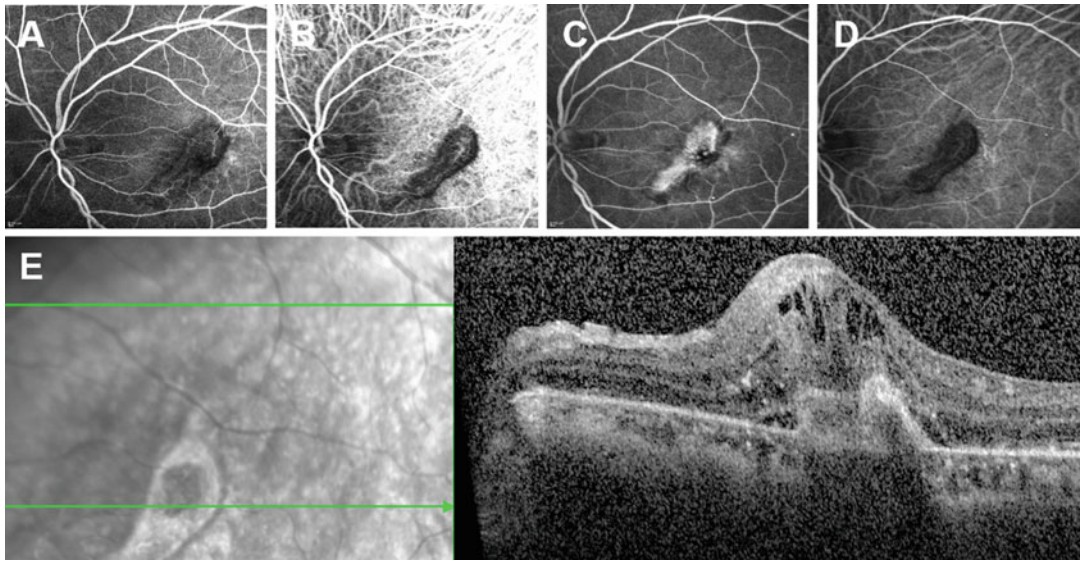

Fig. 3.9 Early and late phase fluorescein angiography (FA) and indocyanine green angiography (ICGA) along with optical coherence tomography (OCT) using Heidelberg SPECTRALIS (Heidelberg Engineering, Heidelberg, Germany) of a patient with advanced wet age-related macular degeneration. (**a**, **b**) Early phase and late (**c**, **d**) FA (**a**, **c**, respectively) ICGA (**b**, **d** respectively) of the left eye show a lacy net of hyperfluorescence with leakage consistent with choroidal neovascular membrane. (**e**) OCT reveals choroidal neovascular membrane with associated intraretinal and subretinal fluid

hemorrhagic PED components. Overlying retina is often intact, with comparably less visual symptoms, but secondary leakage of fluid, blood, or lipids into the retina can result in alteration of retinal structure (Fig. 3.8).

Type 2 neovascularization is located between the neurosensory retina and the RPE, and corresponds to the "classic" CNV appearance on angiography with leakage both under the RPE and into the outer retina (Fig. 3.9). Separation

from the RPE results in atrophy of photoreceptors and other outer layers of the neurosensory retina. Histologically, a reflected layer of inverted proliferating RPE onto the outer surface of type 2 choroidal neovascular membrane (CNVM) is present [71]. Inverted RPE cells may also be present on the inner surface of type 2 CNVM, and occur in association with a similar layer of RPE cells on the external surface of the CNVM [71].

Combined type 1 and type 2 choroidal neovascular growth patterns confer varying degrees of representation of each, resulting in "minimally classic" or "predominantly classic" appearances on angiography [76].

Type 3 neovascularization or retinal angiomatous proliferation (RAP) was described as a type of neovascular process that begins in the retina and not in the choroid, unlike type 1 and 2 neovascularization [77, 78]. Little information on the histopathology of type 3 neovascularization exists, but through clinical observation and optical coherence tomography findings, it reportedly occurs in three stages: (1) an intraretinal neovascular stage arising from the deep capillary plexus of the retina in the paramacular area, (2) a subretinal neovascular stage that may precipitate neurosensory retina and RPE detachments, and (3) choroidal neovascularization with the formation of retino-choroidal anastamoses [77].

3.6.3 Polypoidal Choroidal Vasculopathy

Polypoidal choroidal vasculopathy (PCV) is a distinct variant of AMD from which the primary abnormality lies within the choroidal vasculature [79]. PCV is characterized by an inner choroidal network of vessels that terminate in an aneurysmal bulge, resembling a polyp. Histopathologically, these vessels are located in the sub-RPE space and have been reported to exhibit extensive exudative changes and hyalinization of vessels [80, 81]. Inflammatory cells and positive expression of VEGF have been reported in cases of PCV [80]. Gross dilatation of the choroidal venules and capillaries in the sub-RPE neovascular membrane leads to the characteristic polyp structures, a unique clinical feature in PCV [82]. Recently, PCV pathogenesis is thought to occur through an initial stage mediated by proteolytic degradation of extracellular matrix protein by increased HTRA1 activity and a progression stage driven by inflammatory cascades [83].

Currently, common treatment modalities for PCV include intravitreal anti-VEGF monotherapy [84, 85], indocyanine green angiography-guided photodynamic therapy (PDT) with verteporfin [86, 87] and combined anti-VEGF and PDT [88]. Intravitreal anti-VEGF treatment can effectively reduce intraretinal and subretinal fluid in patients with PCV. In some patients however, choroidal vascular changes may persist, despite intravitreal anti-VEGF treatment [84]. PDT with verteporfin induces local choroidal vascular changes that eventually lead to the thrombosis and regression of PCV lesions. Although PDT has been shown to be effective, it may be associated with complications such as post-PDT subretinal hemorrhage and suprachoroidal hemorrhage [89, 90]. Combination therapy with anti-VEGF and PDT may lead to thrombosis of PCV lesions and regression of associated neovascularization via different mechanisms, and may be especially useful in refractory cases of PCV [88].

3.6.4 RPE Tears or Rips

Intravitreal anti-VEGF therapy has revolutionized the treatment of numerous retinal vascular diseases including AMD. Anti-VEGF pharmacotherapy is particularly effective in improving best corrected visual acuity by decreasing intraretinal and subretinal fluid. However, even though PEDs have been shown to improve with anti-VEGF pharmacotherapy, they are often resistant to anti-VEGF therapy. RPE tears or rips are also recognized as potential complications of anti-VEGF therapy [91, 92]. RPE tears are rare, but are potentially devastating to vision, and may also occur spontaneously or after photodynamic therapy [93, 94] or laser photocoagulation [93]. RPE tears lead to a zone of dehiscence of the RPE with

an adjacent scroll of retracted and irregular RPE monolayer. In AMD, RPE tears are most frequently associated with a vascularized PED, or type 1 neovascularization. It has been proposed that a type 1 neovascular membrane, tightly adherent to its surrounding structures, exerts a contractile effect on the undersurface of the RPE [95]. This contractile force is further increased after anti-VEGF therapy. Moreover, the increased hydrostatic force that contributed to the formation of these PEDs may lead to an acute rupture of the RPE, purportedly typically near the base of the PED. Nevertheless, even after an RPE tear occurs, continued monitoring for exudative changes warranting anti-VEGF therapy may stabilize VA, improve anatomical outcomes, reduce fibrosis, and decrease the risk of developing a large blinding end-stage exudative disciform scar [96].

3.7 Histopathology in Disciform Scarring of CNVM

End-stage neovascular AMD often progresses to a cicatricial stage with the formation of subretinal disciform scars, which are usually vascularized. Disciform scars are areas of fibrous tissue located within the Bruch's membrane, or between the RPE and retina (Fig. 3.10) [97, 98]. The location of the disciform scar with respect to the RPE, however, may become harder to determine due to destruction of the RPE over time. Fibroblasts, RPE hyperplasia, inflammatory cells, and small vessels are found within the fibrovascular tissue. Typically, there is loss of overlying retinal tissue, especially of the outer retinal cell layers, with corresponding visual impairment [99]. Particularly when surrounded by atrophy, disciform scars are generally considered clinically stable.

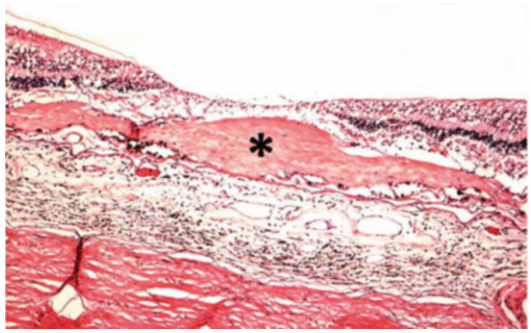

Fig. 3.10 Photomicrograph of an AMD eye with a large disciform scar of dense fibrous tissue in the central maculae (hematoxylin & eosin (H&E), original magnification, ×50)

3.8 Histopathology of Atrophy after Collapse of PED/CNVM as Compared to GA

Atrophy that occurs around disciform scars after the collapse of PEDs and CNVMs differs from atrophy associated with geographic atrophy [100]. Atrophy in geographic atrophy follows a distribution that corresponds to the presence of atrophic rod photoreceptors, drusen, and PEDs. Unlike disciform scars, geographic atrophy is usually confined to the macula, and does not commonly extend significantly further (Fig. 3.5). Atrophy in geographic atrophy is related to patient age, and generally has a later onset and slower progression than disciform scar-associated atrophy [100].

3.9 The Role of the Immune System in AMD

Histopathologically, immune cells have been found to be associated with numerous AMD lesions as detailed above, and play an important role in the pathogenesis of AMD.

3.9.1 Microglia

An extension of the brain, the retina contains many types of neurons and glial cells, including microglia. During development, microglia are distributed throughout the retina but localize to the inner layers as the retina reaches maturity [101]. Microglia form an important part of the retina's immune defense and response to tissue injury and more recently have been shown to also

be involved in the neural and vascular development of the retina [102–104]. Similarly to microglia in the brain, retinal microglia have been shown to express Toll-like receptors (TLRs) and Dectin-1 to recognize and promote clearance of infectious pathogens [105–107]. Activation of such receptors causes microglia to change shape, migrate from the inner to outer retinal layer, and secrete cytokines, chemokines, and neurotoxins [108, 109]. The ability of microglia to phagocytose dying neurons and remove cellular debris as a response to tissue injury has been well described in earlier studies [110, 111]. More recent studies have also shown that activation of TLR4 promotes phagocytosis of photoreceptor proteins and may contribute to retinal neurodegenerative diseases, including AMD [106].

Notably, microglia are absent from the outer layer and subretinal space in the healthy human eye [101]. With aging, however, microglia may migrate from the inner retinal layers to the subretinal space in older adults, resulting in an aberration of their usual distribution and numbers [112, 113]. In animal studies, aged microglia have been shown to have altered morphology, with reduced branching and shorter processes potentially compromising their ability to survey and interact with the surrounding environment and remove cellular debris. Functionally, they seem to have slower responses to tissue injury compared with younger microglia and synthesize excessive amounts of pro-inflammatory cytokines [113, 114]. It has additionally been shown that certain genes involved in neuroprotection are down-regulated in the aging retina [114], supporting a role for microglial senescence in retinal degenerative diseases such as AMD [115].

Consistent with this, activated microglia have been found in the outer retina and subretinal regions in patients with AMD, implying a potential pathogenic role for these cells [116]. In fact, in vivo studies have shown that activated microglia transplanted into the subretinal space caused displacement of additional microglia from the inner to outer layers, implying a potential positive feedback loop that promotes chronic neuroinflammation [117]. Indeed, results from in vitro studies have shown that activated microglia may have the ability to result in injury to healthy photoreceptor cells [118]. Recent studies in mice have supported these earlier findings and have shown that infiltrating retinal microglial cells secrete inflammatory mediators that contribute to the death of living photoreceptors [119, 120]. Genetic variants of the *CX3CR1* gene that produces the microglia chemokine receptor have been previously associated with an increased risk of AMD, and functional studies have suggested that inflammatory cells bearing this risk-conferring variant exhibit altered chemotaxis [121]. These data suggest that impaired microglial migration might contribute to AMD pathogenesis. Overall, dysregulation in migroglial distribution, morphology, and functionality seem to play a central role in the development of AMD.

3.9.2 Macrophages

Macrophages can be found in histological specimens of human AMD lesions, particularly in areas of choroidal neovascularization (CNV). They have similarly been noted in regions of RPE atrophy and breakdown of Bruch's membrane, suggesting a potential role in AMD pathogenesis [108]. Initial evidence implicating macrophages in the development of AMD comes from the studies of mice deficient in the macrophage chemoattractant protein CCL2/MCP-1, which exhibit decreased extravasation of monocytes from the circulatory system into the retina. In their study, Ambati et al. showed that mice with deficient CCL2-mediated macrophage recruitment exhibited AMD-like lesions, including geographic RPE atrophy, CNV, and drusen deposits, suggesting a protective role of macrophages in AMD [122]. Other earlier studies, however, have concluded the opposite, that decreasing CCL2-mediated macrophage recruitment to the retina lowers AMD risk [123], leaving it unclear whether macrophages accumulate near CNV lesions in a causative role or if they serve as an adaptive response in the pathogenesis of AMD.

One possibility is that macrophages play a complex role with both an anti-inflammatory and pro-inflammatory actions. The phenotypic plasticity of macrophages to be polarized into M1 and M2 subsets is well recognized and may contribute to an explanation for these findings. M1 macrophages are generally proinflammatory and secrete M1 chemokines, such as CXCL10. On the other hand, the M2 subset is less inflammatory, facilitating tissue repair and neovascularization, and secreting M2 chemokines, such as CCL2 [108]. In a pilot study of patients with and without AMD, Cao et al. found that increased levels of chemokines from both M1 and M2 subsets were present in AMD eyes as compared to eyes without AMD, suggesting an overall increase in both subsets of macrophage infiltration into the retina in AMD. When comparing eyes with the wet and dry forms of AMD, they found that eyes with the dry form had a greater expression of M1 cytokines, whereas those with the wet form had greater M2 cytokine expression. The authors speculate that the M1 macrophages might be implicated in AMD pathogenesis in the early stages with the M2 subset initially playing a protective role, with an eventual shift to M2 macrophage-induced fibrosis and angiogenesis in the later course of the disease [124]. In support of these findings, Yang et al. found that in mouse models of laser-induced CNV, M1-related markers were transiently upregulated in the early stages with a sustained M2 response in the later stages, leading them to conclude that the M2 subset likely plays a more important role in the development of CNV [125].

Recent studies have also suggested that age-related changes in macrophage function may contribute to numerous diseases of aging, including AMD. Lin et al. showed that miR-50, a microRNA found to be upregulated in macrophages from AMD patients, regulates macrophage-mediated inflammation and may mediate a switch from a healthy to a disease-promoting macrophage phenotype [126]. Indeed, the role of macrophages in the development and progression of AMD is likely multifaceted and may change throughout the disease process.

3.9.3 The Complement System

The complement system consists of over 40 proteins found in the systemic circulation and can be divided into three main pathways: the classical pathway triggered by antibody-antigen complexes, the lectin pathway triggered by polysaccharides (mannose and N-acetyl glucosamine) on microbial surfaces, and the alternative pathway triggered by binding to a host cell or pathogen surface. The function of this system is to create a proinflammatory response to provide defense against pathogens and remove apoptotic cells. It is well recognized that dysregulation of the complement system can lead to immune-mediated damage to host tissue. As such, it has been implicated in a wide spectrum of diseases. The complement system is continuously activated at low levels in the normal eye with tight regulation to maintain activity at a low level, providing microbial defense without host tissue damage [108].

Studies in human AMD eyes have suggested that the complement system may contribute to the pathogenesis of the disease. As noted above, complement components and regulators have been identified in drusen. One component of the system, complement factor H (CFH), seems to play a particularly important role in the development of AMD in both animal and human studies. CFH is known to be expressed in the human eye and acts as a negative regulator of the complement system, impairing activation of the alternative pathway. Impairments in CFH function might therefore result in overactivation of the alternative pathway and a proinflammatory state CFH was the first complement protein to be described in the pathogenesis of AMD following a functional variation in the *CFH* gene that was implicated in AMD in 2005 [47, 127–129]. CFH was also found to accumulate in drusen, RPE cells, sub-RPE cells, the inter-photoreceptor matrix, and the choroid [130]. A large population cohort (Beavers Dam Study) found that *CFH* gene polymorphisms were associated with macular pigmentary irregularities [131]. The Y402H missense variant in the *CFH* gene has been

associated with the presence of soft drusen in an Icelandic population [132], and has also been associated with the presence of bilateral intermediate-to-large drusen [133]. Homozygous individuals with the Y402H variant were also found to have more central and peripheral drusen, although there was no association with drusen size, location, or total area in this study [134]. In another study of 1107 subjects, the Y402H single nucleotide polymorphism was associated with peripheral drusen, while no known AMD-related polymorphisms were associated with the presence of 20 or more small hard drusen [135]. Studying the pathophysiology of the complement pathway and the genetics behind the *CFH* gene variations is important in understanding potential mechanisms for treatment of early AMD.

Animal studies similarly support the role of CFH in AMD pathogenesis. Using CFH knockout mouse models *($cfh^{-/-}$)* Coffey et al. demonstrated that $cfh^{-/-}$ mice had decreased visual acuity and impaired photoreceptor function compared with age-matched controls, suggesting a critical role of CFH for the health of the retina [136]. Similarly, there are variants in complement factor 3 (C3) that reduce its binding affinity to CFH and are associated with the development of AMD [137]. Rare variants in CFI, a factor that converts C3b and C4b to their inactive forms, have also been reported in association with the disease. These variants have been shown to result in diminished CFI production, interfering with the ability to regulate the alternative pathway leading to complement over activation [138]. Geerlings et al. showed that carriers of a C9 variant previously associated with an elevated risk for AMD have elevated concentrations of C9 in serum as compared to non-carriers. They hypothesize that increased C9 level results in elevated complement activation in patients with AMD, which may directly contribute to retinal destruction through lysis of target cells [139].

Given evidence supporting the role of the alternative pathway in AMD pathogenesis, more recent studies have investigated the effect of complement inhibition for the treatment of geographic atrophy (GA) associated with dry AMD. The monoclonal antibody lampalizumab, an inhibitor of factor D, which in turn is an activator of the alternative pathway upstream of CFH, was evaluated in the phase III CHROMA and SPECTRI studies. Both studies failed to meet their primary endpoints of reduction in the mean change in GA size [140]. The phase II FILLY trial investigated APL-2 for the treatment of GA associated with dry AMD. This compound inhibits C3, which plays a central role in all three complement pathways, potentially having a stronger anti-inflammatory effect in comparison to lampalizumab, which inhibits only the alternative pathway. Results from this trial showed a 29% reduction in growth of GA lesions at 12 months in the monthly treatment group (ClinicalTrials.gov Identifier: NCT02503332). As of the writing of this chapter, a phase III trial of APL-2 has begun in 2018. It is clear that the complement system, particularly the alternative pathway, plays a role in AMD pathogenesis. Future studies are needed to explore how selective targeting of this system may offer novel therapeutic strategies for AMD.

3.10 Conclusion

Aging is associated with a number of histological changes in the choroid, Bruch's membrane, RPE, and neuroretina. Outside of the normal physiologic aging spectrum of changes, abnormal deposits such as BlamD, BlinD, and soft drusen are known to be associated with AMD. Progression of AMD to advanced stages involving GA, CNVM, and/or disciform scar can result in debilitating vision loss.

Knowledge of the angiogenic pathway and its components that stimulate neovascularization has led to the development of a new paradigm of intravitreal anti-VEGF pharmacotherapy in the management of neovascular AMD. Currently however, there are no available treatments for the modification of disease progression in non-neovascular AMD, or for the treatment of

geographic atrophy. Further understanding of the histopathology of AMD and the molecular mechanisms that contribute to pathogenesis of the disease may reveal additional therapeutic targets.

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Bruch's Membrane and the Choroid in Age-Related Macular Degeneration

4

Malia Edwards and Gerard A. Lutty

Abstract

A healthy choroidal vasculature is necessary to support the retinal pigment epithelium (RPE) and photoreceptors, because there is a mutualistic symbiotic relationship between the components of the photoreceptor/retinal pigment epithelium (RPE)/Bruch's membrane (BrMb)/choriocapillaris (CC) complex. This relationship is compromised in age-related macular degeneration (AMD) by the dysfunction or death of the choroidal vasculature. This chapter will provide a basic description of the human Bruch's membrane and choroidal anatomy and physiology and how they change in AMD.

The choriocapillaris is the lobular, fenestrated capillary system of choroid. It lies immediately posterior to the pentalaminar Bruch's membrane (BrMb). The blood supply for this system is the intermediate blood vessels of Sattler's layer and the large blood vessels in Haller's layer.

In geographic atrophy (GA), an advanced form of dry AMD, large confluent drusen form on BrMb, and hyperpigmentation (presumably dysfunction in RPE) appears to be the initial insult. The resorption of these drusen and loss of RPE (hypopigmentation) can be predictive for progression of GA. The death and dysfunction of CC and photoreceptors appear to be secondary events to loss in RPE. The loss of choroidal vasculature may be the initial insult in neovascular AMD (nAMD). We have observed a loss of CC with an intact RPE monolayer in nAMD, by making RPE hypoxic. These hypoxic cells then produce angiogenic substances like vascular endothelial growth factor (VEGF), which stimulate growth of new vessels from CC, resulting in choroidal neovascularization (CNV). Reduction in blood supply to the CC, often stenosis of intermediate and large blood vessels, is associated with CC loss.

The polymorphisms in the complement system components are associated with AMD. In addition, the environment of the CC, basement membrane and intercapillary septa, is a proinflammatory milieu with accumulation of proinflammatory molecules like CRP and complement components during AMD. In this toxic milieu, CC die or become dysfunctional even early in AMD. The loss of CC might be a stimulus for drusen formation since the disposal system for retinal debris and exocytosed material from RPE would be limited. Ultimately, the photoreceptors die of lack of nutrients, leakage of serum components from the neovascularization, and scar formation.

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Therefore, the mutualistic symbiotic relationship of the photoreceptor/RPE/BrMb/CC complex is lost in both forms of AMD. Loss of this functionally integrated relationship results in death and dysfunction of all of the components in the complex.

Keywords

Age-related macular degeneration · Bruchs membrane · Choriocapillaris · Choroid · Choroidal neovascularization · Geographic atrophy · Inflammation

4.1 Normal Bruch's Membrane

Bruch's membrane (BrMb) is a pentalaminar extracellular matrix (ECM) that separates retinal pigment epithelium (RPE) from choroid. This thin membrane (2–4 μm thick) extends from ora serrata to ora serrata except at optic nerve head. The anterior-most layer is the basement membrane or basal lamina of the RPE, which is rich in RPE-synthesized laminins that bind RPE integrins [1]. Posterior to the RPE basal lamina is the inner collagenous layer, which is rich in proteoglycans like dermatan sulfate and chondroitin sulfate [2, 3]. The central layer of BrMb is the elastic layer consisting of elastin fibers, fibronectin, and collagen VI [4]. The next layer is the outer collagenous layer that has outward extensions into the intercapillary pillars, which are the structures between choriocapillaris lumens [4]. The most posterior layer is the basal lamina of the choriocapillaris (CC), the capillary system of the choroidal vasculature.

Marshall and associates have documented inactive forms of matrix metalloproteinases (MMPs) 1, 2, and 9 in BrMb [5]. These enzymes are most likely synthesized by RPE and are necessary for remodeling of BrMb, that is, they maintain the homeostatic turnover of this ECM. Diffusion through this membrane is dependent on the local glucose concentration, salts, and pH. Maximum diffusion occurs a pH 5.0. At neutral pH, BrMb has a negative charge [6].

Therefore, the roles of BrMb are to provide basal lamina for RPE and be a semipermeable barrier to control molecular exchange based on size and charge. Any change in the composition or structure of BrMb will affect transport, and subsequently, the function and health of RPE and photoreceptors.

4.2 Normal Choroid

The choroid is the layer immediately posterior to the RPE and BrMb and its outer boundary is the lamina fusca (Fig. 4.1). Choroidal stroma is a dense tissue composed mostly of collagens and glycosaminoglycans. The choroid has a limited population of cells. Its pigmented appearance is due to melanocytes that are throughout the stroma but never in inner choroid in normal subjects. The stroma contains fibroblasts which are probably responsible for generating the glycosaminoglycan and collagen-rich nature of the choroidal tissue. Embedded in the stroma, mostly in the submacula, are ganglion cells (GCs). These GCs cells are only present in primates. Also, in the stroma are inflammatory cells: mast cells, and resident and circulating macrophages.

The relationship with outer retina components is a mutualistic symbiotic forming in essence a photoreceptor (PR)/retinal pigment epithelium (RPE)/Bruch's membrane (BrMb)/choriocapillaris (CC) complex. Each component contributes to the homeostasis and health of the other, and each component is dependent on the other components. The role of the choroidal vasculature is to provide nutrients and oxygen to the RPE/photoreceptor complex and remove waste generated by the RPE. This relationship is quite apparent in the work of Robert Linsenmeier, which demonstrated that photoreceptors live precariously, reaching almost anoxic conditions in the dark. Without the choroid and its vasculature, the photoreceptors could not maintain their high metabolic activity and important function. The juxtaposition of the RPE/BrMb/CC complex permits the CC vasculature to provide 90% of the O_2 consumed by the PR in darkness [8] as well as all of the metabolic needs from serum. This chapter focuses mostly on the human choroid with occasional use of studies in nonhumans where function can be demonstrated.

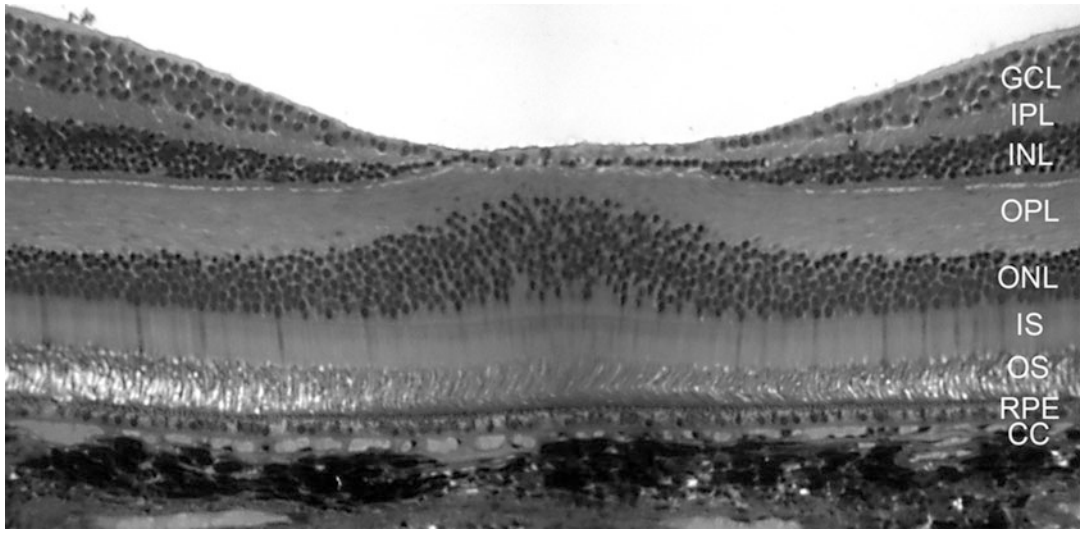

Fig. 4.1 Fovea of a Macaque monkey in cross section illustrates the layers of retina and the morphological relationship of photoreceptor/RPE/BrMb/choroid complex. The layers of the sensory retina are clearly visible to the left and right of the foveal pit (center). The inner most layer of nuclei are the ganglion cells (GCL). The inner plexiform layer (IPL) separates the inner nuclear layer of neurons (INL) from the ganglion cell soma while the outer plexiform layer (OPL) represents the synapses between photoreceptors whose nuclei are in the outer nuclear layer (ONL) and secondary neurons in the INL. The photoreceptor inner segments (IS) are mitochondria-rich and their outer segments (OS) make close contact with the retinal pigment epithelium (RPE), the outer most layer of retina. Bruch's membrane (not discernible at this magnification) separates the RPE from the choriocapillaris (CC). The melanocytes of choroid are the extremely dark structures in monkey below the CC (From Bhutto and Lutty, *Mol. Aspects Medicine* 2012;33;295–317 [7])

4.2.1 The Choroidal Vasculature

The choroidal vasculature is unique in that capillaries are anterior, adjacent to BrMb, and intermediate (Sattler's layer) and large blood vessels (Haller's layer) are posterior to the capillaries. Developmentally, the capillaries form first as blood island-like structures and eventually they anastomose large vessels invading from the central retinal artery [9, 10]. The result is an expansive vasculature that is part the uveal tract, which receives 80% of the blood from the central retinal artery.

4.2.1.1 Choriocapillaris (CC)

The capillary system of choroid is called the choriocapillaris (CC) and it is located immediately posterior to BrMb. The CC is a nonhomogenous network of large (10–38 μm diameter, average diameter 17.7 μm) capillaries. This vascular network is a flattened in the anterior-posterior aspect, and anatomically changes from a dense, honeycomb-like, nonlobular structure in the peripapillary area to a lobule-like pattern in submacular areas and most of the posterior pole and equatorial areas [11]. CC organization in periphery is more fan or ladder-like vascular network and terminates in arcades at the ora serrata [11]. The blood supply from CC is feeding arterioles derived from the short posterior ciliary arteries and it is drained by the collecting venules. These arterioles and venules form Sattler's layer, the intermediate-sized vessels of the choroid, which occupy most of the choroidal stroma. The majority of these vessels in the peripapillary and submacular areas form a 90-degree angle with the posterior aspect of the choriocapillaris. The size of choriocapillaris lobules varies from 0.6 to 1.0 mm.

The idea that functional "lobules" exist and subdivide the choroid into many functional islands is controversial. One hypothesis suggests

that the CC is a single, continuous vascular layer and that anatomical lobules were functionally interconnected. Hayreh [12] has suggested that there are noncommunicating lobules. Using fluorescein angiography, he described a lobule having one containing an arteriole in the middle and a venule at its periphery. Our histological examination of the lobules suggests that lobules are interconnected and arterioles and venules are not uniform in their location within lobules [11].

Between the capillary segments there are intercapillary septa, which are pillar-like glycosaminoglycan (GAG)-rich structures. The CC endothelial cells have peg-like processes, mostly on the RPE side, that protrude through their basement membrane sometimes reaching BrMb [13]. The function of these processes is not clear but they may stabilize the CC vessels, or serve as chemoreceptors or mechanoreceptors [6]. The CC is fenestrated and the fenestrations are so numerous that they resemble a sieve plate when viewed by freeze-fracture replication [13]. The diameter of the fenestrations is 50–65 nm but the pores have diaphragms composed in part of PV-1, the product of the *Plasmalemmal Vesicle Associated Protein* (*Plvap*) gene [14], which divides the pore into eight. So the radial arc is reduced to 5.46 nm, similar to the diaphragms in other fenestrated capillaries, permitting passage of small molecules and solutes only [15, 16]. The pore has a GAG-like glycocalyx cover that is net negative, which was characterized in part by Pino et al. [17]. Most macromolecules like albumen are transcytosed by caveolae [18], which contain receptors for many macromolecules in other tissues [19]. There are also transendothelial cell channels (TECs), coated pits, and vesiculovascular organelles (VVOs), although these latter two structures are rare in normal CC. The suggestion by many authors that CC is leaky and is incorrect [20]. The CC endothelial cells normally have tight junctions and transport is tightly controlled by receptors in its transcytotic transport systems. Another unique characteristic of CC is that it is sided in many ways. The fenestrations are predominantly, but not exclusively, on the retinal side [21]. VEGF receptor-1 and -2 (VEGFR-1 & -R2) are present predominantly on the retinal side of the capillaries [22]. The CC is dependent on RPE-derived VEGF for maintenance of the fenestrations and for its survival [23]. Another unique characteristic of CC is that CC endothelial cells (CC EC) constitutively make intracellular adhesion molecule-1 (ICAM-1) [24]. ICAM-1 is responsible for firm adherence of macrophages and neutrophils that express CD11b/CD18 to CC EC.

4.2.1.2 The Blood Vessels of Sattler's and Haller's Layers

Intermediate (Sattler's layer) and large blood vessels (Haller's layer) that lie posterior to the capillaries are the source of blood for CC. This three-layered vasculature is most apparent in the submacula whereas peripheral choroid has barely two layers and a less dense CC. There are several opinions about the location of arterioles and venules in the lobule depending on the technique used. Shimizu and Ujiie [25] central arterioles and the peripheral location of the venule in lobules. McLeod and Lutty [11], using alkaline phosphatase (APase) stained choroidal flatmounts, observed arterioles and venules located almost randomly in the lobules. Using vascular casts, Fryjcowski found that arterioles and venules had standard positions in the lobule. In the posterior pole, the entry of arterioles into the lobule is sometimes at a right angle. A given arteriole may actually have many secondary arterioles feeding the lobule [26].

Arteries

The three main arterial sources of blood to the choroid are: (1) long posterior ciliary arteries (LPCA, temporal and nasal); (2) short posterior ciliary arteries (SPCA); and (3) anterior ciliary arteries. LPCA have an oblique intrascleral course. The LPCA's send branches from the ora serrata region posteriorly to supply the choroid as far posterior as the anatomical equator and travel in the potential suprachoroidal space. Each LPCA is accompanied by a ciliary nerve and these arteries often suffer from scleritis. There are 15 to 20 SPCAs that supply the choroid from equator to optic nerve. Their distribution is

perifoveal, peripapillary, or the papillomacular oval. The circle of Zinn are the arteries that surround the optic nerve in a wheel-shaped arrangement. Hayreh, one of the pioneers in the study of the choroidal vasculature, has documented choroidal "watershed zones" in primates, areas that normally fills slowly with blood. The radial areas that are supplied by the arteries are separated by triangular-shaped "watersheds" with the apex of the triangle directed toward the fovea. Hayreh has proposed that the choroidal vasculature is strictly segmental, an end-arterial system, and its watershed zones are situated between the short PCAs, the arterioles, and the vortex veins [12]. The end-arterial nature of the choroidal vasculature and the existence of watershed zones in the choroid are of great clinical importance and may play a significant role in the production of ischemic lesions in the choroid. Finally, the anterior ciliary arteries pierce the anterior sclera and send recurrent branches posteriorly to supply the choroid at 3 o'clock and 9 o'clock. All arterial systems rapidly extend internally via arterioles to supply blood to the choriocapillaris.

Choroidal Veins (Vortex Veins)

The main venous drainage of the choroid is provided by four to six vortex veins, which drain into superior and inferior ophthalmic veins. In the macular region, the venous portion predominates over the arterial one. The venous plexus becomes less dense with increasing distance from the macula. The veins of the macular region are characteristically tortuous while in the extramacular region the vessels are straighter. Venous drainage occurs in quadrants, with watersheds oriented horizontally through the disc and fovea and vertically through the papillomacular region. The macula is centered over both arterial and venous watersheds, which may either prevent ischemia through multiple submacular blood supplies or predispose it to relative ischemia. Submacula is the only region of choroid that has a distinct three-layered vasculature.

4.2.1.3 Physiology and Blood Flow in Choriocapillaris

Historically the choroid was thought to lack autoregulation, because there is no metabolic regulation [27]. The CC was not constricted after exposure of neonatal dogs to 100% oxygen for 4 days [28]. The PO_2 in cat choroid is normally around 70 mm Hg but, in hyperoxia, it can increase to 250 mm Hg [29]. Elevated intraocular pressure or systemic hypoxia causes PO_2 to decrease. Recently, there is evidence for some autoregulation, for example in response to change in intraocular pressure change [30] or change in perfusion pressure caused by isometric exercise [31, 32]. The evidence for regulation was reviewed by Nickla and Wallman [33]. The choroid is innervated by parasympathetic and sensory nerves that cause vasodilation while sympathetic nerve fibers stimulate constriction. The innervation of choroid is elaborated in the review by Reiner and associates [30].

The retinal vasculature supplies oxygen to inner retina while CC supplies oxygen to outer retina (photoreceptors and RPE). Approximately 80% of the blood from the ophthalmic artery enters the uveal tract of which the choroidal vasculature is the major component. Using radioactive microspheres [34] or krypton 85 [35], blood flow in cat choroid was found to be at least 10 times higher than retinal blood flow. However, when red blood cell (RBC) velocity in CC was assessed, the velocity was actually 4 times slower than in inner retinal capillaries [36, 37]. This seeming discrepancy is due to techniques employed where the former values are based on flow in all choroidal vessels, whereas, the RBC velocities were measured directly in CC. Choroidal blood volume is quite large, whereas volume has little to do with velocity. CC has no linear vascular segments because of its lobular nature and the arterioles posterior to CC are often at right angles to the capillaries. These characteristics undoubtedly contribute to slow RBC velocities in CC [36, 37]. The shapes of the capillary lumen may also contribute as because they are broad and flat, not round tubes allowing faster flow. Retinal detachment from

choroid is extremely detrimental to proper photoreceptor function and viability. Wangsa-Wirawan and Linsenmeier have suggested that hyperoxia might be used therapeutically to rescue photoreceptors after retinal detachment [8].

4.2.2 Non-vascular Cells of Choroid

There are several nonvascular cells in choroid. Melanocytes and fibroblasts may account for the largest number of nonvascular cells in choroid. Very little is known about the role of melanocytes in choroid. In submacular primate choroid there are ganglion cells, which are mentioned in the next section. Also, in the submacular region in primates there are nonvascular smooth muscle cells. There are also inflammatory cells in choroid, including mast cells and macrophages (resident and circulating), which will be discussed in Sect. 4.3. Mast cells are intimately but not exclusively associated with arteries and arterioles in humans, while macrophages may interact with any choroidal blood vessels [38–40].

4.2.2.1 Innervations of the Choroid

Human choroidal stroma has an intrinsic network of nitrergic ganglion cells that are NADPH-diaphorase and nitric oxide synthase (NOS) positive. These ganglion cells are interconnected and connected to a perivascular network of neurons [41]. This plexus is present in the choroid of foveate animals and are concentrated mainly in the submacular region of the human choroid. These ganglion cells are mostly polygonal and have diameters ranging from 10 to 40 μm. Most are located close to the walls of large arteries and none are observed near the CC [42]. Nitric oxide (NO) is the mediator of endothelium-derived vascular relaxation is in the cat choroid [42–48]. Vasointestinal polypeptide (VIP) is another vasodilator and it colocalizes with NOS [49–52]. Neuronal nitric oxide synthase (nNOS) is scattered cells throughout the choroid but localized mostly to perivascular neurons and the RPE while endothelial NOS (eNOS) was associated with choroidal ECs [53]. NO-induced vasodilation causes a reduction in arterial blood pressure. Parver's work suggested that the vasodilation could provide protection of the retina from thermal damage associated with light exposure [54, 55].

4.3 Pathological Changes in Age-Related Macular Degeneration (AMD)

Ten to eighteen percent of individuals between 65 and 75 have lost some central vision as a result of AMD [56] while 30% of those aged 75 years or older lost vision [57]. There is a decline in sharp, central vision in AMD because the macula is most affected. The diagnosis of AMD is based on visual dysfunction and characteristic pathological macular characteristics. The two major types of AMD are exudative or neovascular AMD (nAMD) and non-exudative or dry AMD. Approximately 10–15% is nAMD while the majority of patients with AMD have the dry form, which progresses more slowly than nAMD. In geographic atrophy (GA), an advanced form of "dry" AMD, the fovea is often spared from degeneration initially. In the studies of Sunness et al., the total atrophy area enlarged a median of 0.9 disc areas per year in GA [58].

4.3.1 Bruch's Membrane in Aging and AMD

There is progressive destruction of BrMb with age. BrMb increases in thickness with age and the changes are more significant in the posterior pole than in periphery [59, 60]. Disease risk is associated with age-related thickness of this structure [61, 62]. John Marshall's lab has demonstrated that BrMb hydraulic conductivity, ease with which fluids flow across BrMb, precipitously declined with age. The maximal capacity for fluid transport was reduced 50% for every 17 years of life [63]. In part, this is due to progressive accumulation of lipids in BrMb [64, 65]. There is a linear increase in lipid content of BrMb with age, while hydraulic conductivity declines with age [4]. As esterified cholesterol

(EC) rises linearly with age from zero in young subjects to variable high levels in aged donors, hydraulic resistivity of BrMb increases [4]. Curcio and associates determined the EC-rich material was lipoprotein-containing apolipoprotein B [66]. The Curcio lab has determined that the bulk of the lipids in BrMb in aging and AMD are of RPE origin, based on their unusual lipid profiles [4, 66, 67]. Rodriguez suggested that lipid was oxidized in BrMb and CC based on his isolation of oxidized lipid in retina [68]. Oxidized LDL is injurious to RPE, can initiate chronic infection, and is a critical contributor to atherosclerosis [69]. By the seventh decade of life, the inner collagenase area of BrMb is filled with lipid, which Curcio has termed the "lipid wall." Not only is there a "lipid wall," but the elastic layer of BrMb begins to calcify. In addition, advanced glycosylation end products (AGEs) form in BrMb and in the intercapillary septa of CC [70]. AGEs promote retention of lipids in BrMb through lipoprotein lipase [70].

One result from these changes in BrMb is a 90% decrease in transport across BrMb with age and proteins larger than 100 kDa have a significantly decreased flux across it [71]. This leads to deposition of material on and in BrMb. Marshall's lab found pro- and active forms of MMP-2 and MMP-9 are exponentially increased in aged BrMb [72]. They hypothesized that these were sequestered in BrMb during aging and may be responsible for the thickening of BrMb in aging. In the mass spectroscopy studies of Yuan et al. on BrMb/CC complex, they found 43 were reduced in AMD compared to aged controls and 56 were elevated in AMD [73]. About 60% of the elevated proteins are involved in immune response. Galectin 3, an advanced glycosylation end product (AGE) receptor, was the most elevated protein in dry AMD. BrMb shows extensive thickening and formation of deposits with aging and more so in AMD with the greatest thickening in the posterior pole compared to the periphery [74]. Karwatowski et al. found that collagen solubility declined with age [75].

Deposition of material in and on BrMb is a risk factor for AMD. The deposition takes the form of basal linear (BlinD) and laminar (BlamD) deposits, as well as drusen, hard and soft. One hypothesis on the genesis of drusen states that the deposit of the debris on/in BrMb is a result of CC insufficiency. Alternatively, debris may accumulates on/in BrMb, preventing transport to CC, which induces atrophy since it is not needed or able to perform transport any more [76, 77]. The proteome Crabb found in drusen of AMD subjects had oxidative protein modifications and carboxyethyl pyrrole (CEP) protein adducts [78]. CEP is formed by the oxidation of docosahexaenoate-containing lipids, which are abundant in the photoreceptor outer segments. AMD-like changes were observed in the mice when Hollyfield and colleagues injected CEP adducts into mice [79–81]. Circulating antibodies to CEP were found in AMD subjects by Gu et al. suggesting that CEP antibodies might be used as a biomarker for AMD [82].

Hard drusen are refractile so they appear as yellow white deposits with fundus photography. They are small (less than 63 um) sharp edged deposits that form between the RPE basal lamina and the ICL. This is the same compartment as the "lipid wall" proposed by Curcio. We and others have found that hard drusen are not over CC lumens but rather over intercapliary septa almost exclusively [83], suggesting that this material accumulates on/in BrMb where transport is least likely. Mullins et al. found that the incidence of drusen was inversely associated with CC density, that is, the number of drusen increased with CC dropout [84]. Numerous hard drusen are independent risk factors for vision loss in AMD [85, 86]. All drusen contain esterified and unesterified cholesterol, as well as phosphatidylcholine and other phospholipids [87, 88]. Soft drusen are much larger, have indistinct edges, and appear fluffy in cross section. Sarks referred to the contents as "membranous debris" [89] but, more accurately, it could be called "lipoprotein-derived debris" [4]. Large confluent soft drusen are independent risk factors for AMD [90]. Interestingly, there can be spontaneous resolution of soft drusen in GA.

A third kind of deposit, basal linear deposit (BLinD), has been associated specifically with

progression and severity of AMD. These are not visible clinically or even by light microscopy. Green and Enger observed BLinD with Transmission Electron Microscopy (TEM) as a thin sub-RPE layer of membranous profiles [91]. Sarks et al. hypothesized that they were membranous bodies released from the basal plasma membrane of the RPE and became entrapped in BrMb, because they were unable to enter the collagen fibril meshwork of the ICL [89]. Starita et al. found that the ICL imparted the major resistance to fluid movement between RPE and choroid [92] explaining the location of BlinD. Curcio and Millican demonstrated that BLinD and large drusen with membranous contents are strongly associated with early AMD compared with basal laminar deposit (BLamD) [93]. BlinD have high lipid content including apoB and apoE [88, 94]. With TEM this deposit is observed between the ICL of BrMb and the basal lamina of the RPE.

BLamD consists of diffuse heterogenous material that lies internal to the RPE basal lamina. BLamD is easily identified with PAS staining as a flocculent material with light microscopy. Only TEM permits one to distinguish between BLamD and BLinD because BLamD is between the RPE cytoplasmic membrane and the basal lamina of RPE, while the BLinD is found between the basal lamina of RPE and the ICL [93]. TEM analysis demonstrates that long spaced collagen is a dominant constituent of BLamD whereas membranous debris is the major constituent of BLinD [89, 93].

4.3.2 Changes in Choroid in AMD

Before Optical Coherence Tomography (OCT), the role of choroid in AMD was controversial. Using enhanced depth imaging (EDI) spectral domain OCT, Spaide has observed a 16 μm decrease in choroidal thickness per decade of life [95, 96]. A more extreme choroidal thinning (5.4 μm reduction per year) was observed by Ding et al. [97]. On the contrary, no difference in choroidal thickness between nAMD and control subjects was observed by Kim et al. [98]. The change in retinal thickness found by Wood et al. in AMD subjects compared to controls probably was due to photoreceptor loss, but they found no significant difference in choroidal thickness between cohorts [99]. The accuracy of measuring choroidal thickness with spectral domain OCT has been questioned [100]. In our histologic specimens, we find that choroidal thinning is present in almost all of the AMD subjects (Imran Bhutto and Gerard Lutty, unpublished data, 2011).

Friedman hypothesized that loss of PR and RPE in AMD was due to vascular insufficiency [101]. He also hypothesized that accumulation of waste at and on Bruch's membrane, that is drusen formation, was due to lack of transport by CC [102]. The diameter and density of the CC and intermediate choroidal vessels substantially decline with age, suggesting a decrease in choroidal blood flow and blood volume [61]. This was confirmed by Grunwald and associates who found using laser Doppler flowmetry that foveolar blood flow declines in aging and declines further in AMD [103–106]. Friedman championed the hypothesis that AMD is a hemodynamic sequela of atherosclerotic changes in the postcapillary resistance of the choroidal vasculature [107]. Other vascular insufficiencies observed with ICG angiography in hypertension could explain angiographic choroidal filling defects in AMD [108, 109].

4.3.2.1 Changes in the Choroidal Vasculature in Early AMD

We recently developed a flat mount technique for confocal imaging of the human choroidal vasculature [110]. The choroids were stained with *Ulex europaeus* agglutinin lectin (UEA), which Mullins had previously shown to stain only viable choroidal blood vessels [84]. We found a 20.5% reduction in vascular density in early AMD subjects in submacular CC (Fig. 4.2) [110]. Furthermore, hypercellular capillaries that appeared to be "buds" of neovascularization were present in these areas of submacular capillary dropout in 22% of the early AMD eyes and 40% of the intermediate AMD eyes. We have subsequently determined that some of these "buds" are the earliest form of CNV [111]. Interestingly, the areas with CC loss often had arteriosclerotic and

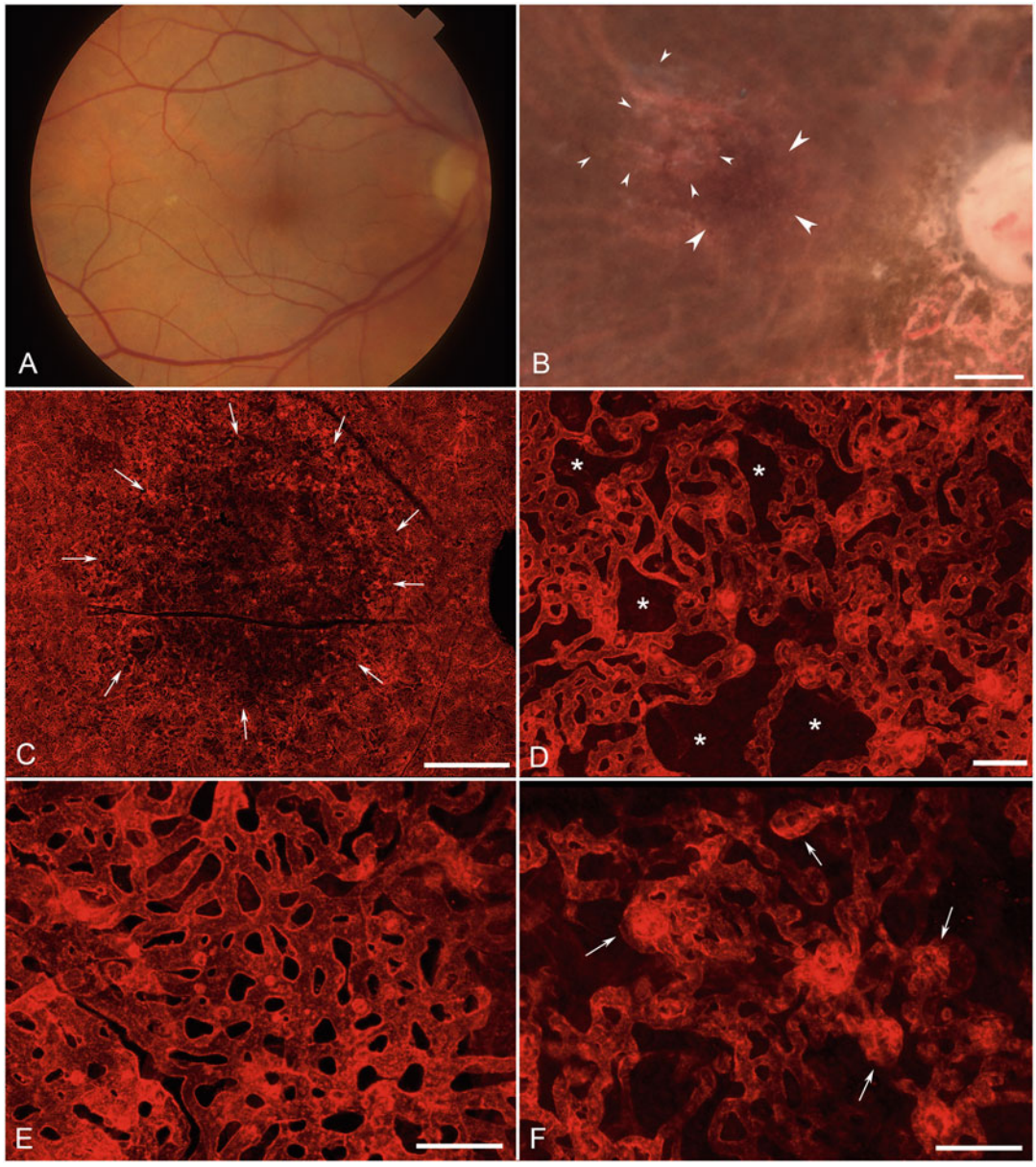

Fig. 4.2 An 86-year-old Caucasian female with a history of early stage AMD. The fundus photo (**a**) shows drusen in the macula. Postmortem gross photo of the eye cup after removing the retina (**b**) shows confluent drusen (small arrowheads) and pigmentary abnormalities (large arrowheads). After gross photographs were taken, the RPE was removed with EDTA so that lectin and antibody staining could be done. Low magnification confocal micrograph of the UEA (*Ulex europaeus* agglutinin lectin) stained choroidal flat mount shows a submacular region of CC pathology (arrows) that was 10.47 mm^2 in size (**c**). The same area at higher magnification demonstrates loss of interconnecting capillary channels (asterisks) and narrowing of the CC lumen in submacula (**d**). Compare that to CC in the perimacular region where CC has broad diameter lumens that freely interconnect (**e**). Small apparent neovascular buds (arrows) were observed at the border of submacular CC atrophy (**f**). The mean %VA was 43 ± 8.7% in the submacula versus 76 ± 2.8% in the perimacular region. The mean CC diameter was 13.51 ± 0.72 μm in the submacular region compared to 17.35 ± 1.76 μm in the perimacular region (Scale bars: **b**, **c** = 1 mm, **d**–**f** = 50 μm) (From Seddon et al., *Jama Ophthalmol*. 2016;134:1272–1280 [110] with permission)

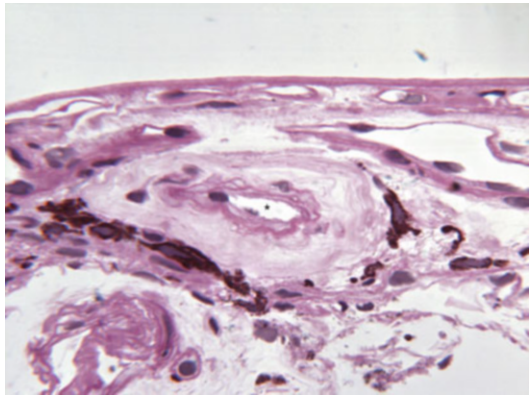

Fig. 4.3 Arteriole from a 90-year-old hypertensive Caucasian male with early AMD. After analysis as in Fig. 4.2c–f, this choroid was embedded in JB4 polymer and sectioned. In this area with early loss of CC, there is an arteriole with a sclerotic wall and constricted lumen in Sattler's layer (*). The artery in bottom left has hypertensive changes, that is, reduplication of the elastin. (PAS and hematoxylin)

hypertensive changes in Sattler's layer arterioles (Fig. 4.3). Esmaeelpour has recently observed with OCT thinning of Sattler's layer arterioles and Haller's layer arteries [112]. We hypothesize that these early changes may represent the flow voids that have been documented by swept source OCT [113]. Flow voids are areas where RBC velocity is reduced in CC. If these flow voids are actually the areas of early CC loss, they may be biomarkers for subsequent advanced AMD.

4.3.2.2 Changes in CC During GA

GA is an advanced type of "dry" AMD, which is characterized by a sharply defined focal areas of RPE atrophy that are associated with varying degrees of loss of the CC. Early in GA pigmentary abnormalities and drusen are observed in the macula. As GA advances, retina thins and the photoreceptors in the macula. There is no known therapy for GA but the progression of GA may be slowed by antioxidants and zinc [114, 115]. Bird proposed that photoreceptors degenerated first in a majority of cases of GA because photoreceptor loss is seen outside the area of atrophy and in eyes without RPE loss [116], but this is still unsettled.

Staining of choroid for endogenous alkaline phosphatase activity (APase), an indicator of endothelial cell viability and functionality, has allowed us to quantify changes in RPE and CC in choroids from postmortem human eyes of subjects with AMD [11, 117]. The choroids were partially bleached so that choroidal melanocyte melanin was completely and RPE melanin was beige and bleached (Fig. 4.4). The loss of RPE and CC was quantified by using two techniques for illumination to capture images, transmitted light to view blue APase activity and epi-illumination to view partially bleached RPE (Fig. 4.4). Adobe Photoshop and Image J software were then used to determine the number of blue APase pixels in viable choroidal blood vessels (area of vasculature or percent vascular area) or the number of tan pixels in RPE yielding percentage of Bruch's membrane covered by RPE [117]. This permitted the loss of CC and RPE to be correlated. These choroids were the embedded flat in glycol methacrylate so that areas of interest could be sectioned following image analysis, permitting areas documented by image analysis to be documented in cross section.

In classic GA, as documented by Sarks and Sunness [58, 119], the RPE and photoreceptor degeneration occur in a horse shoe-shaped pattern surrounding the fovea, often sparing the fovea initially, as shown in Fig. 4.4a. The loss of CC appeared to be a secondary event in their studies [58, 119]. We found a linear relationship between the loss of CC and RPE using our APase technique on GA subjects. A 15% reduction in vascular area was found in regions of complete RPE atrophy in GA but no area was completely devoid of CC [118] (Fig. 4.4). The border of the RPE atrophy was clearly delineated and coincided closely with the area of decreased choroidal vascular density; however, there were areas with RPE loss at the border that had normal appearing CC pattern. This suggested that RPE loss occurred in advance of CC death. This agrees with the experimental observations of Korte et al. that loss in RPE results in loss of CC [120] and basic science studies in which RPE expression of VEGF is eliminated and CC die [23].

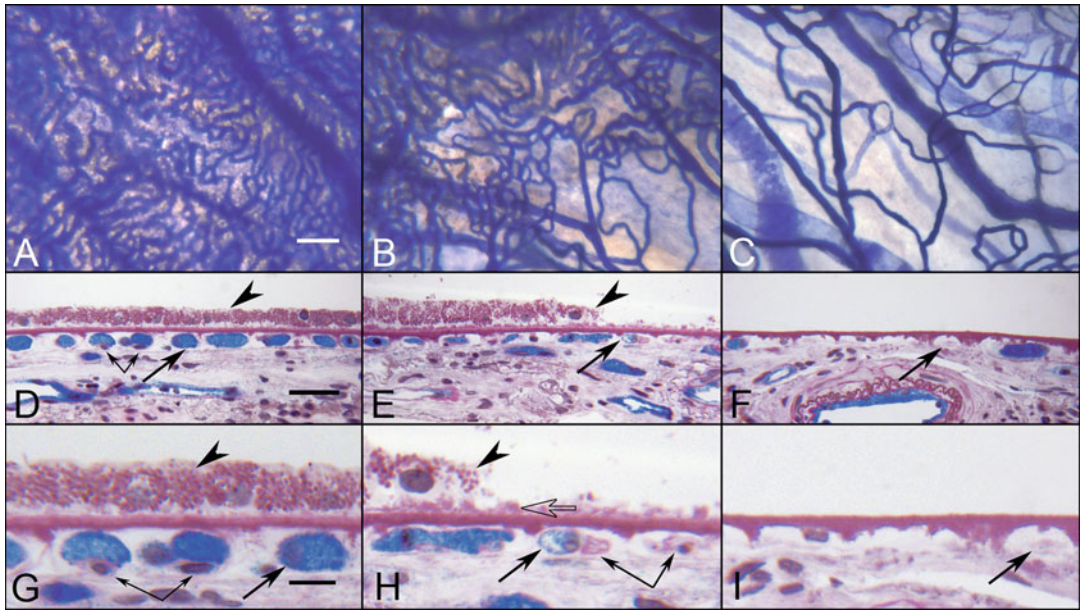

Fig. 4.4 Choroid from an 88-year-old Caucasian male with GA that was incubated for endogenous APase-activity (blue) that demonstrates a nonatrophic region (**a**, **d**, **g**), border region (**b**, **e**, **h**), and atrophic region (**c**, **f**, **i**) in flat perspective viewed with transillumination prior to embedment (**a**–**c**) and in cross sections stained with PAS and hematoxylin (**d**–**i**). (**a**) CC in the nonatrophic region (**a**) has broad diameter lumens filled with serum APase (arrow in **d**, **g**). The APase^{+} endothelial cells and pericytes are under viable RPE (arrowhead in **d**, **g**). RPE appears hypertrophic in the border region (arrowhead in **e**, **h**), and CC appears constricted (arrow in **e**, **h**) with some being completely degenerated (no APase, paired arrows **h**). A thin basal laminar deposit is associated with Bruch's membrane (open arrow) in this area. Many capillaries in the atrophic region have degenerated leaving only collagenous tubes and remnants of basement membrane material (arrows in **f**, **i**). (Scale bar = 100 μm in **a**–**c**, 30 μm in **d**–**f**, 10 μm in **g**–**i**) (Fig. 4.10 from McLeod et al., *Invest. Ophthalmol. Vis. Sci 50*:4982–4991, 2009 [118] with permission)

The surviving viable capillaries in the area of complete RPE loss appeared highly constricted but expressed APase [118] (Fig. 4.4). Morphometric analysis of the viable CC in the RPE atrophy area demonstrated that CC had significantly smaller diameters compared normal areas of the GA eyes and control subjects and in ($P < 0.0001$). Bhutto et al. found a significant reduction in vascular eNOS as well as nNOS in neurons and RPE in AMD choroid [53]. The severe constriction in these surviving capillaries could be explained by the presumed reduction in NO in AMD. The extreme constriction of surviving CC undoubtedly appears as a lack or poor perfusion of CC in the atrophic area using OCT angiography (OCTA) [121, 122]. Using TEM and OCT, we have observed choroidal venules at the level of CC in areas of atrophy, suggesting a collapse forward of choroidal vasculature as CC is lost and choroid thins [123].

We observed clinically undetected CNV in the periphery and even the macula in some of our GA subjects, using the APase flat embedding technique. These CNV formations were always associated with surviving RPE cells. Sunness also observed CNV [124]. Eighteen percent of GAS subjects developed CNV in the study eye by 2 years and 34% by 4 years, if patients had CNV in the fellow eye. RPE cells associated with CNV in our GA specimens suggests that RPE cells may provide a stimulus for new vessel formation or stabilization.

4.3.2.3 Changes in CC During Neovascular AMD (nAMD)

Neovascular AMD (nAMD) is characterized by formation of abnormal choroidal neovascularization (CNV) from the submacular choroidal vasculature (Fig. 4.5). The CNV leaks fluid into the retina and the subretinal space. CNV in nAMD was divided into classic or occult CNV historically. Classic CNV, now called type 2, was defined as distinct or well demarcated with fluorescein angiography (FAG) whereas occult CNV (type 1) was obscure or poorly demarcated with FAG. These abnormal blood vessels eventually lead to disciform scar, which leads to permanent loss of central vision.

APase analysis as well as the UEA staining technique of nAMD subjects yielded a very different picture in terms of CC viability compared to GA [110, 118]. We observed CC loss adjacent to CNV with both techniques (Fig. 4.5) [110, 118], which we previously observed in diabetic choroidopathy using our APase technique [11, 126]. In nAMD, there were large areas with reduced $APase^{+}$ CC/UEA+ CC vascular segments around the CNV that were completely covered with RPE (Fig. 4.5a, b, d, g). Viable RPE was always associated with the significant attenuation of viable CC. Areas without RPE had greatly reduced viable CC as we observed in GA. The anterior tips of the viable CNV channels had intense APase activity (transmitted light, Fig. 4.5b) and there was a 15% reduction in viable CC adjacent to the CNV [118]. So, the percent vascular area was 40% instead of 8% seen in controls in submacula, while the RPE density was 100%. This suggested that nAMD had a vascular etiology.

Disciform scar is the final pathologic insult after CNV formation and leakage of serum proteins. CNV was often present within the scars but these vessels appeared to be stabilized, that is, not leaking and not growing. The components of scar may stabilize the CNV. Two endogenous inhibitors of angiogenesis, PEDF and thrombospondin-1, were prominent scars [127].

In summary, every active CNV formation (high APase activity and intense UEA staining) had surviving RPE associated with it as represented by the schematic Fig. 4.6. Loss of CC occurs in the presence of RPE in nAMD. We hypothesize that CC loss results in ischemic RPE and then RPE produces hypoxia-inducible angiogenic factors like VEGF, which stimulate growth of neovascularization from CC or venules which invades BrMb and grows subretinally (Fig. 4.6).

4.3.3 Angiogenic Factors and CNV

The growth of neo blood vessels from the choroid into or sub-RPE or the subretinal space is called choroidal neovascularization. CNV occurs in a variety of chorioretinal diseases [128] including diabetic choroidopathy [126]. AMD is the most frequent cause of CNV [129], yet the exact mechanism for CNV is not yet understood. The progression of CNV is as follows: (1) endothelial cells (EC) from the CC proliferate and migrate toward the retina through the BrMb in the initiation stage; (2) when CNV expands this is the active stage; (3) finally, when the CNV becomes fibrotic and forms a disciform scar this is the involution stage [130].

Angiogenesis, the growth of neovessels from preexisting blood vessels, occurs when the balance between factors that stimulate and factors that inhibit vessel growth are tipped toward stimulators or angiogenic factors. Inhibitory factors, anti-angiogenic factors, predominate and vessels remain quiescent in most tissues. In contrast, in states like nAMD, neovascularization occurs because of decreased production of anti-angiogenic and/or increased production of angiogenic factors [131]. Matrix metalloproteases and other enzymes are required in angiogenesis to digest the surrounding ECM or basement membrane, permitting EC migration from the existing blood vessels into the surrounding tissue.

VEGF is assumed to be the major angiogenic factor stimulating CNV and, indeed, anti-VEGF therapy controls the spread of CNV in the majority of cases. However, there are many patients

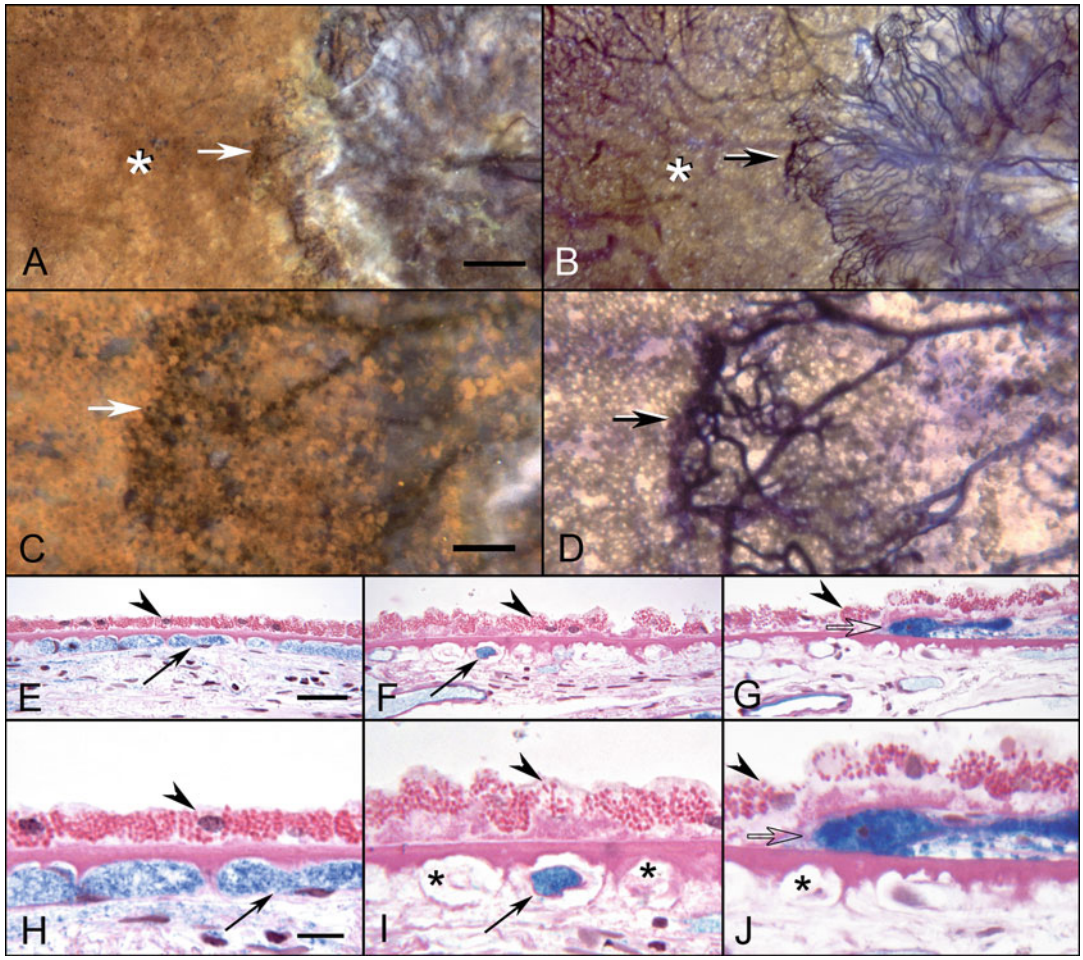

Fig. 4.5 APase-incubated choroid from the 81-year-old Caucasian female with nAMD showing submacular CNV using epi-illumination (**a**, **c**) and transillumination (**b**, **d**). The edge of the CNV is closely associated with viable RPE (arrows **a**, **b**). Areas of CC dropout (reduced APase activity) are evident at the edge of the CNV (asterisks **a**, **b**). The equatorial region (**e**, **h**) in PAS and hematoxylin stained sections has broad capillaries (arrows) containing serum APase with both $APase^{+}$ endothelial cells and pericytes. The RPE has a normal morphology (arrowhead) with no deposits associated. One mm beyond the CNV (**f**, **i**) only a few capillaries are viable (arrows) in sections and many degenerative capillaries are present (asterisks in **i**). Hypertrophic RPE (arrowheads) are present and as well as a basal laminar deposit. Sections taken through the edge of the CNV (**e**, **h**) demonstrate degenerative capillaries (asterisk in **j**), sub-RPE neovascularization (open arrow), which has hypertrophic RPE overlying this leading edge of the CNV. (Scale bar **a**, **b**, 2 mm; **c**–**e**, 30 μm; **f**–**h** 10 μm) (modification of Figs. 4.6 and 4.11 from McLeod et al., *Invest. Ophthalmol. Vis. Sci 50*:4982–4991, 2009 [118] with permission)

who do not respond to anti-VEGF therapy. Many other growth factors like the fibroblast growth factor (FGF) family members, insulin-like growth (IGF) factors, and angiopoietins could contribute to angiogenesis. Muller cells, ganglion cells, EC, pericytes, photoreceptors, and RPE are potential sources of VEGF [132–136]. Secretion of VEGF by RPE is polarized, the highest secretion is basal toward BrMb whereas apical secretion toward photoreceptors is less in normal subjects [22]. VEGF is both a specific EC mitogen and promoter of vascular permeability. Immunohistochemical expression of VEGF has been shown in surgically excised CNV [133, 134, 137, 138] and

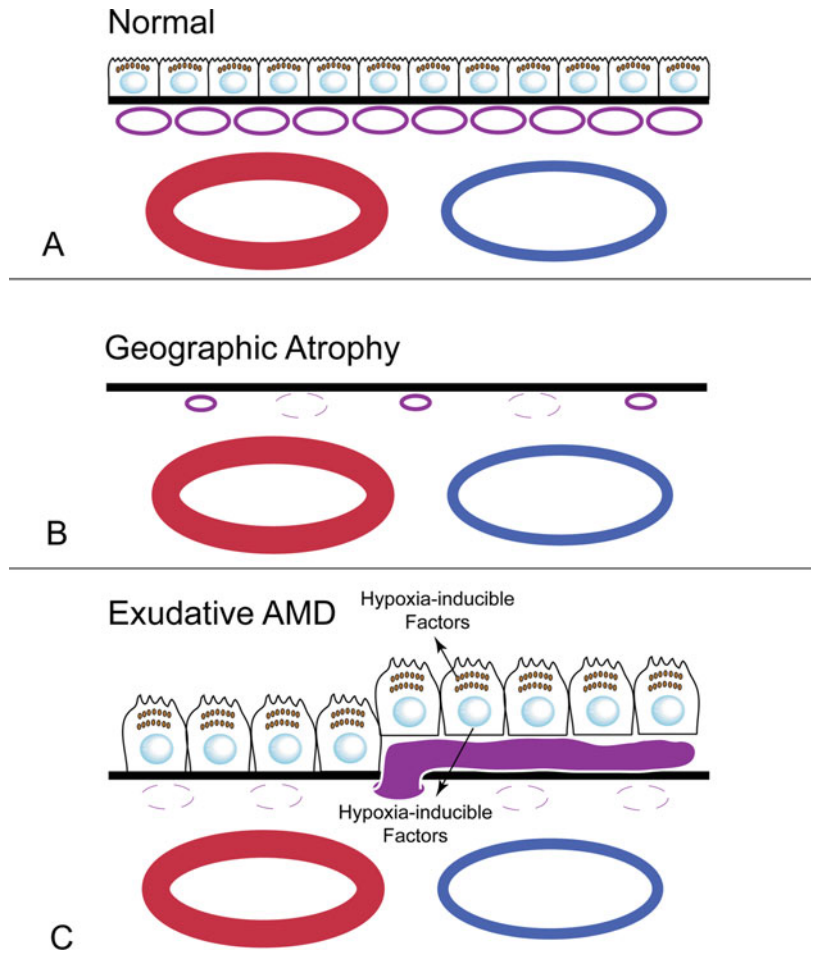

Fig. 4.6 Schematic of a normal RPE/BrMb/CC complex (**a**) and the changes that occur in GA (**b**) and nAMD (**c**). (**a**) The CC (purple) is under BrMb (Black line) and below the CC are large choroidal blood vessels (blue/red). RPE resides on top of BrMb. (**b**) RPE is lost in GA and then the CC becomes attenuated but some CC segments survive but are highly constricted. (**c**) CC is lost while RPE remains in nAMD. We hypothesize that the RPE becomes hypoxic and produces hypoxia-inducible growth factors like VEGF, which stimulates the formation of CNV (solid purple blood vessel) (Fig. 4.5 from Bhutto and Lutty, *Mol. Aspects Medicine* 2012;33;295–317 [7] with permission)

the vitreous levels of VEGF were significantly higher in patients with nAMD [139].

Macrophages may be important players in the initiation stage due to production of cytokines. Macrophages and EC produce MMPs, which, in turn, could degrade BrMb allowing CNV infiltration of retina [140]. It is unknown whether macrophages invade the affected areas after the CNV breaks through BrMb or they actively cause breaks in BrMb (via production of collagenase/elastase) [140]. CNV enlargement may depend on the presence of infiltrating macrophages responding to cytokines in the area or producing cytokines in an autocrine/paracrine manner. Interleukins 2, 6, and 10 may contribute to CNV expansion, but their exact roles have not been investigated thoroughly yet [141, 142]. As mentioned in the previous section, RPE is probably hypoxic due to loss of CC and make VEGF or other hypoxia-inducible growth factors.

The most important molecules for the involutional stage of CNV, may be TGF-β and TIMP-3, which are both are produced by RPE. These molecules affect both the secretion of ECM and the tissue remodeling. Maturation of vessels and formation of scar tissue are the final outcome. The process of subretinal fibrosis is not yet completely understood. It is known that RPE cells directed by TNF-α, TGF-β, and other growth factors, dedifferentiate and proliferate (a process called epithelial to mesenchyme transition) and, together with choroidal fibroblasts, initiate wound repair or scar formation [143]. Anti-angiogenic factors discussed in the next section are present in scar and may act to stabilize the neo blood vessels that remain in the scars.

4.3.4 Loss in Anti-Angiogenic Factors in the BrMb/CC Complex

The stability of blood vessels is due to a balance between angiogenic factors and anti-angiogenic or angiostatic factors. Blood vessels are quiescent and stable as long as the angiogenic factors do not overpower the angiostatic inhibitors. Inflammation or ischemia can tip the balance toward angiogenic factors that are released by the injured or hypoxic cells [144, 145].

Three endogenous antiangiogenic inhibitors found in the eye are thrombospondin-1 (TSP-1), endostatin, and pigment epithelium-derived factor (PEDF) [127, 146–148]. These angiostatic factors are often require proteolytic processing for their activation and are components prevalent in extracellular matrix or they bind to the matrix [149]. We have observed a decrease in endostatin, TSP-1, PEDF, in BrMb/CC complex during AMD possibly yielding BrMb susceptible to new blood vessel invasion [127](Fig. 4.7).

PEDF is a product of RPE and a member of the serpin family; however, it lacks serine protease inhibitor activity. PEDF has many biological activities including: gliastatic, neurotrophic, neuroprotective, immunomodulatory, anti-angiogenic, and antivasopermeability properties [150–152]. These diverse activities are modulated by different domains of PEDF. All of the functions of endogenous PEDF in the eye are still not completely understood. Intravitreal delivery of viral vectors that express PEDF or recombinant PEDF have been found to inhibit choroidal or retinal neovascularization [153, 154]. High doses of PEDF, however, were stimulatory for neovascularization. In our IHC investigation of VEGF and PEDF in aging and AMD, there was a shift in the balance toward angiogenesis in AMD specimens [145, 155]. PEDF localization in AMD was significantly reduced in BrMb, RPE cells, and choroidal stroma [147]. VEGF immunoreactivity, however, was not significantly increased in the RPE/Bruch's membrane/CC complex except near the tips of growing CNV. The most intense VEGF immunoreactivity was observed in large cells assumed to be choroidal mast cells or leukocytes.

TSP-1 is also a 180 kDa, secreted glycoprotein that has many domains and functions, including neuroprotection, axon guidance, and inhibition of angiogenesis [156]. TSP-1 the scavenger receptor, CD36, on endothelial cells. Developing TSP-1 knockout mice have increased retinal vascular density [157]. ECs in mature vessels are not affected by PEDF or TSP-1 but selectively induce apoptosis in ECs in neovascularization. RPE in vitro produces TSP-1 [158, 159] and in vivo TSP-1 is found in vitreous and aqueous humor [156]. Our IHC investigation of TSP-1 in aging and AMD eyes demonstrated that TSP-1 in BrMb dramatically declines with age [160] and was almost absent in BrMb and CC basement membrane of AMD eyes (Fig. 4.7). Additionally, TSP-1 and PEDF were observed at extremely high levels in disciform scars where quiescent CNV are present.

Endostatin is antiangiogenic activity only after proteolytic cleavage. Endostatin is generated by the cleavage of collagen XVIII [161]. Cleavage of the C-terminal noncollagenous domain (NC1) of collagen type XVIII by cathepsin L and elastase produces endostatin. Adenoviral vectors containing an expression construct for endostatin administered intravenously resulted in prevention of laser-induced CNV in mice, demonstrating its anti-angiogenic activity [162]. We have observed a reduction in endostatin in AMD choroid compared while collagen XVIII levels were comparable between AMD and control subjects [146].

In summary, three of the angiogenic agents and found BrMb/CC complex were significantly reduced or absent in AMD [127] (Figs. 4.6 and 4.7). This suggests that these anti-angiogenic factors endogenous to RPE/BrMb/CC complex may play a significant role in nAMD in that this makes BrMb susceptible to invasion by CNV. The presence of at least three anti-angiogenic factors in BrMb and intercapillary septa (Fig. 4.7) suggests that these angiogenic inhibitors with different molecular mechanisms may work synergistically for treatment of CNV.

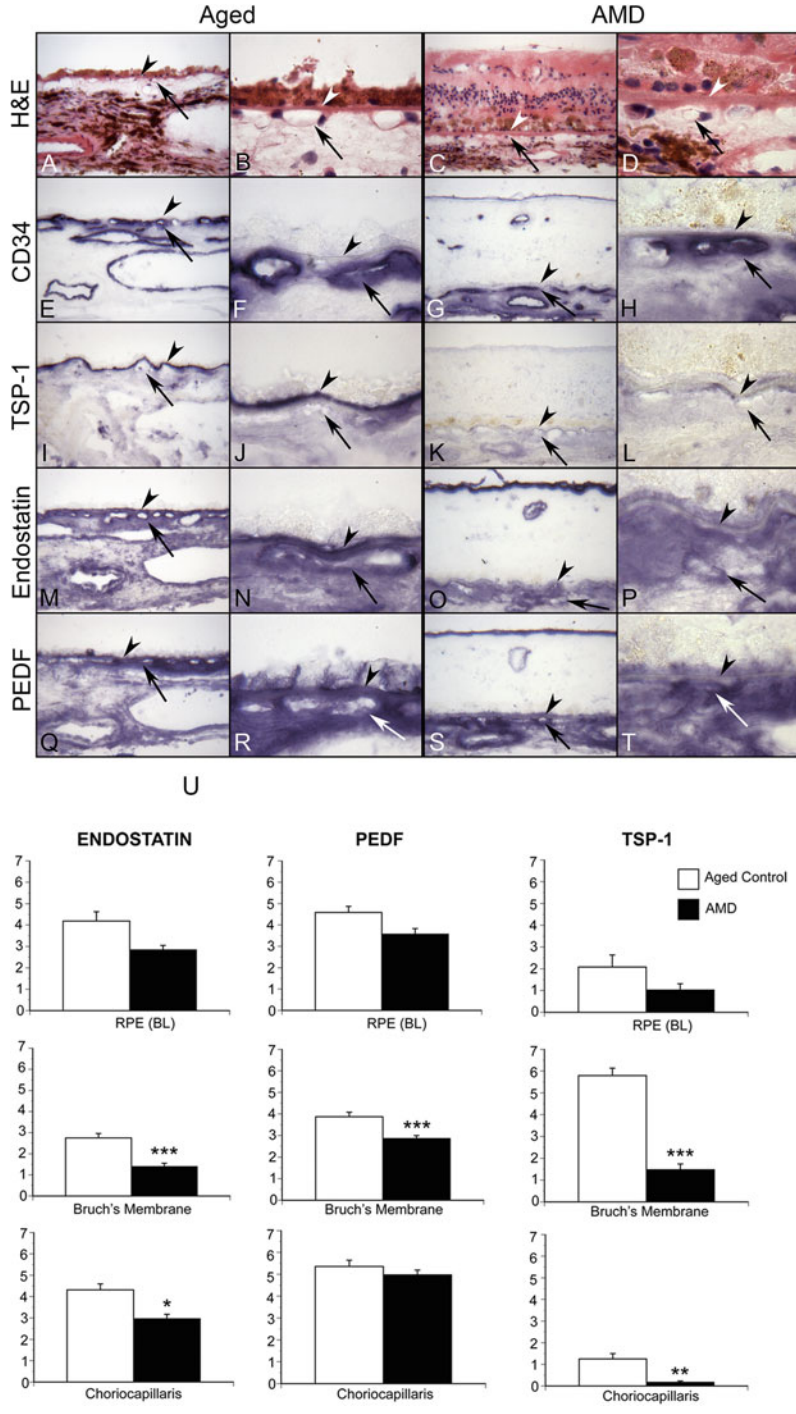

Fig. 4.7 Serial sections (**a**–**t**) of submacular choroid from normal aged control (left) and from an AMD subject (right) in which TSP-1, endostatin, and PEDF were localized by immunohistochemistry (IHC). Right panels are high magnification photos of left panels. (**a**–**d**) Hematoxylin and eosin (**h**, **e**, top) staining show morphological features of choroid in the control and retina and choroid in the AMD subject (low magnification, third column). In the AMD subject, migration of RPE cells into the AMD retina is apparent (**c**, **d**). Pigment in IHC sections was bleached from RPE and choroidal melanocytes. Immunostaining of CD34 (**e**–**h**) is associated with the retinal and choroidal

4.4 Role of Inflammation in AMD

4.4.1 Choroid Is a Proinflammatory Milieu

The pathogenesis of AMD remains unclear despite intensive basic and clinical research. There is significant evidence indicating that the complement system plays a key role in the etiology of AMD. If inflammation plays a role in AMD, it is not a classical inflammatory disease per se but rather evidence suggests that chronic, abnormal inflammatory response and immunologic events play a key role in progression of AMD [163]. Components of the complement system including C3 and C5, and C5b-9, the membrane attack complex (MAC), are present in ocular drusen and the intercapillary septa of the CC layer [78, 164, 165]. These and other proinflammatory molecules that are found in the vitreous of AMD subjects [166].

The complement system is part of the innate (or non-adaptable) immune system. The complement system promotes inflammation, eliminates pathogens, and enhances an individual's immune response. This complex system has more than 20 proteins that are generally synthesized by the liver; these circulate as pro-proteins or inactive precursors. The final component is MAC, which creates perforations within cellular membranes, killing the cell. There are three distinct complement pathways: the alternative pathway, classical pathway, and the mannose-binding lectin pathway.

Antigen-antibody complexes trigger the classical complement pathway. The alternative and mannose-binding lectin pathways do not require antibodies for activation (nonspecific immune response). The common goal of all three pathways is to deposit clusters of C3b on target pathogens. C3 is cleaved by C3-convertase, creating C3a and C3b. C3b binds to the pathogen surface, which leads to internalization by phagocytic cells like macrophages. Recruitment of inflammatory cells can be induced by C5a, which is an important chemotactic protein. Both C3a and C5a have anaphylatoxin activity and this induces mast cell degranulation leading to increased vascular permeability. The membrane attack pathway starts with C5b, which leads to the formation of the end product of the complement cascade: the MAC. Mullins and associates have found that there is MAC even in young choroids around CC but the level increases in AMD eyes that have the high risk CFH genotypes [167].

The complement system is continuously activated at low levels in the normal eye. Spontaneous complement activation is prevented by regulatory elements: CD35, CD46, and CFH, which maintains complement activity at a level that promotes elimination of potential pathogens without damaging healthy tissue. Overactive or dysregulated complement activity can cause immune-mediated ocular damage. Janet Sparrow has suggested that complement can be activated by photo-oxidation of A2E, a component of lipofuscin [168]. Weismann and associates have demonstrated that CFH binds malondialdehyde epitopes and protects against oxidative stress [169].

Using DNA sequence data from the Human Genome Project, three independent groups

Fig. 4.7 (continued) blood vessels including CC (arrow). TSP-1 immunoreactivity (**i**, **j**) in the aged control choroid is intense especially in BrMb (arrowhead). Both endostatin (**m**, **n**) and PEDF (**q**, **r**) are prominent in RPE basal lamina, BrMb, and CC basement membrane and show similar pattern and intensity with IHC. In contrast, expression of TSP-1 (**k**, **l**), endostatin (**o**, **p**), and PEDF (**s**, **t**) are greatly reduced in the AMD choroid compared to the aged control. The reaction product of endostatin and PEDF appears more diffuse in choroidal stroma (arrowhead, BrMb; arrow, CC). (**u**) Numerical data from grading IHC reaction product in 8 aged control subjects and 12 AMD subjects (in **a**–**t**). The scores (0–7) on the *Y*-axis represent the mean scores from three masked observers. Aged control subject data is represented by open bars and AMD subjects by black bars. A significant difference in BrMb scores (***, $P < 0/01$) was found for all three inhibitors between control and AMD subjects (Modified from Figs. 4.6 and 4.7 in Bhutto and Lutty, *Mol. Aspects Medicine* 2012;33;295–317 [7] with permission)

demonstrated that a polymorphism (Tyr402His) in the CFH gene increases the risk for developing AMD [170–172]. CFH functions mainly to regulate the alternative complement pathway activation in plasma and at sites of tissue inflammation in host cells and tissue. CFH acts as a cofactor for cleavage of C3b to its inactive form and weakens the active complex that forms between C3b and factor B. The mutation in CFH (Tyr402His) reduces the affinity of CFH for CRP and specific GAGs. This change appears to result in reduced ability of CFH to regulate the alternative pathway permitting it to run uncontrolled. The failure of CFH-Y402H to bind to CRP, could result in high levels of unbound CRP in the choroid, which we have observed [97], making choroid permissive for chronic inflammation [173, 174].

CRP is an acute-phase protein that activates the complement system [175]. It is considered to be a nonspecific serum biomarker for subclinical inflammation and is considered as a risk for cardiovascular disease [176], adult-onset diabetes (NIDDM) [177] and, more recently, AMD [178–180]. CRP is present in drusen and other sub-RPE deposits [166, 181]. However, the association of CRP and AMD has been questioned by others [182, 183]. CRP can be deposited at sites of tissue damage [184] and can form soluble complexes with certain lipoproteins containing apolipoprotein B (apoB) [185]. We investigated the IHC expression pattern of the CRP and CFH in the submacular RPE/BrMb/CC complex in eyes with early, nAMD, and GA and compared it to localization in aged control subjects [97] (Fig. 4.8). CRP immunoreactivity was prominent in and around CC and in individual cells in choroidal stroma in aged control subjects (Fig. 4.8). CRP was more intense and significantly increased in the RPE/BrMb/CC complex in early and nAMD, especially in the intercapillary septa (ICS). In contrast, CFH immunoreactivity was significantly reduced in the BrMb/CC complex including the ICS in eyes with early and nAMD [97]. Furthermore, there was a significant inverse correlation between the CRP and CFH levels in eyes with nAMD and GA (Fig. 4.8). We hypothesize that high levels of CRP and insufficient CFH in choroid may lead to uncontrolled complement activation with associated cell and tissue damage. Another inhibitor of complement, CD46, was also reduced in the AMD RPE suggesting that the normal regulators of complement are not present or reduced in AMD choroid [186].

4.4.2 Innate Immunity During AMD

Forrester and colleagues propose that chronic para-inflammation contributes to the initiation of AMD [187]. Para-inflammation is a state between basal inflammatory state and true inflammatory state in response to noxious stress or cellular dysfunction [188]. AGEs, dead cells, oxidative stress, and oxidized lipoproteins are triggers for para-inflammation and all of these exist in the photoreceptor/RPE/BrMb/CC complex in early AMD. Choroidal para-inflammatory may contribute to abnormalities in choroidal melanocytes, changes in choroidal thickness, and fibrosis of choroidal tissue. Resident choroidal mast cells and macrophages are part of the innate immune system in choroid.

4.4.3 Choroidal Inflammatory Cells in AMD

We have recently demonstrated the recruitment of mast cells (MCs) and macrophages to disease affected areas in eyes with AMD. Staining of choroidal flat mounts from aged donors with no ocular disease or various stages of AMD with were incubated for endogenous APase activity (blood vessels) and nonspecific esterase (stains MCs and granulocytes) demonstrated that MCs were increased across the choroid in early AMD and in the paramacular area of eyes with GA and nAMD [38]. Furthermore, a greater number of choroidal MCs were degranulated in all eyes with AMD (Fig. 4.9). Degranulated MCs were observed mostly in areas with CC loss. MCs also appeared to migrate from Sattler's and Haller's layers where they reside in control choroids to the CC layer in eyes with GA and nAMD. We hypothesized that mast cell

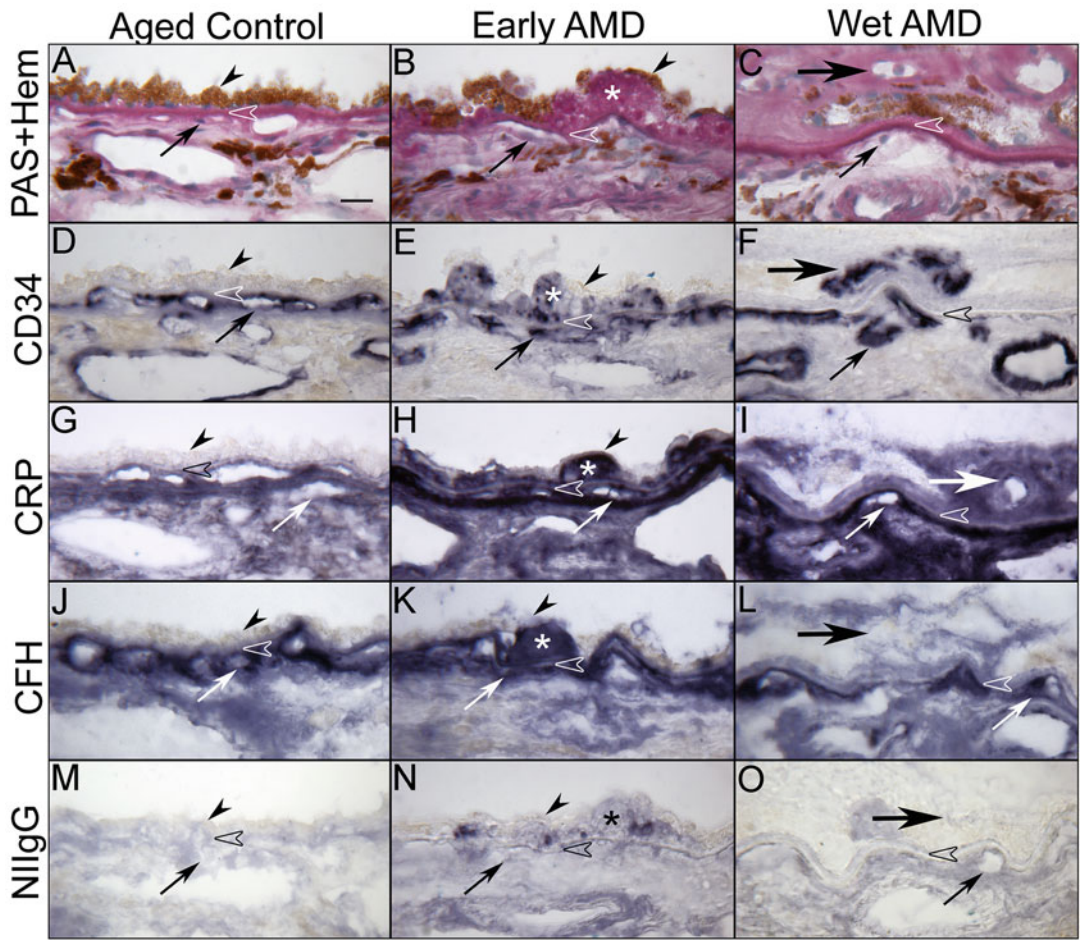

Fig. 4.8 Immunolocalization of C-reactive protein (CRP) and complement factor H (CFH) in submacular choroid from aged control, early and late nAMD eyes. Periodic acid-Schiff's (PAS) and hematoxylin (Hem) staining shows morphological features of the choroid from aged control (**a**), drusen (asterisk) in early AMD (**b**), and CNV (large arrow) anterior to RPE in nAMD (**c**). Pigment in IHC sections was bleached from RPE and choroidal melanocytes. CD34 IHC shows CC (small arrow) and large choroidal vessels which appear morphologically normal with broad lumens in aged control (**d**); however, CC lumens appear irregular and constricted in early (**e**) and nAMD (**f**). CRP (**g**) and CFH (**j**) are prominently localized to the CC, intercapillary septa (ICS) and BrMb (open arrowhead) in aged control choroid. CRP immunoreactivity is significantly increased in early (**h**) and nAMD (**i**) choroids compared to the aged control and the immunoreaction product appears more diffuse in choroidal stroma. CFH in early AMD (**k**) is comparable to aged control, whereas it is significantly decreased in nAMD (**l**). Drusen are intensely labeled with CRP and CFH (**h**, **k**). Note that in nAMD the CNV (large arrow), intensely labeled with CD34 antibody (**f**), has more CRP and less CFH (**i**, **l**). Nonimmune rabbit IgG (NIIgG) yields a very weak to negative reaction product except in drusen (**m**–**o**). (Fig. 4.3 from Bhutto et al. *British Journal of Ophthalmology* 95:1323–1330, 2011 [97] with permission)

recruitment and degranulation may contribute to choroidal thinning and CNV formation. A follow-up study investigated the expression of tryptase, the primary protein released early in mast cell degranulation, in eyes with either no AMD or GA [125]. Tryptase in control eyes was confined to mast cells which were located in Sattler's and Haller's layer. By contrast, tryptase-positive mast cells were observed in the CC layer and near BrMb in eyes with GA. There was also strong

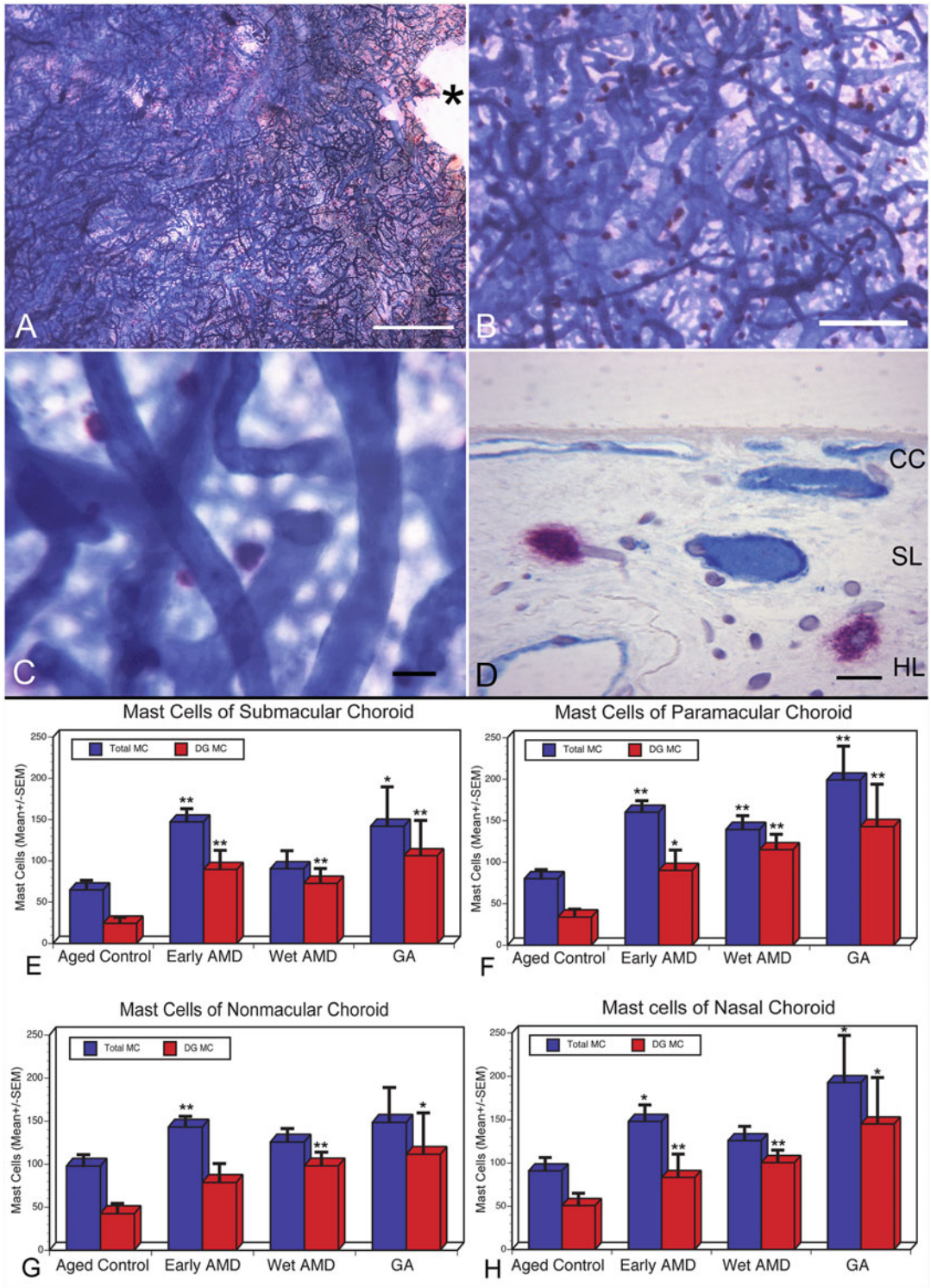

Fig. 4.9 Submacular choroid flat mount from an aged control subject stained with APase and nonspecific esterase (NSE). (**a**, **b**) Choroidal vessels are stained blue with APase^{+} reaction product and NSE-positive MCs are stained red. (**c**) CC is out of focus in this image where the MCs were focused on because they are largely

tryptase staining in BrMb of eyes with GA in atrophic areas, at the border of atrophy, and in nonatrophic regions (Fig. 4.10) [125]. Tryptase can activate MMPs and digest collagens, causing degradation of BrMb and CC basement membrane as well as result in choroidal thinning, a hallmark of AMD, which is not understood as yet. We recently demonstrated that just activating and degranulating choroidal mast cells can result in a GA like-phenotype in a rat model [189].

We also recently compared the number of macrophages in AMD choroids to that in aged control choroids [190]. There was a significant increase in ionized calcium-binding adapter protein-1 positive ($IBA1^+$) macrophages in choroids of donors with early/intermediate AMD compared to controls. Furthermore, there was a significant increase in the number of human leukocyte antigen–antigen d-related positive ($HLA\text{-}DR^+$) activated macrophages in all forms of AMD. This activation was confirmed using image analysis of the IBA^+ cells, which showed that macrophages in early, nAMD and GA choroids were rounder (increase in sphericity) and were smaller, both signs of macrophage activation (Fig. 4.11) [190].

4.5 Conclusions

Studies of the choroidal vasculature demonstrate that there is attenuation of the CC in early AMD, nAMD, and GA. However, nAMD and GA may have different etiologies in regard to the death or dysfunction of the choroidal EC. In nAMD, the loss of choroidal vasculature may be the initial insult to the RPE/BrMb/CC complex. We have observed loss in CC with an intact RPE monolayer in wet AMD [118]. This may be due to reduction in blood supply because of intermediate and large vessel dysfunction and eventual stenosis [101]. Firm adhesion of activated neutrophils and other $CD11b/CD18^+$ leukocytes is always possible in CC because the CC constitutively express ICAM-1 [24]. Furthermore, the milieu around CC, BrMb, and ICS, is a proinflammatory with accumulation of complement components [166] as well as proinflammatory molecules like CRP [97]. Mullins recently reported a relationship between acellular capillaries and drusen [84]. CC die or become dysfunctional in this toxic milieu making adjacent RPE hypoxic. Hypoxic RPE would then produce angiogenic substances like VEGF that are hypoxia-inducible, stimulating growth of CNV. This loss of CC might also be a stimulus for drusen formation since the disposal system would be limited. Ultimately, the photoreceptors would die from lack of nutrients, leakage of serum, and scar formation.

It appears that large confluent drusen formation and hyperpigmentation (presumably dysfunction in RPE and/or melanocytes) are the initial insult in GA and the resorption of these drusen and loss of RPE (hypopigmentation) can be predictive for progression of GA [115], that is, RPE and BrMb appear to be dysfunctional first. In our studies and the work of the Curcio lab, it appears that the RPE died first in GA and were hypertrophic and multilayered at the edge of the atrophy [118]. However, Biesemeier found that

Fig. 4.9 (continued) distributed in the intermediate and deep choroid. (**d**) Histological section of the choroid shown in (**a**) demonstrates MCs (red) are associated with Sattler's layer (SL) and Haller's layer (HL) blood vessels, which are $APase^+$. (*, optic nerve; Bar = 1 mm in **a**; 200 μm in **b**; 50 μm in **c**; and 20 μm in **d**). (**e**–**h**) Number of MCs (total MCs, blue bar; degranulated or DG MCs, red bar) present per mm^2 of choroid. MCs were counted in the flat perspective before embedding for sectioning in aged control and AMD subjects. MC numbers/mm^2 from four areas of posterior choroid are represented [submacular (**e**), paramacular (**f**), nonmacular (**g**), and nasal (**h**)] and compared to the aged control in those regions. Total numbers of MCs as well as degranulated MCs significantly increased in almost all areas examined in AMD choroids compared to the aged control. The significance of the difference between aged control and AMD ($P < 0.05$) is indicated using the Students *t*-test (*) and Wilcoxon rank-sum test (**) (Fig. 4.1 from Bhutto et al. *Brit. J. Ophthalmol.* 2016.100:720–726 [38] with permission)

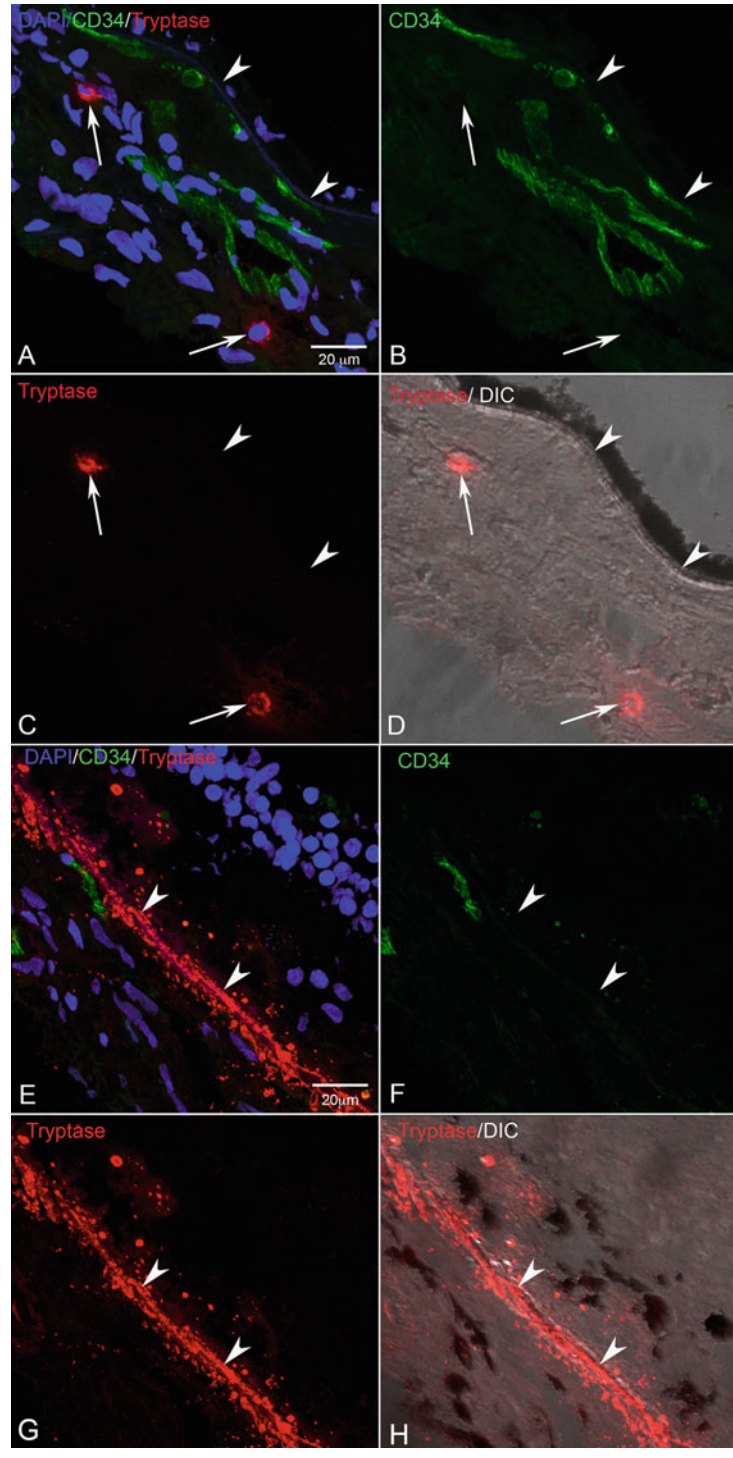

Fig. 4.10 Tryptase localization (red) in a control subject (**a**–**d**) and in a GA subject (**e**–**h**). In the aged control subject tryptase is confined to the mast cells (red). Blood vessels (green) were stained with anti-CD34 and nuclei are stained blue with DAPI. The DIC/tryptase image (**d**) is presented so RPE (black) can be seen. In the GA subject at the border of atrophy, tryptase granules (red) are present in BrMb as well as RPE cells migrating up into retina (**h**). CC is attenuated (green, **f**)

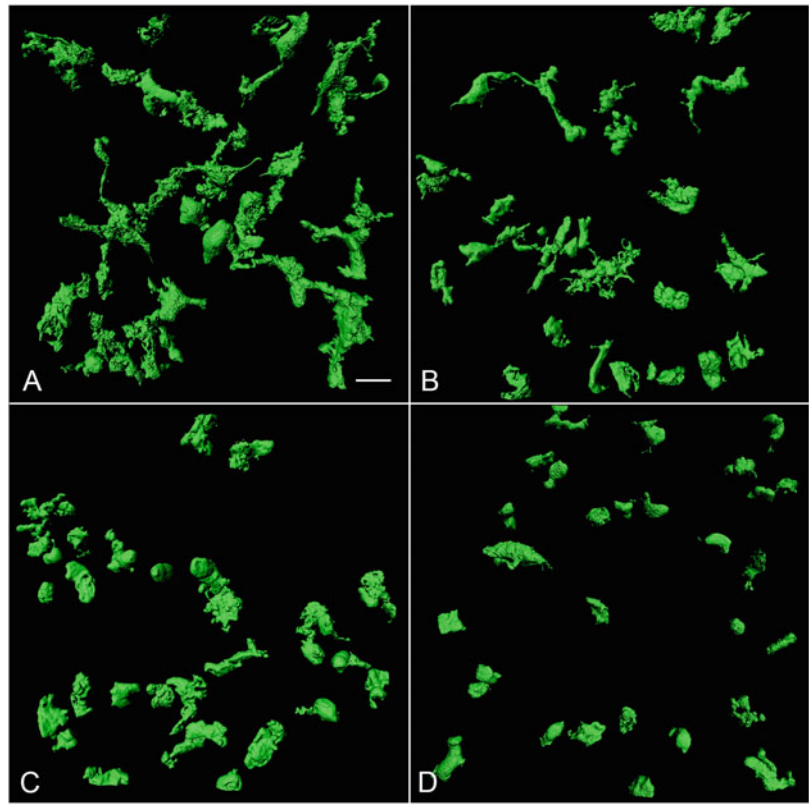

Fig. 4.11 Representative volume renderings of IBA1$^+$ macrophages in the submacular choroid of an aged control eye (**a**), early AMD eye (**b**), an eye nAMD eye (**c**), and an eye with GA (**d**). Macrophages in the aged control have a ramified cellular morphology and a large cell volume. Macrophages in early AMD eyes have fewer processes and reduced cell volume compared to controls. Macrophages in advanced AMD, whether nAMD or GA, are more rounded, have very few processes, and are much smaller in size (Fig. 4.4 from McLeod et al. *Invest. Ophthalmol. Vis. Sci.* 2017;58:5887–5896 [125] with permission)

CC died before RPE in their TEM study [191]. Another reason for RPE death and dysfunction may be toxic products accumulated within the RPE [168, 192, 193]. These toxic products could activate the complement cascade, which could be the cause of death for many components in the photoreceptor/RPE/BrMb/CC complex. A hurdle to successful treatment may be the inability of the new RPE/progenitor cells to repopulate an aged, thickened BrMb.

Therefore, the mutualistic symbiotic relationship within the photoreceptor/RPE/BrMb/CC complex is lost in both nAMD and GA. Loss of this functionally integrated relationship results in death and dysfunction of all of the components in the complex. Perhaps, restoration of the relationship can be accomplished therapeutically by targeting the initial insult. Control of inflammation could prevent loss of CC and degradation of BrMb by MC degranulation and release of tryptase.

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Innate Immunity in Age-Related Macular Degeneration

5

Yikui Zhang and Wai T. Wong

Abstract

Multiple lines of investigation have demonstrated that inflammation plays significant roles in etiology of age-related macular degeneration (AMD). Although interventional trials in AMD therapy targeting inflammatory pathways have been conducted, they have not yet been successful and a detailed understanding as to why some have failed is still elusive. One limitation is the relative dearth of information on how immune cells interact with retinal cells to generate AMD phenotypes at each disease stage. Here, we summarize current research evidence and hypotheses regarding potential pathogenic roles of innate immune cells in the eye, which include resident retinal microglia, macrophages derived from infiltrating systemic monocytes, and macrophages resident in the choroid. We relate recent findings regarding the physiology, function, and cellular interactions involving innate immune cells in the retina and choroid to AMD-related processes, including: (1) drusen formation and regression, (2) the onset and spread of degeneration in late atrophic AMD, and (3) the initiation, growth, and exudation of neovascular vessels in late "wet" AMD. Understanding how innate immune cells contribute to specific AMD phenotypes can assist in generating a comprehensive view on the inflammatory etiology of AMD and aid in identifying anti-inflammatory therapeutic strategies and selecting appropriate clinical outcomes for the planned interventions.

Keywords

Age-related macular degeneration · Inflammation · Innate immune cells · Resident retinal microglia · Choroid macrophage · Infiltrating monocyte · Drusen · Geographic atrophy · Neovascularization

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5.1 Inflammatory Changes Involving Innate Immune Cells Are Centrally Involved in AMD Pathobiology

Multiple lines of evidence, some dating back several decades [1], have demonstrated the presence of inflammatory changes in the AMD retina in close spatial proximity to the hallmark lesions of the disease. Recent research findings have generated increasing consensus that these inflammatory changes exist not only as a response to retinal changes in AMD but also contribute to driving disease initiation and progression. The causality of these associations, as well as the cellular mechanisms and molecular pathways underlying their effects, are subjects of scrutiny in ongoing studies and therapeutic strategies [2]. One approach towards a greater understanding of the inflammatory etiologies of AMD is to inquire about the cellular mechanisms underlying pathogenesis, asking specifically how innate immune cells interact with retinal cells at the photoreceptor–RPE–Bruch's membrane complex to influence disease progression. This review summarizes the current ideas and findings regarding how innate immunity may play a role in AMD. First, it may be instructive to consider the evidence linking innate immunity to AMD pathogenesis, some of which is summarized below:

5.1.1 Genetic Risk Factors for AMD Involve Inflammatory Pathways Operational in Innate Immune Cells

Genome-wide association studies (GWAS) conducted on large numbers of AMD patients and controls have identified 52 independently associated variants at 34 genetic loci that together account for much of the genetic risk for AMD [3]. Among these loci are genes whose products are expressed in innate immune cells in the healthy and pathological retina and feature in multiple inflammatory pathways [4]. The association between AMD risk and these inflammatory genes suggests that variations in innate immune cell responses may confer differential risks for AMD initiation and progression. This association also highlights the possibility that these genes, and other genes in the same pathways in which they act, may constitute targets for therapeutic AMD risk reduction or treatment.

Most prominent among these inflammatory risk genes involve those in the complement pathway, including *CFH*, *C2/CFB*, *CFI*, *C3*, and *C9*. Traditionally understood as being a key part of the innate immune system and evolutionarily conserved for host defense against pathogens, the complement system has more recently been discovered to play important homeostatic, as well as pathological, roles in the CNS [5, 6]. In the retina, expression levels of complement-related gene products are upregulated prominently in aging and retinal degeneration [7], with innate immune cells constituting an important cellular source for these products [8–11]. Besides complement molecules, genetic risk loci include inflammation-related molecules that are expressed by innate immune cells, including: *TGFBR1*, a receptor for TGFβ signaling that constitutes a key regulatory pathway regulating homeostatic versus pathologic effects of innate immune cells in the brain [12] and the retina [13]; *APOE*, a molecule involved in systemic lipid transport and also the regulation of innate immune cell survival and activation in the retina [14, 15], and *PILRB/PILRA*, type I transmembrane immunoglobulin-like receptors that mediate activating/inhibitory intracellular signaling that regulate inflammatory gene expression [16]. Together, this prominent representation of innate immune cell-expressed inflammatory genes among AMD genetic risk factors strongly implicates the agency of innate immune cells in disease pathogenesis.

5.1.2 Histopathologic AMD-Related Lesions Are Spatiotemporally Associated with Innate Immune Cells

Another source of evidence linking inflammation to AMD arises from histopathological studies that demonstrate the presence of innate immune cell

changes at the photoreceptor/RPE/Bruch's membrane complex, the locus of AMD pathology in the retina. In the normal young healthy retina, the subretinal space, recognized as a specialized zone of immune regulation, is largely devoid of innate immune cells [17]; this contrasts with the inner retina and the choroid of young healthy animals which contain resident populations of innate immune cells that carry out constitutive dynamic immune surveillance [18, 19]. In eyes with large drusen, the hallmark of intermediate AMD, innate immune cells, also termed as mononuclear phagocytes (MPs), can be detected in the outer retina, localizing to positions both overlying and within drusen [20–23]. Histopathological analysis of eyes with geographic atrophy (GA) has detected cells immunopositive for myeloid markers in both the atrophic zone and the surrounding transition zone [14]. Adaptive optics imaging in eyes of GA patients has also revealed mobile hyporeflective clumps in and around atrophic lesions [24] which may correspond to migrated microglial cells, as they are capable of phagocytosing hyperreflective melanin and demonstrating dynamic motility in response to RPE damage [25]. Innate immune cells, in the form of activated macrophages, are also spatially associated with the neovascular form of late AMD, including subclinical choroidal neovascularization (CNV) lacking exudative change [26], surgically-excised choroidal neovascular membranes [27, 28], and neovessels from exudative CNV lesions [29, 30]. The spatial and temporal proximity of activated innate immune cells to the hallmark lesions of AMD demonstrate their presence "at the scene of the crime" and suggest that they can act locally to influence the formation and progression of AMD-related disease lesions.

5.1.3 Animal Models of AMD Demonstrate AMD-Related Phenotypes in Association with Innate Immune Cell Changes

Although rodent models of AMD have limitations in that the murine retina lacks a macula and does not form large soft drusen closely resembling those in human AMD [31, 32], they have arguably been useful in generating insight into pathological mechanisms underlying how candidate causative genes or processes relate to histopathological changes observed in AMD. In these models, inflammatory changes in the retina have been apparent. In models simulating increased retinal oxidative stress, a factor implicated in AMD progression [33], AMD-related pathological changes were induced alongside inflammatory changes in the outer retina. Examples of these include: (1) a model involving immunization with carboxyethylpyrrole-adducted proteins (CEP), which is formed from the oxidation of docosahexaenoic acid in the retina [34], and (2) a model of increased light stress [35, 36], in which retinal degenerative changes induced from the oxidative insults were accompanied by complement deposition and the infiltration of innate immune cells into the outer retina. In models of RPE degeneration induced by sodium iodate to simulate retinal degeneration in atrophic AMD [37, 38], degenerative changes in the outer retina were also accompanied by prominent microglial migration and accumulation in the subretinal space [39], which appear to augment photoreceptor degeneration [40]. One interpretation that arises from these models is that cellular injury may trigger, and in turn be exacerbated by, inflammatory responses mediated by innate immune cells. Support for this interpretation has been provided by the ability of immunomodulatory interventions to ameliorate the extent of resulting degeneration [41, 42].

Strengthening this causal connection between inflammation and AMD pathogenesis are rodent models in which primary perturbations in innate immune cells, such as those involving cell-specific genetic alterations, have produced phenotypes that simulate aspects of AMD pathobiology. Examples include: (1) a mouse model involving the ablation of CX3CR1, a myeloid cell-specific receptor, which demonstrates subretinal MP accumulation and associated photoreceptor degeneration [20], and (2) a mouse model in which macrophage-specific deletion of ABCA1 and ABCG1 resulted in impaired MP cholesterol clearance, and induced subretinal

drusenoid deposits reminiscent of reticular drusen observed in AMD [43]. These rodent models provide support for the idea that primary immune cell changes can contribute to producing AMD-like phenotypes in the retina.

In addition to rodent models, AMD animal models have included non-human primates, which possess a macula akin to humans. The maculae of aged macaques have demonstrated anatomical changes suggestive of human AMD including: (1) the prevalent development of sub-RPE drusenoid deposits that share morphological similarities to drusen in intermediate AMD [44, 45], (2) the association of sub-RPE deposits with some degree of degenerative change in the RPE and Bruch's membrane [46, 47], (3) similarities in the composition of drusenoid deposits to those found in human AMD [48], and (4) similarities in genetic susceptibility factors as those identified for human AMD [49]. Interestingly, ultrastructural analyses in the aged rhesus monkey have also found the consistent presence of lipofuscin-containing macrophage-like cells in the vicinity of drusen [50], implicating the involvement of innate immune cells at the locus of disease.

5.1.4 Innate Immune Cells in the Retina Demonstrate Aging Changes

Among patient-related factors associated with AMD risk, aging is the most influential, with disease prevalence being nearly absent in patients <50 years of age, regardless of genotype or environmental exposure, but rising sharply with increasing age into the seventh, eighth, and ninth decades of life [51]. The mechanisms underlying this strong age association, which is shared with other neurodegenerative conditions such as Alzheimer's disease and Parkinson's disease, are incompletely understood but has been related to the marked changes that occur in the innate immune system during aging [52, 53]. In the retina, there is evidence that the innate immune cell population undergoes progressive age-related changes that may relate to AMD pathobiology. Microglia in the retina increase in number but demonstrate features that suggest decreased constitutive function, such as decreased ramification and motility; their dynamic responses to tissue injury also demonstrate slowed onset but a more prolonged duration, indicating less nimble and more chronic responses [54]. In aged mouse models, retinal microglia demonstrate increased displacement into the subretinal space, where they accumulate lipofuscin [55] and oxidized lipids [56], and upregulate markers of immune activation. On a molecular level, aging retinal microglia also demonstrate changes in their transcription profile that indicate altered homeostatic function and activation, in particular with respect to the expression of complement genes C3 and CFB [8]. Outside the retina in the choroid, choroidal macrophages also increase in number with aging both in mouse models [18] and in the human choroid [57]. These aging changes within innate immune cells in the retina and choroid posit that differential inflammatory responses of these cells, altered as a function of aging, may increase AMD risk and aid disease progression [58].

5.2 Innate Immune Cell Types Potentially Involved in AMD Pathobiology

The study of how innate immune cells of the myeloid lineage contribute to AMD pathobiology is made more challenging by the recognition that they derive differentially from distinct subsets of myeloid cells. These cells are located in different parts of the eye and the body, and are distinguished from each other in terms of ontogeny, cellular morphology, gene expression profile, and physiological function [59]. As a result, their specific involvement with AMD pathobiology, their contribution towards driving or adapting to pathological change, as well as the means by which they may be targeted therapeutically, may also demonstrate potential differences. We provide some background information regarding each of the three main populations of innate immune cells that have been associated with AMD: retinal microglia, monocyte-derived retinal macrophages, and resident choroidal macrophages (Fig. 5.1).

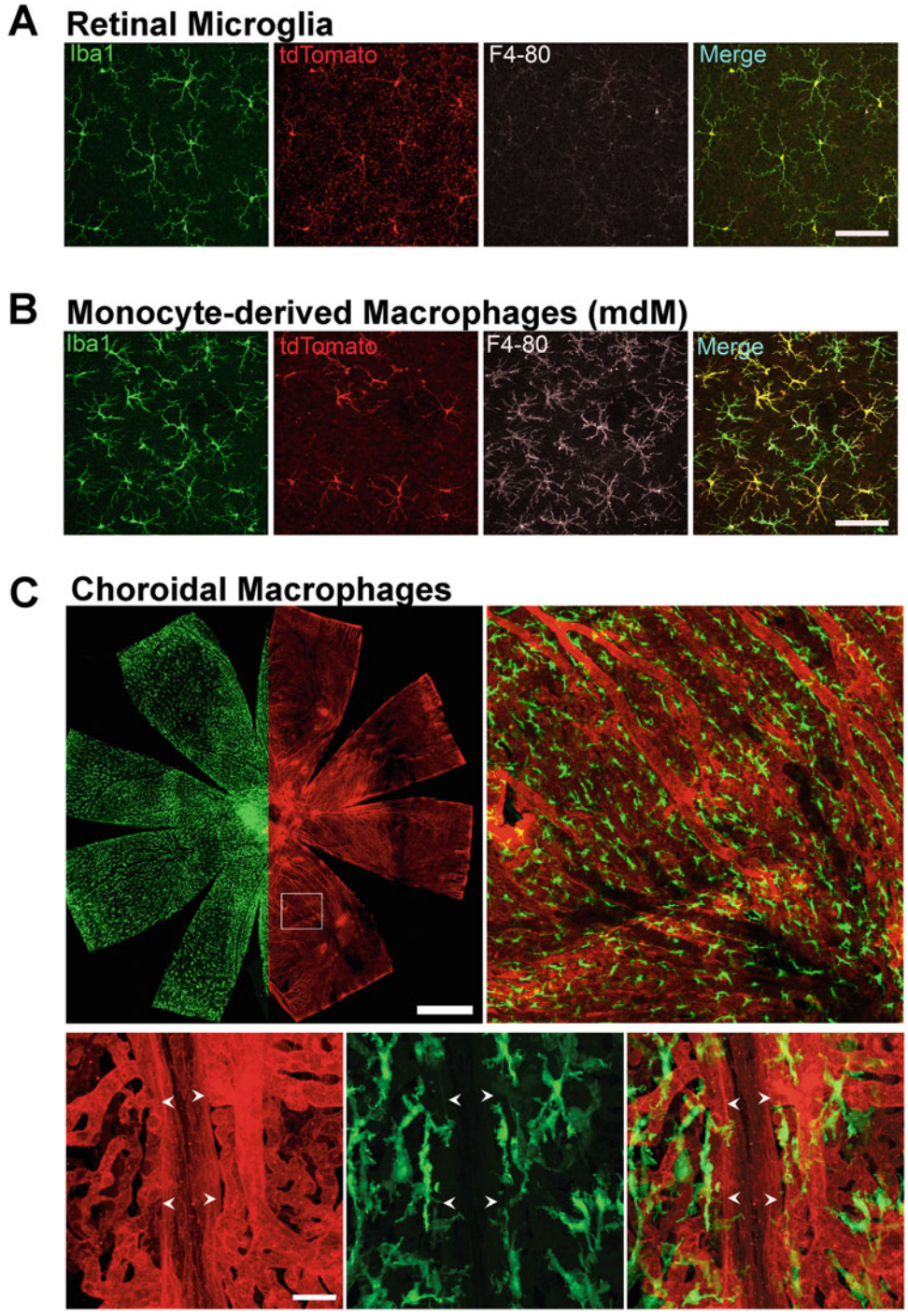

Fig. 5.1 Innate immune cell populations in the adult mouse retina and choroid. (**a**) Retinal microglia: Flatmounted preparation of a retina from an adult CX3CR1$^{CreER/+}$: Rosa26-flox- STOP-flox-tdTomato mouse in which tamoxifen had been systemically administered to induce expression of tdTomato in CX3CR1-expressing cells [39]. Resident retinal microglia in the absence of injury are uniformly immunopositive for IBA1, express CX3CR1, negative for the activation marker F4/80, and comprise the entire innate immune cell population in the retina. Scale bar = 60 μm. (**b**) Monocyte-derived macrophages (mdM): Adult CX3CR1$^{CreER/+}$: Rosa26-flox- STOP-flox-tdTomato mice were administered systemic tamoxifen to induce

5.2.1 Retinal Microglia

Retinal microglia constitute the population of innate immune cells normally resident within the neural parenchyma of the retina and separated from the systemic circulation and extra-CNS environment by the blood-retinal barrier [60]. They are distinct from systemically circulating monocytes and extra-CNS macrophages in that they derive from primitive hematopoietic progenitors from the extra-embryonic yolk-sac that colonize the retina during early development [61], akin to microglia resident within the brain parenchyma [62, 63]. In the absence of intraparenchymal disease or other factors that compromise of the integrity of the blood-CNS barrier, they constitute a population of long-lived cells [64] that are maintained separately without ongoing contributions from circulating monocytes or other extra-CNS macrophages [65]. Within this closed system, they maintain their numbers and homeostasis via signals from neurons and macroglia, including CSF1, IL34, IL1β [61, 66–68], and CX3CL1, and are capable of endogenous regeneration and recovery from perturbations via cellular division and migration, such as when endogenous retinal microglia are subjected to depletion [69].

In the healthy adult retina, microglia are small, stellate-shaped cells, with flat, branching morphologies oriented in a plane horizontal to the laminated structure of the retina [70, 71]. Individual microglial cell somata are distributed in a regular mosaic pattern, with their ramified processes concentrated in the inner and outer plexiform layers that tile the retina via non-overlapping processes. Microglial processes also demonstrate constitutive and rapid surveying movements, enabling physical coverage of much of the surrounding extracellular space via their dynamic behavior [19], which is regulated as a function of retinal neuronal activity [72] and chemokine signaling [73]. Their constitutive functions under healthy conditions appear related to the maintenance of retinal synapses; when microglia are depleted from the retina over the long term, degeneration in the structure and function of synapses, including those of photoreceptors, occurs, leading to a decline in electrophysiological responses to light stimuli [74].

In the absence of photoreceptor/RPE injury, microglia are typically ramified and are largely excluded from the outer retinal layers, including the outer nuclear layer and subretinal space. However, upon the onset of neuronal injury and structural perturbation, retinal microglia detect danger- or pathogen-associated molecular patterns (DAMPs, PAMPs) and transition from their constitutive physiological state to one or more injury-associated perturbation states, acquiring functions related to cellular migration, proinflammatory cytokine production, and phagocytosis [75], and interact with other retinal cell types in an attempt to restore homeostasis. These homeostatic effector mechanisms triggered in retinal disease and injury have been found to facilitate adaptive effects, such as in the clearance of apoptotic cells and cellular debris [11, 61, 76]. However,

Fig. 5.1 (continued) expression of tdTomato in CX3CR1-expressing microglia and circulating monocytes. Animals were kept for an additional 3 months under standard conditions to allow for the turnover of labeled monocytes and their replacement by new unlabeled tdT- monocytes. Long-lived retinal microglia remained tdT+. Animals were administered intraperitoneal sodium iodate to induce retinal injury and stimulate the infiltration of systemic monocytes into the retina. Innate immune cells in the retina were analyzed in retinal flatmounts 3 months post-injury. Monocyte-derived macrophages (mdM) can be observed in the retina as IBA1+, tdT-, F4/80+ cells. These macrophages are now long-lived within the retina and demonstrate a ramified morphology similar to nearby microglia cells (IBA1+, tdT+) which are chronically activated and F4/80+ in a postinjury context. Scale bar = 60 μm. (**c**) Choroidal macrophages: choroidoscleral flat-mount from a 3-month-old adult CX3CR1$^{+/GFP}$ mouse that had been perfused intravascularly with lipophilic dye, DiI (*red*), showing CX3CR1-expressing choroidal macrophages distributed throughout the choroid. Scale bar: 500 μm. Higher magnification views show the ramified morphology and perivascular distribution of choroidal macrophages, scale bar: 25 μm

depending on the context, they are also associated with maladaptive consequences, such as the production of neurotoxic proinflammatory cytokines and the clearance of stressed but still-living neurons by the process of phagoptosis [42, 77, 78]. As a function of their constitutive presence in the retina and constant interaction with retinal cells, microglia are poised to detect and respond to perturbations within the retina; as such, their response to dyshomeostatic features in the AMD retina such as the formation of drusen, including soft drusen in the sub-RPE space and reticular drusen in the subretinal space, as well as progressive photoreceptor/RPE disorganization such as found in exudative AMD and GA, may be critical and consequential. The molecular mechanisms underlying these microglial responses and how they result in adaptive versus maladaptive consequences will be central questions to be tackled in future studies.

5.2.2 Monocyte-Derived Macrophages (mdMs)

Under healthy conditions, monocytes found in the systemic circulation do not gain entry into the CNS, including the retina, in any appreciable numbers [65], however, with intra-CNS injury or compromise to the blood-brain/blood-retina barrier, classical Ly6C^{hi}CCR2+ monocytes gain the ability to infiltrate into the neuronal parenchyma of the brain [79] and retina [39, 80], and differentiate into monocyte-derived macrophages (mdMs). Clear distinction between endogenous microglia and mdMs within the CNS using immunohistochemical markers has proved challenging, but recent techniques, including cell-fate mapping methods, have been helpful in this regard, as recently reviewed [81]. In some injury models, mdMs have a transient tenure following infiltration, disappearing during the course of disease evolution [82], while in other injury models, they show an enduring presence, taking up long-term residence in neural tissue alongside endogenous microglia [39]. One of the routes for monocyte entry into the retina appears to be via the retinal vasculature of the inner retina; CCR2+ mdMs had been were found occupying the inner retinal layers following photoreceptor/RPE injury in light-injury and sodium iodate-induced injury models, possibly as a repopulating response [83] centered in the inner retina following the mobilization of endogenous microglia to the outer retina [39, 80]. Entry may also occur via the outer choroidal vasculature and the RPE monolayer [84] with CCR2+ monocytes located in the subretinal space in the inherited retinal degeneration models [42, 85] and the CX3CR1-deficient mouse model of photoreceptor degeneration [23]. In a light-inducible arrestin-deficient mouse model of photoreceptor degeneration, monocytes have been thought to enter via the inner retinal vasculature and migrate into the outer nuclear layer traversing the thickness of the retina [86].

Upon entry into the CNS, mdMs differentiate from a monocytic phenotype to one that is more macrophage-like, to resemble microglia in terms of morphology and some of their cellular markers; however, they remain distinguishable from endogenous microglia in terms of their transcriptome and expression of particular markers [80, 83, 87]. Given their somewhat separate status and their potential functional distinction from microglia [83], the key AMD-relevant issues that pertain to mdMs include: (1) the nature of the signals that attract them into the retina, (2) the mechanisms by which they move across the inner or outer blood-retinal barriers, and (3) their immune effects on nearby retinal cells and if these effects are different from or akin to those exerted by other innate immune cell types. In the retina, these infiltrating monocytes appear to be recruited at least in part via CCL2-CCR2 [23, 86, 88, 89] and IL33-mediated signaling [90], with Muller cells implicated as a cellular source of these ligands. The nature of the contributions that mdMs make to the progression of retinal neurodegeneration appear to vary across different injury models; measures that reduce monocyte infiltration have been largely reported to result in decreased neurodegeneration [23, 89, 91–93], but a converse increase in neurodegeneration [84] or an absence of positive or negative effects [86] have also been reported in different injury models. It is likely that mdMs have effects that are differ from

those of microglia; in the rd10 model of photoreceptor degeneration, CCR2+ mdMs are largely confined to the subretinal space and do not demonstrate dynamic phagocytic behavior while CCR2-negative putative microglia are primarily located in the outer nuclear layer and engage in dynamic phagocytosis of photoreceptors [42].

The potential involvement of mdMs in AMD pathobiology is significant as they may make a separate contribution to disease progression that is distinct from those arising from innate immune cells normally resident within the eye (microglia and choroidal macrophages). Because these mdMs derive from circulating monocytes originating from bone-marrow derived precursors, they may represent a connection between systemic immunity and local inflammatory changes occurring in the eye in AMD pathogenesis. A fuller understanding of this connection can provide a foundation for conceptualizing systemic biomarkers and systemic immunomodulatory interventions in clinical approaches to AMD.

5.2.3 Choroidal Macrophages

The locus of AMD pathology in the retina is not only confined to the photoreceptors and RPE cells but also extends outside the blood-retina barrier to the sub-RPE space, Bruch's membrane, and the choroid vasculature. As such, a consideration of the contribution of the innate immune system to AMD pathology necessarily extends to macrophages that are normally resident within the choroid. The vascular choroid contains a rich population of myeloid cells that express a host of macrophage markers (CD68, CD163), are resident in a perivascular location, and demonstrate a dendritiform or fusiform morphology [94, 95]. These cells are closely associated with choroidal vessels, including the choriocapillaris, which they engage in a polarized manner, being closely associated with the sclerad but not the vitread surface of the capillary network [18, 57]. Choroidal macrophages also demonstrate rapid surveying movements in their processes without extensive cellular migration, showing an extensive interaction with their environment that suggests an immune surveillance or homeostatic function [18]. Choroidal macrophages resemble macrophages resident around the blood-brain barrier at the central nervous system interfaces located around the dural and subdural vasculature and the choroid plexus. These so-called barrier-associated macrophages are found to demonstrate unique transcriptomes and different ontogenies and developmental patterns depending on their anatomical niche [96, 97]. However, their constitutive functions under healthy conditions and their participation in neurodegenerative disease in the CNS are not yet clearly understood.

5.3 Potential Mechanisms by which Innate Immune Cell Populations Contribute to AMD Pathobiology

Although there is substantial evidence linking inflammatory mediators to AMD, a pathogenic "worldview" that depicts the precise cellular contributions from different innate immune cell populations to AMD pathological changes at each stage of the disease, is still quite incomplete. Indeed, the different stages of AMD, from the early to the advanced, from the exudative to atrophic forms, are markedly dissimilar in their anatomical phenotype and in the nature of the tissue changes involved. As a result, the potential inflammatory processes involved at each stage are likely to be mechanistically distinct. In the sections below, we regard the AMD pathological phenotypes and transitions present at each stage and speculate on how innate immune cell populations may contribute to each phenotype/transition.

5.3.1 Potential Immune Influences Driving Drusen Formation in Early/Intermediate AMD

The formation of drusenoid deposits in the form of soft drusen in the sub-RPE space and reticular drusen in the subretinal space (otherwise termed subretinal drusenoid deposits) are the hallmark of early/intermediate AMD [98] and necessary

precursor stages for progression to advanced stages of AMD for both the atrophic form, termed geographic atrophy (GA), and the neovascular form (nvAMD) [99]. Therefore, processes that lead to the formation, secretion, and accumulation of drusen components, which comprise a diverse collection of proteins and lipids [100–102], constitute key pathologic steps in disease progression relevant to therapeutic considerations of early AMD prevention. While RPE cells grown alone in culture can secrete extracellular deposits containing a subset of drusen components, underscoring RPE cells as a key contributing cellular source [103, 104], proteonomic analyses suggest that drusen are additionally contributed to by blood-derived components [105] in the form of serum proteins. It has also been suggested that innate immune cells can contribute to the protein content of drusen, and/or induce physiological changes in RPE cells that promote drusenogenesis [106, 107]. Innate immune cells have been found to be spatially associated with drusen and even located within soft drusen in the sub-RPE space [21–23, 26, 108] and in close contact with reticular pseudodrusen in the subretinal space [109]. At least some of these cells are thought to originate from infiltrating monocytes, as they express the C-C chemokine receptor type 2 (CCR2) marker absent in microglia or choroidal macrophages [23]. Drusen proteins such as complement components, Complement Factor H (CFH), and Apolipoprotein E (APOE), are expressed by innate immune cells of the CNS, particularly in pathological situations, indicating these cells as potential sources of drusen-associated proteins. It has been hypothesized that drusenogenesis begins as an initial deposit formed in the sub-RPE space that then elicits a local inflammatory response in which immune cells organize drusen components as concentric rings around a central core, driving drusen enlargement [110].

In addition to the extracellular secretion of drusen components, the process of drusen formation has also been thought to be aided by a deficit in clearance mechanisms that normally serve to remove drusen components as they are formed. One such clearance mechanism has been attributed to phagocytosis by innate immune cells. While the outer retina (from the outer plexiform layer to the RPE layer) in the young healthy animal is largely devoid of immune cells, there is a monotonic accumulation of outer retinal myeloid cells with increasing age [55, 111], which originate from endogenous microglia migrating from the inner to the outer retina [39, 54]. These subretinal cells can phagocytose material produced by photoreceptors and RPE cells including lipofuscin [55, 112], oxidized lipids [56], and vesicles shed from photoreceptors [113], illustrating their potential for drusen clearance functions. As such, it has been proposed that subretinal drusen accumulation may be exacerbated by a diminished microglial clearance function, the efficiency of which may be regulated by AMD risk genes [114]. This function is also invoked in the mechanism proposed to underlie the ability of laser treatment to induce drusen regression [115, 116], namely that applied laser energy induces the activation of nearby resident innate immune cells, stimulating increased phagocytotic clearance of drusen [117, 118]. Another clearance mechanism demonstrated by innate immune cells is by mediating cholesterol efflux that removes cholesterol from the retina back to the liver through the bloodstream [119]. Specific impairment of this reverse cholesterol transport by the genetic deletion of cholesterol ABC transporters, ABCA1 and ABCG1, specifically in myeloid cells resulted in the induction of subretinal deposits and associated photoreceptor dysfunction and neurodegeneration that recapitulated features of AMD pathology [43]. Taken together, it is possible that innate immune cells may directly contribute to drusenogenesis by the production of drusen components and/or by a deficiency in clearance mechanisms that normally serve to prevent drusen accumulation.

Drusen formation in early AMD may also be influenced by innate immune cells indirectly via their negative effects on the choroidal vasculature. Insufficiency of the choroidal vascular supply to the region of Bruch's membrane is another factor that has been related to drusen formation, specifically that an impaired vascular drainage of

Bruch's membrane retards the constitutive clearance of drusen deposits via the circulation. The structure of the choroid, in terms of its overall thickness and degree of vascularity, has been negatively correlated with advancing age [120] and increasing drusen presence [121, 122], with the local choriocapillary vascular bed directly beneath drusen demonstrating a decreased density of patent vessels [123, 124], supporting the hypothesis that the loss of choroidal vasculature may promote drusen formation [125]. There are a number of indications that this age- and AMD-related vascular loss may result from inflammatory influences: (1) choriocapillary vascular density loss was correlated with increasing numbers of choroidal macrophages in both mouse models [18] and human AMD tissue with non-advanced disease [57], (2) increased complement activation, evident as increased local formation of membrane attack complex (MAC), a factor capable of inducing endothelial cell injury [126], was associated with increasing age and higher risk genotypes for AMD [127] and potentially modulated by innate immune cells [10], and (3) mast cell number and mast cell degranulation in the choroid, which can influence microglia/macrophage activation reciprocally [128, 129], were elevated in AMD eyes, even at the early stage in the disease [130]. These suggestive correlative associations are currently explored in studies involving the experimental manipulation of macrophage presence or activation that can ascertain causal relationships between innate immune cells and choroidal vasculature.

Taken together, a body of evidence suggests that innate immune cells are closely associated with drusen in AMD and demonstrate cellular mechanisms that can be influential to drusenogenesis via direct and indirect mechanisms. In addition, there are indications that retinal microglia may also react to drusen presence early in disease progression, showing upregulation of the major histocompatibility complex II (MHCII) expression [131], and perhaps contributing to a non-cystic swelling of the retina [132] and choroid [121] at the intermediate stage. The reaction of innate immune cells to drusen, either from attempts to clear them and/or stimulation from drusen components, when sustained may result in longer term effects on the retina that can contribute to further AMD advancement. These effects are discussed in the following sections.

5.3.2 Potential Immune Influences Driving RPE Disorganization in Intermediate AMD

Besides the presence of large drusen, another clinical phenotype in intermediate AMD that portends increased risk for progression to advanced disease (both GA and nvAMD) is the presence of hyper- and hypopigmentary changes in the retina [133, 134]. These pigmentary changes correspond to small areas of intraretinal RPE disorganization and migration as revealed on optical coherence tomography (OCT) imaging [135], indicating that abnormalities in RPE organization and integrity may be precursors to overt retinal atrophy or neovascular changes. There are experimental findings that indicate that innate immune cells in the aging retina may help promote these early RPE changes. The age-dependent migration of microglia into the outer retina characterized in rodent models show that microglia-RPE contacts and interactions which are rare in the young mouse, are prevalent in the aged retina. While microglia in the subretinal space has been described as potentially beneficial to the RPE cell layer in some rodent injury models [61], there is also evidence that subretinal microglia with altered activation states can alter the local immune environment and exert deleterious effects on RPE integrity [20, 136, 137]. In vitro experiments also show that microglia activated with lipopolysaccharide stimulation can induce changes in the RPE monolayer, including loss of tight-junctional structure, increased RPE cell migration, and increased expression of pro-inflammatory cytokines [58, 138]. The initiating cause for increased microglia migration into the subretinal space with aging is unclear, but has been associated with age-related accumulation of substances in the outer retina including amyloid beta [139], oxidized lipids [56], lipofuscin-related compounds [112, 140]. As such, innate immune

cell interactions with the RPE cell layer may induce early defects in RPE integrity that, together with drusen formation, drive progression to the advanced atrophic and neovascular forms of AMD.

5.3.3 Potential Immune Influences Driving Photoreceptor and RPE Atrophy in Geographic Atrophy

The advanced or late stage of AMD, which is primarily responsible for central vision loss associated with the disease, takes two phenotypic forms: (1) geographic atrophy (GA) featuring enlarging areas of macular atrophy that extend contiguously across retinal layers, involving photoreceptors, RPE cells, and the choriocapillaris, and (2) neovascular AMD, in which neovascular growth of vessels from the choroid into the retina culminates in exudative changes that disrupt retinal structure, leading in the long term to retinal fibrosis and widespread cellular loss. In geographic atrophy, large soft drusen and pigmentary changes are anatomical risk factors for the initial emergence of the atrophic lesion; longitudinal studies have demonstrated that significant regression of large soft drusen is followed by deleterious physiological changes in RPE cells in the same local area [141, 142], progressing to overt RPE and photoreceptor loss [143–145]. Once arisen, the area of the GA lesion increases slowly but monotonically with time via the contiguous expansion of its borders [146, 147]. Concurrently, the underlying choriocapillaris vasculature, evidently already decreased in structure and blood flow at the intermediate AMD stage [123, 148], undergoes further degeneration upon the emergence of GA [149].

While the causes for the initial emergence of the GA lesion and its subsequent expansion over time are likely multifactorial, it may be instructive to consider in the potential contribution that innate immune cells may make to these phases of GA evolution. Multiple clinical studies have targeted immunomodulatory mechanisms in attempts to prevent and retard GA evolution [150–152], but none have yet shown efficacy. A current limitation in these efforts is a lack of clarity regarding how immune mechanisms contribute to the specific phenotypes in GA evolution and growth and how mechanisms targeted in clinical trials contribute to actual anatomical changes in the AMD eye that constitute the primary clinical outcome on which the success or failure of the trial hinges.

One relevant pathogenic question in targeting GA concerns how regression of large soft drusen, the initial event leading to GA emergence, is induced and whether innate immune cells may play a role in this step. The agency of microglia and perivascular macrophages of the brain in clearing extracellular amyloid β (Aβ)-containing deposits in Alzheimer's disease (AD) is well-described [153, 154]; these cells express a host of phagocytic cellular machinery, including scavenger receptor A (SR-A), complement C3, cluster of differentiation 36 (CD36) and the receptor for advanced glycation end products (RAGE), to engage their extracellular targets [155]. Although this clearance by innate immune cells is thought to be helpful initially in maintaining homeostasis in the AD brain, it is hypothesized that these cells following prolonged exposure to Aβ become chronically stimulated and metabolically imbalanced, escalating their production of proinflammatory cytokines and neurotoxic factors [156, 157]. In the absence of accessible AMD animal models that exhibit actual drusen formation and regression, we may extrapolate from processes in AD models to hypothesize that innate immune cells are analogously drawn to the outer retina by drusen presence and actively contribute to drusen regression. Subsequently, through prolonged exposure to drusen contents via phagocytotic clearance, outer retinal microglia may be converted to a pathological neurotoxic state and begin to exert a deleterious effect on nearby photoreceptors and RPE cells. In clinical OCT imaging of AMD, the presence and prominence of intraretinal hyperreflective foci, which have been correlated to microglia [158], have been associated with an increased risk of progression to GA [159, 160], implicating microglia at the site of pathology. On histopathology, Iba1+ innate immune cells have been found in the vicinity of reticular pseudodrusen and RPE damage [109], and also within and on the

transitional zone of GA lesions [14], confirming their proximity to regions of GA initiation and spread.

Despite the inability of mouse models to recapitulate drusen regression, there has been multiple models linking the presence of microglia/mdM to increased photoreceptor and RPE degeneration. As previously reviewed [161], the outer retina is an immunosuppressive environment in which multiple molecular signals, some of which are related to AMD genetic risk factors such as CFH [162] and ApoE [14, 15], serve to limit the number and survival of microglia/mdM in the subretinal space. A surplus of innate immune cells in the subretinal space, particularly when associated with a dysregulated state of activation, such as in CX3CR1 deficiency [20], has been associated with deleterious effects in the outer retina, likely mediated by increased production of proinflammatory cytokines such as IL1β [21, 41, 42] and tumor necrosis factor α (TNFα) [163, 164]. These observations taken together posit that the onset and spread of GA may be initially triggered by the homeostatic clearance of drusen by phagocytic innate immune cells; when this clearance activity is chronic and sustained, or exceeds a particular threshold, these cells begin to exert a proinflammatory, neurotoxic, and dyshomeostatic influence in their surroundings, potentially driving AMD-related RPE and photoreceptor degeneration. Identification and clarification of the relevant immune cell-to-retinal cell intercellular interactions underlying the phenotypic transitions in GA onset and expansion may perhaps be the necessary foundation for planning for how the activity of implicated molecules (e.g., complement, Htra1, or ApoE) should be therapeutically enhanced, inhibited, or modulated.

5.3.4 Potential Immune Influences Driving the Growth and Exudation of Choroidal Neovascular Vessels

The other late form of AMD, nvAMD, like GA, develops in the context of intermediate AMD but demonstrates marked differences from GA in terms of its development and phenotype. Earlier histopathological studies have suggested that non-exudative neovascular vessels, termed choroidal neovascularization (CNV), emerge from the choriocapillaris and extend into or beneath Bruch's membrane as the first step in CNV development [165]. More recently, these clinically "silent" initial vessels have been documented by optical coherence tomography angiography (OCTA) imaging in enhanced anatomical detail in AMD patients in a clinical setting [166], permitting their longitudinal progression to be studied [167, 168]. These and other studies have revealed that CNV emerges in eyes with intermediate AMD in the context of choriocapillaris vessel loss [120, 169] and flow impairment [170]. It has been hypothesized that when choriocapillaris loss and subsequent outer retinal ischemia occur in the setting of preserved RPE structure, increased vascular endothelial growth factor (VEGF) production induces initial neovascular buds to form from existing choriocapillaris vessels [171] that can subsequently enlarge and invade the retina. At a later point, these initially quiescent vessels develop altered permeability and exudative activity, although the mechanism underlying this transition remains obscure [167].

Given this pathogenic sequence, what is the role of the innate immune cells in the retina and choroid in the evolution of CNV? There are numerous studies that indicate that CNV membranes in clinical AMD are accompanied by a local increase in activated innate immune cells; these are prominent in histopathological studies of postmortem AMD eyes [1, 29], as well as found in neovascular membranes excised surgically from patients with AMD [172, 173]. Other non-AMD inflammatory diseases of the retina, in which innate immune involvement is prominent, can also drive the formation of phenotypically-similar CNV membranes [174]. Experimental studies of CNV in animal models have also documented the local increase in immune cells [175]; manipulations that increase innate immune cell number and activation in the outer retina can increase CNV [56, 138], while those that deplete them or inhibit

their activation can exert the converse effect [176–178]. The precise identity and type of innate immune cells that exert these pro-angiogenic influences are not completely defined; altered number and activation status of choroidal macrophages have been found in nvAMD eyes [26, 57], implicating the role of resident macrophages in the choroid, while animal studies manipulating circulating monocytes to alter their number and activation at the location of the CNV demonstrated effects on lesion size, implicating involvement of infiltrating mdMs [179–181].

In considering the possible innate immune mechanisms contributing to CNV formation, recent anatomical and clinical studies have indicated that CNV evolution may indeed also be a multistage process. Choriocapillaris and choroidal vascular loss in the context of intermediate AMD may constitute a necessary precondition for CNV formation; this can be contributed to by innate immune mechanisms, potentially involving complement activation and MAC deposition, as detailed earlier. In this potentially proinflammatory and ischemic anatomical context, de novo neovascular budding, growth, and extension into the retina may be initiated, possibly as an initial adaptive response. In this process, angiogenic mechanisms, including those involving immune cells may be involved. The molecular mechanisms inducing neovascular growth initiation likely include VEGF upregulation; innate immune cells have been shown to secrete VEGF directly [182, 183], or induce RPE cells to upregulate VEGF secretion [138, 184]. Other potential pro-angiogenic effectors mediated by innate immune cells include growth factors such as angiopoietin 2 (Ang2) [185], and placental growth factor (PGF) [186, 187] which can signal to vascular cells, as well as inflammatory cytokines and mediators that can regulate immune cell activation and recruitment, including TNFα [188, 189], TGFβ [13], and interferon-β [190].

Following the initiation of this initial neovascular complex, the CNV membrane gains pathological significance when it grows to invade Bruch's membrane and acquire exudative features. Innate immune cells associated with CNVs have been documented to express matrix metalloproteinases [191] which may mediate extracellular matrix breakdown in Bruch's membrane to enable their invasive capability [192, 193]. The transition from subclinical CNV to exudative CNV may be potentially related to changes in pericyte coverage [165, 194]; as such, future studies examining the interaction between pericytes and macrophages in the context of CNV [195, 196] may be instructive in elucidating the mechanism underlying the development of exudative membranes.

In summary, innate immunity may play an important role in multiple stages of "wet" AMD pathogenesis, as suggested by clinical studies showing the efficacy of anti-inflammatory steroid treatment as an ancillary treatment in exudative AMD [197]. A more detailed mechanistic understanding of inflammatory mediators to each stage of CNV initiation, growth, and exudation, can uncover more targeted and effective treatments for CNV prevention and management.

5.4 Conclusion

Our overall understanding of AMD as an inflammatory disease is currently undergoing steady evolution we accumulate more information regarding: (1) the distinct innate immune cell populations that are influential in the retina, (2) the cellular and molecular mechanisms regulating the physiologies of these innate immune cell types, and how these mechanisms relate to genetic risk factors for AMD, and (3) how innate immune cell interactions with retinal cell types such as RPE cells, photoreceptors, and vascular cells in the choroid, drive the initiation and evolution of anatomical changes that characterize each separate stage of AMD. Progress in these areas would enable us to clarify how imbalances in specific implicated molecular pathways (e.g., complement dysregulation, HTRA1 overactivity) may contribute mechanistically to the stage-by-stage progression of AMD, so that the correct stage of the disease may be targeted in interventional clinical studies, and the relevant corresponding

anatomical outcomes measures selected, so as to maximize the chances of finding efficacious treatments for AMD treatment and prevention.

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Immunological Aspects of Age-Related Macular Degeneration

6

Michael J. Allingham, Anna Loksztejn, Scott W. Cousins, and Priyatham S. Mettu

Abstract

Increasing evidence over the past two decades points to a pivotal role for immune mechanisms in age-related macular degeneration (AMD) pathobiology. In this chapter, we will explore immunological aspects of AMD, with a specific focus on how immune mechanisms modulate clinical phenotypes of disease and severity and how components of the immune system may serve as triggers for disease progression in both dry and neovascular AMD. We will briefly review the biology of the immune system, defining the role of immune mechanisms in chronic degenerative disease and differentiating from immune responses to acute injury or infection. We will explore current understanding of the roles of innate immunity (especially macrophages), antigen-specific immunity (T cells, B cells, and autoimmunity), immune amplifications systems, especially complement activity and the NLRP3 inflammasome, in the pathogenesis of both dry and neovascular AMD, reviewing data from pathology, experimental animal models, and clinical studies of AMD patients. We will also assess how interactions between the immune system and infectious pathogens could potentially modulate AMD pathobiology via alterations in in immune effector mechanisms. We will conclude by reviewing the paradigm of "response to injury," which provides a means to integrate various immunologic mechanisms along with nonimmune mechanisms of tissue injury and repair as a model to understand the pathobiology of AMD.

Keywords

Age-related macular degeneration · Drusen · Choroidal neovascularization · Geographic atrophy · Pathobiology · Immunology · Innate immunity · Macrophages · Monocytes · T cells · B cells · Autoimmunity · Complement · NLRP3 inflammasome

6.1 Introduction

Age-related macular degeneration (AMD) is a progressive degenerative disorder, involving the

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retinal pigment epithelium (RPE), neurosensory retina, Bruch's membrane, and choriocapillaris, and represents the leading cause of vision loss among the elderly [1, 2]. AMD pathogenesis is complex and multifactorial and includes aging, genetic, systemic health, and environmental risk factors [3–11]. While the specific mechanisms mediating disease onset and progression remain unknown, increasing evidence over the past two decades points to a pivotal role for immune mechanisms in AMD pathobiology.

This chapter will explore immunological aspects of AMD, with a specific focus on how immune mechanisms modulate disease phenotype and severity and how components of the immune system may serve as triggers for disease progression. We will briefly review the biology of the immune system, defining the role of immune mechanisms in chronic degenerative disease and differentiating from immune responses to acute injury or infection. We will explore current understanding of the roles of innate immunity, adaptive immunity, and immune amplifications systems, especially complement activity and the NLRP3 inflammasome, in pathogenesis of the various stages of AMD. Note that the role of resident retinal microglia is beyond the scope of this chapter and will be discussed in a separate dedicated chapter. Finally, we will conclude by reviewing the paradigm of "response to injury," which provides a means to integrate various immunologic mechanisms along with nonimmune mechanisms of tissue injury and repair as a model to understand the pathogenesis of AMD.

6.2 Biology of Immunology Relevant to AMD

In general, there are two types of immune responses: innate and adaptive immunity [12–14]. Innate immunity includes stereotyped, nonspecific responses to various components, including microbes, foreign substances, products of cellular injury, or other potentially offending stimuli. Adaptive immunity includes antigen-specific responses that are designed to rid the body of very specific foreign (i.e., not "self") components, using targeted and coordinated antibody and cellular responses. While innate and adaptive immune mechanisms are typically studied in the context of acute responses to infection injury, both types of immune responses have potential relevance to chronic degenerative diseases such as AMD. We will review specific biologic aspects of innate and adaptive immunity that are relevant to AMD.

6.2.1 Innate Immunity

Innate immunity includes specific immune cell types, especially macrophages and neutrophils, that respond to various stimuli via a pattern recognition response. Such stimuli include microbe-associated molecules (e.g., pathogen-associated molecular patterns, or PAMPs), toxins, or cellular debris resulting from injury (e.g., damage-associated molecular patterns, or DAMPs) [15–17]. Innate immune cells bind to these stimuli via specific pattern recognition receptors (PRRs), (e.g., families of PRRs such as Toll-like receptors (TLRs)). Activation of PRRs on innate immune cells subsequently triggers a stereotyped, antigen-independent signaling, and cellular response. Innate immune cells then [1] phagocytose such stimuli, sequestering, degrading, and processing stimuli, [2] generate biochemical mediators that affect the surrounding microenvironment and recruit additional inflammatory cells, and [3] serve as antigen-presenting cells (APCs), displaying processed antigen on the cell surface to other immune cells, especially cells of the adaptive immune system [18]. PRRs are also frequently found on the cell surface of many parenchymal (nonimmune) cell types, (e.g., as with TLRs on the surface of RPE cells), so stimuli such as PAMPs and DAMPs can also directly stimulate PRRs on parenchymal cells to initiate a direct cellular response from nonimmune cells [19, 20].

In the innate immune response, the PRR signaling mechanisms in monocytes, macrophages, and neutrophils have been genetically and evolutionarily predetermined to recognize conserved molecular patterns on different triggering stimuli. These molecular patterns frequently include specific amino acid sequences, certain lipoproteins,

certain phospholipids, or molecular components. While there is tremendous heterogeneity for such stimuli, the key feature of innate immune response is that these varied stimuli frequently trigger the same stereotyped cellular response. Thus, PRRs s, and the innate immune response they activate, are generally conserved among individuals within a given species [18].

Classically, the fundamental innate immune response is considered within the context of acute infection. For example, in endophthalmitis, or infection within the eye, microbial-derived molecules (which are frequently toxic to host tissue) or cellular debris arising from damaged host tissue bind PRRs, activating neutrophils and monocytes, which in turn phagocytose stimuli, produce inflammatory cytokines, and stimulate the recruitment of additional immune cells [18]. Microbial-derived stimuli can also activate receptors on retinal neurons, exacerbating cellular injury. The PRR-activated mechanisms and effector responses to bacteria such as *Staphylococcus* are nearly identical to those of other organisms, since they are determined by the recognition of conserved patterns or motifs that may be present across different families of stimuli of microbes or cellular debris in the microenvironment. Of course, the innate immune response in the setting of chronic degenerative diseases such as AMD is substantially different from the response encountered in the setting of acute infection or injury.

6.2.1.1 Monocytes and Macrophages

The monocyte, innate immune cell circulating in the blood and originating from the bone marrow, and the macrophage, phagocytic innate immune cell in the tissue, are vital immune cells [15]. Monocytes are relatively large cells (12–20 μm in suspension, but up to 40 μm in tissues) and traffic through many normal sites. Upon tissue infiltration, they can give rise to blood-derived macrophages. Macrophages include two populations: tissue-resident macrophages and blood-derived macrophages. Tissue-resident macrophages are comprised of either precursor cells that migrated into the tissue during embryonic development (i.e., "yolk-sac" or fetal liver derived) or monocyte-derived cells that migrated into a tissue weeks or months previously; the relative proportion of embryonic precursor-derived vs. monocyte-derived is tissue-dependent and at least partially determined by ease of access of circulating monocytes to the specific tissue compartment [21]. The primary function of tissue-derived macrophages is tissue-resident macrophages typically acquire tissue-specific properties and may be distinguished by specific cellular markers. In many tissues, resident macrophages have been given tissue-specific names (e.g., microglia in the brain and retina) [22–24]. In contrast, blood-derived macrophages represent monocytes that have recently migrated from the blood into a tissue locus, usually within a few days and typically in the setting of inflammation or injury, having transformed into macrophages from the monocyte but still maintaining many properties of the circulating cell [21].

Macrophages serve three primary functions: as scavengers to clear cell debris and pathogens while limiting significant tissue damage, as antigen presenting cells for T lymphocytes, and as inflammatory effector cells. Conceptually, macrophages exist in different levels or stages of metabolic and functional activity, each representing different "programs" of gene activation and mediator synthesis. There are multiple paradigms that have been put forth to understand differential biology of monocyte and macrophages, the majority of which is focused on differences in inflammatory and effector functions. In the *conventional paradigm*, infiltrating "quiescent" M0 monocytes can become differentiated into classically activated ("M1") or alternatively activated ("M2") macrophages within inflamed tissues [25–27]. Typical activational stimuli that promote activation of M1 macrophages include bacterial toxins (such as lipopolysaccharide (LPS)), antibody-coated pathogens, complement-coated debris, or certain cytokines [28–30]. While M1 macrophages are proinflammatory, M2 macrophages mediate tissue repair, angiogenesis, and resolution of inflammation [31–35]. M2 macrophages contribute to physiologic processes

such as fibrosis, wound repair, extracellular matrix turnover, and angiogenesis [36–44]. However, these processes can contribute to chronic injury in the absence of inflammatory cell infiltration or widespread tissue destruction. For example, M2 macrophages play important roles in the pathogenesis of atherosclerosis, glomerulosclerosis, osteoarthritis, keloid formation, pulmonary fibrosis, and other noninflammatory disorders, indicating that the "repair" process is not always beneficial to tissues with complex morphologies with precise structure-function requirements [45–49]. The M1-M2 dichotomy in this conventional paradigm derives primarily from in vitro studies and presupposes that the fate and effector function of recruited monocytes are determined in bulk populations of cells by differentiation factors within an inflamed tissue microenvironment, and that macrophages can be "switched" from M1 to M2, and vice versa, either by manipulating factors in the tissue microenvironment or by altering intracellular signaling or transcriptional activity within macrophages that regulate the cell's M1 or M2 identity.

Investigators from the monocyte ontogeny field have proposed an alternative paradigm: Functionally distinct monocyte subsets can be identified in the blood according to specific cell surface markers [50, 51], which include Ly6C in mice and CD14 and CD16 in humans. In mice, "classical" Ly6C^{hi} monocytes (~80% of normal blood monocytes) have high expression of CCR2, low expression of CX_3CR1, and are inflammatory, expressing high levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide synthase 2 (NOS2), and proteases [52]. This subset tends to predominate in the normal physiologic unperturbed "steady state." "Nonclassical" Ly6C^{lo} monocytes (~10%) have low expression of CCR2, high expression of CX_3CR1, and in the setting of infection, injury, or illness, serve a reparative function, expressing profibrogenic factors such as TGF-β, osteopontin, insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), connective tissue growth factor (CTGF), and others [52–54]. This alternative paradigm presupposes that monocytes are pre-programmed to one or the other subset within bone marrow, spleen, or lymphoid tissue. Thus, circulating monocytes in the blood are committed to specific effector functions and are triggered to mediate these functions upon tissue entry. While Ly6C^{hi} monocytes enter tissue early in response to injury or infection, Ly6C^{lo} monocyte tissue entry is delayed (i.e., during a second phase) [52, 55], creating a biphasic response of early inflammatory response followed by later reparative response. In humans, these subsets include classical CD14^{++}CD16^{-}, intermediate CD14^{+} $^{+}$CD16^{+}, and nonclassical CD14^{+}CD16^{+} monocytes [56, 57]. However, considerably less is understood about the biology of these subsets in humans, with investigations suggesting that that nonclassical and intermediate subsets may serve pro-inflammatory roles, and the classical subset serving more reparative roles [56, 57].

To add to the complexity, some studies suggest a third blended paradigm. Dichotomous blood monocyte subsets are present, but tissue Ly6C^{lo} macrophages are presumed to be derived from Ly6C^{hi} infiltrating monocytes, which undergo a poorly understood "phenotype switch" into a Ly6C^{int}-Ly6C^{lo} subset [58, 59].

Importantly, all of these paradigms are not mutually exclusive and may apply variably to different tissues and different disease states, including various stages of AMD disease. For the purposes of simplicity and convenience and to account broadly for these paradigms, this chapter will refer to macrophage subsets as *scavenging, inflammatory, and reparative* macrophage subsets to distinguish macrophages based on effector function and on the potential contributions of each to AMD disease.

6.2.1.2 Dendritic Cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) that serve the primary function of processing antigens and presenting antigen components to T cells DCs are distinct from monocytes and macrophages and comprise approximately 0.1–1% of blood mononuclear cells [60]. Within tissue, DCs grow in size (15–30 μm) that form extensions 23 times the diameter of the cell, similar in appearance to the dendritic structure of neurons (hence the name of

the cell). Nonlymphoid and lymphoid organs recruit DCs by defined migration pathways, and within these sites, DCs function as accessory cells that process and present antigens to T cells. Specifically, DCs serve as APCs for naïve lymphocytes and trigger responses in these cells, and are potent activators of T-cell dependent immune responses. In contrast, to macrophages, the function of DCs is primarily focused on antigen presentation; DCs have limited or no capacity as phagocytic scavenger (repair) cells nor are they major producers of inflammatory mediators. While DCs can be found in the retina and the choroid [61, 62], they are not thought to be significant contributors to AMD pathobiology, as they are not readily detected in histopathology studies of human AMD or in preclinical (e.g., mouse) models of AMD.

6.2.1.3 Basophils and Mast Cells

Basophils are a type of circulating granulocyte that can be recruited from the circulation into the tissue and have been shown to play roles in different types of inflammatory reactions, especially allergic disease. Mast cells are another type of granulocyte that has similar appearance and function; both cells store and release histamine. Once thought to be blood-borne and tissue equivalents, ontogeny studies have demonstrated that basophils and mast cells have distinct hematopoietic origins.

There are two major subtypes of mast cells, connective tissue versus mucosal subtypes. Both subtypes can release preformed granules following activation of receptors on the cell surface, and both subtypes can produce certain mediators de novo [63, 64]. Mast cells of the connective tissue subtype predominantly have granules with histamine and heparin; upon stimulation, they can also produce prostaglandin D2. Mast cells of the mucosal subtype also form granules but need T cell-derived cytokines to enable formation of granules; for this reason, they typically contain lower levels of histamine. Mucosal mast cells are also functionally distinguished in that they synthesize primarily leukotrienes upon cellular stimulation. Specific granule content and effector function can be dependent on tissue location, but the regulatory mechanisms for each are poorly understood. In contrast to other granulocytes such as neutrophils and eosinophils, mast cells have unique granule contents and also have on their cell surface high-affinity Fc receptors for IgE. As such, mast cells are the primary effector cells in in IgE-mediated immune responses, including allergic disease, asthma, atopy, hay fever, and immediate hypersensitivity. However, mast cells can also contribute to other aspects of cell-mediated immunity and wound healing [65, 66], and stimuli beyond IgE, including complement and certain effector cytokines, can mediate mast cell activation and promote cellular degranulation [67]. Mast cells can also secrete other inflammatory cytokines such as TNF-α, thereby mediating cellular injury and acute inflammatory processes. For example, mast cells have been shown to promote neuronal degeneration and death in the setting of thiamine deficiency and certain metabolic diseases [64]. Mast cells have also been detected in atheromatous plaques have been found to be co-localized with angiogenic mediators, such as platelet-derived endothelial growth factor (PDGF) [67–73].

Mast cells (of the connective tissue subtype) have been found in abundance in the choroid [61, 74]. The contributions of mast cells in AMD are not fully understood, though there is some evidence to suggest they may play limited roles. In histopathology studies, degranulated mast cells have been identified near foci of geographic atrophy (GA) [75]. Mast cells have also detected at sites of incipient new vessel formation and have been detected near Bruch's membrane in all stages of AMD [76]. Release of mediators such as heparin, metalloproteinases, and VEGF from mast cells may also activate endothelial cells and promote the early steps of angiogenesis [77–79]. Oral tranilast, a drug that inhibits mast cell degranulation, has been shown to suppress CNV in the rat model of laser-induced CNV [80].

6.2.2 Adaptive Immunity

Adaptive immunity, also referred to "antigen-specific" or "acquired" immunity, is a distinct aspect of the immune response, in which there is a generated response to a specific portion, or

epitope, of an antigen; this is fundamentally distinct from the broad, genetically pre-determined, and stereotyped response of innate immunity [12–14]. In adaptive immunity, there is specific "recognition" by cells of the immune system of a unique antigen as "foreign" to the body and therefore distinct from "self" components. Once recognized and bound, specialized cells of the immune system, called antigen-presenting cells (APCs), display antigenic components on the cell surface and interact with a host of adaptive immune cells. Once the adaptive immune cell (T and B lymphocyte) that recognizes the specific antigenic component is engaged, this subsequently results in the activation of these unique antigen-specific immunologic effector cells (T and B lymphocytes), with production of unique antigen-specific soluble effector molecules (antibodies). The goal of this targeted response a specific antigen is to effect the removal of the specific antigen (and the source from which the antigen derives) from the body in a specific and targeted fashion, other irrelevant antigenic stimuli (including "self" antigens). In the adaptive immune response, these cells must generate, de novo, a specific receptor, which, in turn, must recognize a unique molecular structure in the antigen for which no pre-existing gene was present. As such, the adaptive immune system has evolved a mechanism for the generation of new antigen receptor genes in T and B lymphocytes through recombination, rearrangement, and mutation of the germline genetic structure. As a result, the adaptive immune cells produce a "repertoire" of new antigen receptors, producing a diversity of immune cell recognition capacity within and among individuals.

Classically, the adaptive immune response is vital for response to viral pathogens that can mutate or change over time. The host that is infected by a given virus cannot a priori evolve the requisite receptors to recognize novel mutations in that virus. However, upon infection, novel antigens produced by these mutations in the virus can then stimulate an antigen-specific immune response by the host to the virus, which will recognize the virus producing that antigen, but not other viruses that do not produce this novel antigen.

While adaptive immune mechanisms traditionally have not been thought to contribute to AMD, emerging clinical evidence as well as several preclinical models suggest potential plausible roles for adaptive immunity in various aspects of AMD disease (see following sections below).

Lymphocytes of the adaptive immune system derive from precursors cells within the bone marrow [14, 81, 82]. Full functionality of lymphocytes requires subsequent maturation in peripheral lymphoid organs, where recognition of specific antigens takes place. Lymphocytes are subdivided based upon function and on the presence of specific detectable cell surface proteins (i.e., surface markers). These "markers" are related to functional and molecular activity of individual subsets. Three broad categories of lymphocytes have been determined: B cells, T cells and non-T, non-B lymphocytes.

6.2.2.1 T Lymphocytes

Thymus-derived lymphocytes, which are commonly referred to as T cells, are comprised of several subsets, functioning as effector cells to mediate antigen-specific inflammation and immune responses [83, 84]. Helper T cells (T_h cells), which are recognized as CD4+ T cells, assist in the processing of antigen for antigen-specific immunity within lymph nodes; specifically, CD4+ T cells are vital in assisting B cells to produce antibody in response to specific antigenic components and in sensitizing antigen-specific cytotoxic T cells. Outside the lymph node, CD4+ T cells can also serve as direct effector cells through production and release of certain mediators (e.g., interferon-γ and TNF-β) [85]. In this setting, CD4+ T cells can home into a particular tissue, recognize antigen and APCs, upon recognition fully activate, and then release cytokines and mediators to amplify the immune response. In certain instances, CD4+ T cells can also become activated in an antigen-independent fashion, called *bystander activation* [86–88]. This phenomenon may account for the occasional presence of T lymphocytes detected in CNV specimens from AMD eyes [76, 89].

Cytotoxic T cells (T_c cells), which are recognized as CD8+ T cells, serve as a second subset of T cells, which mediate effector biology to kill virally infected host cells or to recognize and kill tumor cells via release of cytotoxic mediators that are highly injurious or by specialized pore-forming molecules that compromise the structural integrity of target cells. CD8+ T bind MHC class I molecules (which are present on the surface of nearly all nucleated cells) and recognize specific antigenic targets; upon activation, CD8+ T cells produce IL-2 and IFN-γ, cytokines that modulate effector functions of other immune cells (e.g., macrophages, NK cells). It is possible though unlikely that CD8+ T cells contribute to AMD pathobiology.

A third type of T cells, T helper 17 (T_h17) cells, are pro-inflammatory T helper cells that produce IL-17. While T_h17 cells have been shown to play important roles in host defense at mucosal surfaces and in pathogen clearance, they have also been shown to contribute to chronic inflammation and autoimmune disorders, especially in diseases such as rheumatoid arthritis [90]. As will be discussed below, there is some evidence suggesting a contribution of T_h17 cells to AMD.

6.2.2.2 B Lymphocytes and Antibody

B lymphocytes, or B cells, are responsible for the humoral component of the adaptive immune system, producing antibodies that recognize specific antigens. Developmentally, B cells originate from and mature in the bone marrow, though they may complete development to maturity in the spleen. Antibodies, which can include various immunoglobulins isotypes, are soluble antigen-specific effector molecules that mediate the adaptive immune response [14, 81, 82]. B cells express B cell receptors (BCRs) on their cell surface, which enables recognition of specific antigens. Following antigen recognition with the assistance of CD4+ T cells in lymphoid organs (such as lymph nodes), B cells are activated to produce and secrete After appropriate antigenic stimulation with T cell help, B cells secrete antibodies (initially IgM isotype and then later others) at lymphoid organs, which pass into efferent lymph fluid and eventually into the venous circulation. Once in bloodstream or within tissues, antibodies can then mediate a diverse spectrum of immune effector activities by binding to the specific antigen it recognizes and targets.

There are at least four potential mechanisms by which antibodies mediate immune responses within specific tissue microenvironments. Circulating antibodies in the bloodstream (derived from B cells in lymphoid organs) can bind antigen to form circulating immune complexes, which can then deposit into tissues. In a second mechanism, B cells from the circulation can entire specific tissues sites and produce antibodies locally, which then form immune complexes locally following binding to recognized antigens. Third, the Fc portion of a secreted antibody can bind to an innate immune cell, especially macrophage, neutrophil or mast cell (via Fc receptor on cell the surface of that cell), which can result in a combined antibody and cellular effector immune response. None of these three mechanisms are likely to contribute to AMD pathobiology.

It is possible, however, that a fourth antibody-mediated mechanism may contribute to AMD: circulating antibodies (typically the IgG isotype), previously produced and secreted by B cells in lymph nodes and other tissue sites, may passively leak into tissue with fenestrated capillaries, like the choriocapillaris. Antibodies that accumulate within the tissue may then form an immune complex with antigens within the tissue, triggering the onset of a specific effector mechanism [14, 81, 82, 91–94]. We will consider several such effector mechanisms here (Fig. 6.1):

(a) *Immune complexes with extracellular matrix-bound antigens:* Free antibody passively entering the tissue can bind to recognized antigen trapped, or bound, within the extracellular matrix of the tissue, forming antibody-antigen immune complexes. Such complexes can in some instances trigger activation of the complement system, which can produce local cellular injury as well as production of

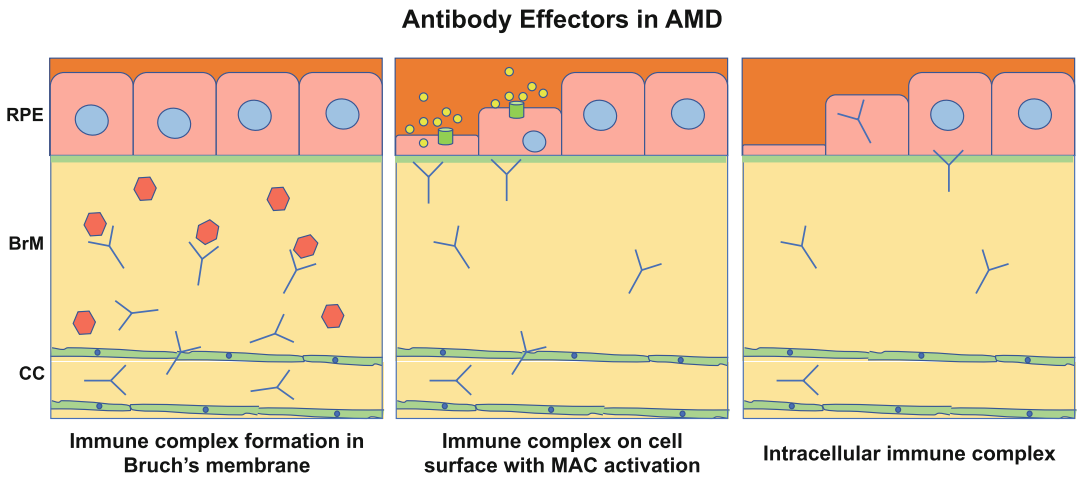

Fig. 6.1 Possible mechanisms for antibody effectors in AMD, including immune complex (IC) formation in Bruch's membrane (BrM), IC formation on retinal pigment epithelial (RPE), or choroidal endothelial, cell surface with membrane activation complex (MAC) activation, or endocytosis of antibody with subsequent intracellular IC formation with antigen within cell. *CC* choriocapillaris

complement components called anaphylatoxins, which serve to recruit additional inflammatory cells. In chronic disease such as AMD, such formation of in situ immune complexes may occur at a low-grade level, which could be consistent with the modest (and variable) immune cellular infiltration observed in AMD.

(b) *Immune complexes with cell-surface antigen:* Free antibody passively entering the tissue might also bind to antigen on the surface of cells within tissue, and this antigen-antibody binding could itself trigger complement activation and specifically formation of the membrane attack complex (MAC), which can then induce cellular injury, a phenomena that has been observed in certain immune-mediated diseases (e.g., hemolytic anemia). Given the genetic associations of AMD with the complement system, it is plausible that this mechanism may contribute to cellular injury (e.g., at RPE cells) in the setting of AMD (Fig. 6.1).

(c) *Immune complex with intracellular antigen:* Another potential means by which free antibody may mediate cellular injury is via immune complex with intracellular antigen [93, 94]. In this setting, an antibody is bound and then internalized into cells independent of antigen biding. The antibody functionally becomes an autoantibody (i.e., antibodies recognizing self-antigens) directed against a specific intracellular antigen, which could be in various organelles, including the ribosome or nucleus (Fig. 6.1). The bound autoantibody-antigen can alter cellular signaling pathways or cellular metabolism, creating cellular injury. Such a mechanism is well described in the setting of systemic lupus erythematosus and has been implicated in cancer-associated retinopathy (CAR), wherein specific autoantibodies directed against intracellular antigens in photoreceptors may promote cellular injury and death in rod and/or cone photoreceptors [95]. This is of direct relevance to AMD since emerging data demonstrates the presence of retinal auto-antibodies in some patients with AMD, suggesting the possibility that this mechanism may also contribute to a subset of AMD disease.

6.2.3 Mechanisms for the Activation of Immune Responses in Degenerative Diseases

6.2.3.1 Activation of Innate Immunity

There are multiple potential mechanisms for activation of innate immunity; we will review several of them here.

(a) *Cellular injury as a trigger of innate immunity:* As noted, the innate immune system is a broad and conserved response to specific stimuli, including injury to parenchymal (nonimmune) cells [14, 81, 82, 96–98]. Broadly, cellular injury can be defined as any exposure or triggering stimulus that perturbs the cell's normal function and elicits a cellular response, evident as activation of signaling pathways, alterations in biochemical mediators, or change in specific cellular functions. Such stimuli may include immune effector mechanisms, cytokines, oxidants, chemical mediators, alterations in ambient pH, hypoxia, increased temperature (heat), light, or mechanical injury [98]. Cellular responses to injury include activation of signaling pathways, morphological changes, cellular migration, proliferation, cytokine production, alteration in gene expression, or alterations in cellular metabolism. These cellular responses to injury can promote the recruitment and activation of immune cells or the activation of immune amplification systems, such as the complement system, via production of inflammatory mediators and/or upregulation of cellular adhesion molecules. A highly relevant cellular response to injury that can promote activation of innate immune mechanisms is extracellular deposit accumulation [98, 99], which can serve as a nidus for macrophage infiltration and phagocytosis, especially in the setting of oxidant injury and modification of deposits by oxidation or other forms of chemical alteration.

(b) *Infection as a trigger of innate immunity:* Microbial invasion is a well-established trigger of innate immunity in active infection, usually by the release of microbial-derived molecules (i.e., endotoxins, exotoxins, cell wall components) that can directly activate receptors expressed on the cell surface of monocytes, macrophages, neutrophils or, in some cases, on parenchymal cells. Active infection is characterized by microbes that invade the tissue microenvironment, replicate, and cause tissue injury via direct effects or indirectly, via activation of the immune response [100].

There has been considerable interest over the past several decades in the concept that certain chronic degenerative diseases may be caused by direct microbial infection at a given locus of disease [97–100]. One such example is peptic ulcer disease, wherein the gram-positive bacterium called *Helicobacter pylori* directly infects the gastric subepithelial mucosa and results in chronic tissue injury [101]. In vascular biology, direct and chronic infection of vascular endothelial cells by bacteria or viruses has been suggested as a potential etiology for certain types of atherosclerotic disease. There is limited data to suggest a similar phenomenon in AMD, including with latent CMV infection and *C. pneumoniae* infection [102–104]. Another possibility is that infection can indirectly promote cellular response to injury. Chronic exposure to microbial components can prime, or partially activate, monocytes in spleen, lymph node, bone marrow, or other sites of exposure or surveillance, altering the expression of certain cytokines and mediators and committing exposed cells to specific effector functions [105–107]. These functions then become fully manifest upon recruitment to the disease locus (i.e., RPE and retina) and transformation into fully activated macrophages. These concepts as they relate to AMD will be discussed in greater detail in Section II, Part D [102–104, 108].

6.2.3.2 Mechanisms for the Activation of Normal and Aberrant Adaptive Immunity

(a) *Activation of adaptive immunity*: In the "immune response arc," interaction between antigen and the adaptive immune system is comprised of three distinct phases: afferent arm (peripheral tissue site of antigen recognition), processing (within the immune system, e.g., lymph node) and effector arm (cellular and humoral response to the antigen at the original site, completing the arc) [14, 81, 82]. The *afferent* arm transmits information on the antigen to the lymph node for processing by two distinct mechanisms. In the first, the APC captures, digests, and presents antigenic fragments, and then carries the fragments to the lymph node, where interaction with T cells occurs [83, 84, 109]. Alternatively, in the second mechanisms, the intact antigen is transmitted directly to the lymph via lymphatic circulation, where it can interact with specific B cells that recognize components of the antigen [14, 81, 82].

Processing of the antigen occurs in the lymph node, where antigenic component, APC, T cell, and B cells interact in a specific fashion to activate the adaptive immune response. The spleen serves as an alternative site of processing for tissues such as the retina and choroid that are not served by draining lymph nodes. Once processing occurs, antigen-specific B cells can be activated to produce antibodies and antigen-specific T cells can be activated to produce cellular mediators. These activated cells and their products are then released into efferent lymphatics and the blood circulation, where they eventually return to the original tissue site and mediated a specific *effector* response (e.g., immune complex formation or delayed hypersensitivity reaction). Compared to most tissue sites, the immune response arc of the retina and choroid is not well characterized is influenced by specific aspects of the retina and choroid, including immune privilege, blood-retinal barrier, unique anatomical features, and these are discussed elsewhere [110, 111].

(b) *Aberrant activation of adaptive immunity:* Aberrant activation of adaptive immunity may play a role in the pathogenesis of chronic degenerative diseases such as AMD. Among potential autoimmune mechanisms, two in particular may have relevance to AMD: molecular mimicry and desequestration. Additionally, potential third and fourth mechanisms include the generation of "neo-antigens" against which immune responses are directed and true foreign antigens that become trapped within normal tissues.

In *molecular mimicry*, certain regions (epitopes) of an unrelated foreign antigen share similar structure to self-antigens [112]. In this setting, there may be the generation of an appropriate afferent, processing, and efferent immune reaction against a true foreign antigen of a microbe, but antibodies directed against the foreign antigen or antigen-specific T cells may inappropriately cross-react with a self-antigen that shares similarity with this foreign antigen, generating an unwanted autoimmune response. This response would include directed tissue injury where this self-antigen is recognized, causing additional lymphocytes responses that may be directed against other self-antigens and recruiting additional effector immune cells. Molecular mimicry has been identified as a potential mechanism for anti-retinal autoimmunity [113].

In most cases, inappropriate activation of adaptive immune system is prevented by a process of active tolerizing to self-antigens (which occurs by several mechanisms). However, in other cases, antigens must be sequestered within specific cellular or tissue compartments away from exposure to components of the adaptive immune system. *Desequestration* occurs when such molecules are released from such

compartments, where they are mistaken by the immune system to be foreign antigens [114–116]. For example, certain nuclear proteins and ribosomal enzymes are sequestered; in the event of cellular injury, such proteins or enzymes can be extruded into extracellular space and upon exposure to an immune cell, may be recognized as an autoantigen [115]. Of relevance to AMD, nonlethal RPE cell injury is associated with active blebbing or extrusion of cytosolic or membrane components the extracellular space. Upon their release into the extracellular environment, previously intracellular peptides can become desequestered, becoming antigens that can auto-activate the adaptive immune system [110, 116].

Formation of "neo-antigens" represents another mechanism for aberrant activation of adaptive immunity. In this mechanism, there is chemical modification of normal self-proteins that are trapped or deposited within tissues [117]. For example, oxidative modification of peptide components of large proteins (e.g., apolipoproteins) trapped within Bruch's membrane can produce neo-antigens, and citrullination of cellular peptides in the setting of cellular injury or exposure to oxidants is a biochemical modification that is increasingly recognized as a cause of neo-antigen formation [118]. In both cases, resultant neo-antigens can trigger specific T cell responses and antibodies that are reactive to the modified protein.

Antigen trapping represents the fourth and final mechanism for aberrant adaptive immunity activation [119]. In this mechanism, true foreign components of a particular size or charge become inappropriately trapped within the substratum of this tissue, leading to inappropriate activation of the adaptive immune system at the site of trapped antigen. In AMD, this may occur when foreign antigen passes through fenestrated capillaries of the choriocapillaris, becoming lodged within the connective tissue of the choroid or within extracellular matrix of Bruch's membrane. While this mechanism has been put forth as a potential trigger of ocular inflammatory disease and certain conditions such as ocular histoplasmosis [120, 121] and this could be a plausible trigger for AMD, there is minimal supportive evidence for this in AMD pathobiology.

6.2.4 Amplification Systems for Immune Responses in Chronic Degenerative Diseases

Although injury or inflammation can be directly induced by either innate or adaptive immunity, in most cases, a process of amplification is required for overt clinical signs of disease to manifest. Several potential amplifications systems contribute, including: [1] plasma-derived enzyme systems, which include complement, kinins, and fibrin; [2] lipid mediators (prostaglandins, leukotrienes, other eicosanoids and platelet activating factors); [3] vasoactive amines (histamine and serotonin); and [4] cellular inflammatory signaling programs that serve to integrate both immune and nonimmune responses, which includes the NLRP3 inflammasome. This chapter will focus on the complement system and the NLRP3 inflammasome, since both have been identified as potential modulators of the immune response in AMD.

6.2.4.1 Complement System

The complement system serves to amplify the effects of both innate and adaptive immune system and mediate various injury responses [122–124]. Components of the complement system, which include over 30 different protein molecules, are synthesized in the liver and circulate in the blood as inactive precursors and become activated at sites of injury, in response to infection or inflammation. However, some specific complement components can also be produced locally within certain tissues; within the eye, this includes the cornea, sclera and retina [123]. For example, certain complement-related proteins, including C3, factors B, H, H-like

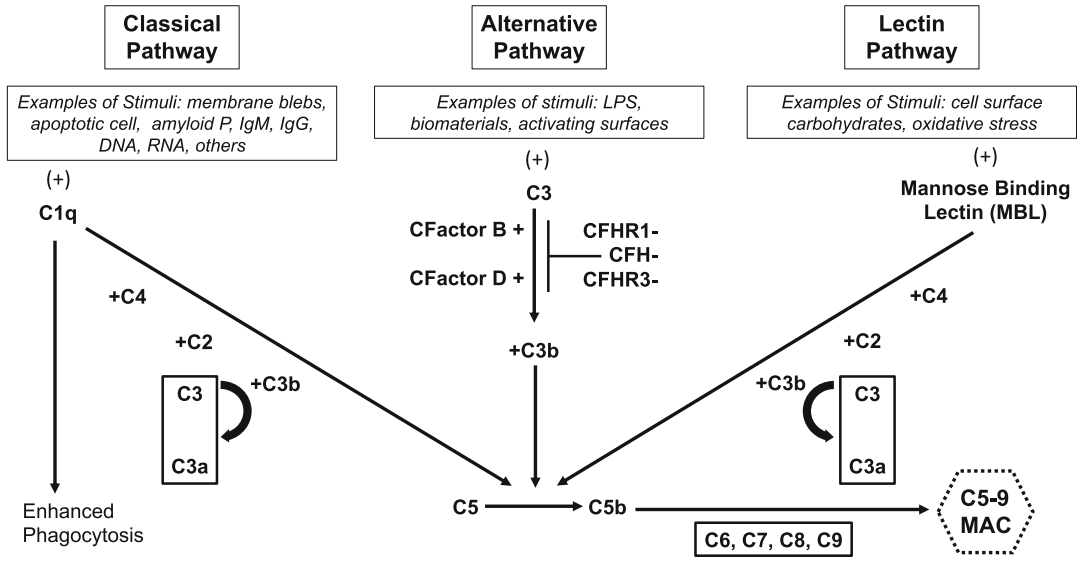

Fig. 6.2 Schematic of the components and fragments of the complement cascade indicating three primary sources of activation via the classical, alternative or lectin pathway

1, CD46, CD55, CD59, and clusterin, can by produced by RPE cells [125].

Components of the complement system serve a collective amplifier function by interacting in a sequential cascade to produce a number of different cellular (and noncellular) phenomena. This may occur by one of three specific pathways that activate the complement system: classical pathway, alternative pathway and the lectin pathway (Fig. 6.2).

The classical pathway can be activated by adaptive immune mechanisms, including by certain antigen/antibody (immune) complexes, referred to as pentraxins, especially those formed by IgM, IgG1 and IgG3 [122–124]. However, activation of the classical pathway, and ultimately C3 and C5 activation, can also be triggered by certain innate immune stimuli, including DNA, RNA, insoluble deposits of abnormal proteins (e.g., amyloid P) or apoptotic cells [126–129].

Activation of the lectin pathway, which includes activation of C2 and C4, occurs via mannose-binding lectin (MBL), or ficolin, that identifies DAMP molecule regions on apoptotic cells or microbial-derived PAMPs, that are typically certain types of sugars [130]. MBL typically does not recognize self-components; however, oxidant modification or glycosylation modification of molecular structures, as can occur in degenerative diseases such as AMD, may alter surface protein expression or the presence of sugar moieties that are not typically present but that are recognized by MBL, leading to MBL deposition and complement activation via C3 [131–134].

Finally, the alternative pathway can be activated by innate immunity, primarily by activating C3. This may occur through innate immune recognition of specific moieties on microbial-associated molecules (e.g., LPS), activated surfaces (e.g., implanted medical devices) [126], or oxidant-modified cellular byproducts (e.g. the photo-oxidative products of A2E, bis-retinoid pigment that may accumulate in the RPE in AMD) [135].

Ultimately, activation of any one of the complement pathways leads to the same common points of activation and generation of the same activated complement byproducts. Activated complement byproducts may amplify injury or inflammation by at least three distinct mechanisms: [1] C3b, which is a specific fragment of C3, can opsonize, or coat, antigenic or pathogenic surfaces, to enhance phagocytosis by

macrophages or neutrophils [2]; the terminal complement components C5–9, called the membrane attack complex (MAC), can be activated to form transmembrane channels that disrupt cell membrane of target cells, leading to cellular injury, loss of cytoplasm (lysis), or death [3]; production of anaphylatoxins (C3a, C4a and C5a), small pro-inflammatory polypeptides, that can induce many inflammatory mediators and recruit inflammatory cells. Complement activation is regulated by specific inhibitors, such as decay accelerating factor (CD55), CD59, CD46, complement factor H (CFH), complement receptor 1 (CR1), and others that serve to block, resist, or modulate the induction of various activation pathways especially in degrading the C5b-9 MAC. As will be discussed below, the role(s) of CFH and potentially other inhibitors (e.g., CR1) may be relevant to pathobiology of AMD [122–124].

As noted, the RPE is capable of producing various complement components locally in situ. Thus, C3 and other complement components can be cleaved to produce activated fragments by various enzyme systems, in the absence of the entire complement cascade, which in turn can activate certain specific cellular functions [136, 137]. Additionally, inhibitors of complement activation, which can also be produced locally by cells such as the RPE, serve to regulate the function of complement, protecting against tissue injury mediated by inappropriate complement activation [138].

6.2.4.2 NLRP3 Inflammasome

The NLRP3 inflammasome is a multimeric complex of cellular cytosolic proteins that assembles in response to specific exogenous and endogenous danger signals, including damaged cell-intrinsic components (e.g., DAMPs such as dsRNA, cytoplasmic DNA), microbial-associated molecules (e.g., PAMPs such as LPS), oxidants, and other moieties, integrating cellular responses to such injury stimuli by specific upregulation of the cellular inflammatory response [139]. The NLRP3 inflammasome has traditionally been described as present in innate immune cells such as macrophages and microglia and may also function in part to integrate aspects of the innate and adaptive immune responses [140]. More recent work has described the role of the NLRP3 inflammasome in nonimmune cells, where its assembly is triggered in response to cellular perturbations such as mitochondrial dysfunction, aberrant ion (e.g., calcium) flux, lysosomal dysfunction, and proteasome insufficiency [141]. Indeed, as will be detailed below, the NLRP3 inflammasome may play a vital role in RPE cellular function and response to injury in the setting of AMD.

6.3 Specific Immune Contributions to AMD Pathobiology

Unlike with acute inflammatory diseases, primary autoimmune diseases, or uveitis, immune mechanisms in the setting of AMD are a secondary response to a primary disease process at the RPE and neurosensory retina, which serves to amplify or alter the disease phenotype. Considerable data from human pathology of AMD and preclinical animal models have clarified specific roles for the immune system in both dry and neovascular AMD, which this section will elaborate in greater detail.

6.3.1 Innate Immunity

6.3.1.1 Macrophages and Dry AMD

Early histopathologic studies using immunofluorescence and electron microscopy noted the presence of macrophages in close proximity to Bruch's membrane, drusen, and the basal aspect of RPE in eyes with dry AMD [142, 143]. However, macrophages were also observed in the choroid of eyes from age-matched controls without AMD, raising questions about whether the presence of these cells was specific to disease or instead representative of age-related alterations in the local immune microenvironment. Sarks and colleagues assessed the presence of macrophages in choroid and outer retina of a broad range of ocular specimens from normal aging, early AMD, NVAMD, and late AMD

with GA and found that thinning of Bruch's membrane, areas of deposition of membranous debris from the RPE, and drusen deposits were all associated with macrophages, which were frequently observed engulfing fragments of outer collagenous zone of Bruch's membrane or membranous debris [142]; they hypothesized that the accumulation of phospholipid membranous debris and focal concentration of lipid in drusen served as attractants to recruit macrophages. Subsequent work found that the presence of extracellular deposits, specifically soft drusen as well as thick continuous basal laminar deposits was associated with increased frequency of macrophages recruited to the inner choroid and Bruch's membrane, and that choroidal macrophages from eyes with the various stages of AMD were characterized by expression of iNOS, while choroidal macrophages from normal eyes did not express iNOS [144]. This observation highlights a potential key difference between choroidal macrophages recruited in the context of dry AMD versus typical resident choroidal macrophages [144]. Interestingly, in this study, macrophages present in Bruch's membrane in AMD eyes did not express iNOS, suggesting either immunomodulatory differences between choroid and Bruch's membrane, or distinct subsets of macrophages present at each site. Subsequent studies have affirmed that early AMD with drusen and basal laminar deposits is associated with a significant increase in IBA1+ macrophages and HLA-DR+ subset of activated macrophage as compared to age-matched control eyes [145]. In addition, AMD eyes with thick basal deposits, CNV, and GA were all associated with a marked increase in the frequency and size of CD163+ cells in the outer retina, subretinal space, and subRPE space in the macula, while such cellular infiltration was not observed in healthy aged controls without AMD [146] (Fig. 6.3). In particular, the findings of the latter study suggest that the frequency of macrophages in AMD may have been underestimated due to use of markers that do not readily detect macrophage cells in tissues, and that macrophages may play a key role in all aspects of AMD disease. Multiple studies have now demonstrated that macrophages, labeled by a variety of markers including RCA-1, CX3CR1, CD18, IBA1, CCR2, CD163, and CD14, are found in association with large drusen, within atrophic areas, or RPE cells adjacent to atrophy [147].

The precise role(s) that macrophages play in the setting of AMD likely depend on the predominant effector function of the infiltrating cells, and whether those cells manifest *scavenging, inflammatory, or reparative* effector biology. Presently, our ability to differentiate specific effector functions is limited because cellular markers do not necessarily distinguish functionally distinct subsets and because presently available techniques to label effector cytokine expression in situ are inconsistent, particularly for postmortem specimens. At the same time, our general lack of understanding of the specific mechanisms that mediate progression to advanced stages of AMD disease, especially GA, limits our ability to interrogate effector biology of macrophages in appropriate context. However, there is considerable inferential evidence from human histopathology and supporting data from preclinical mouse models that suggest potential hypotheses for how macrophages contribute to dry AMD.

(a) *Scavenging and homeostatic function:* As noted above, macrophage phagocytosis of lipid-rich membranous debris along with macrophages associated with accumulated drusen are readily apparent by histopathologic studies of AMD, suggesting that macrophages may serve a housekeeping role in clearing debris from Bruch's membrane and the subRPE space and in dynamic turnover of drusen [142, 143]. Mice with genetic deletion of P2X7 receptor, which has function as scavenger receptor on macrophages, develop progressive thickening of Bruch's membrane, RPE cell loss, retinal functional deficits, and accumulation of inflammatory macrophages in the subretinal space [148]. The development of pathologic features that mimic dry AMD suggest that scavenging function of choroidal macrophages may be essential to provide a homeostatic function. Indeed, patients with

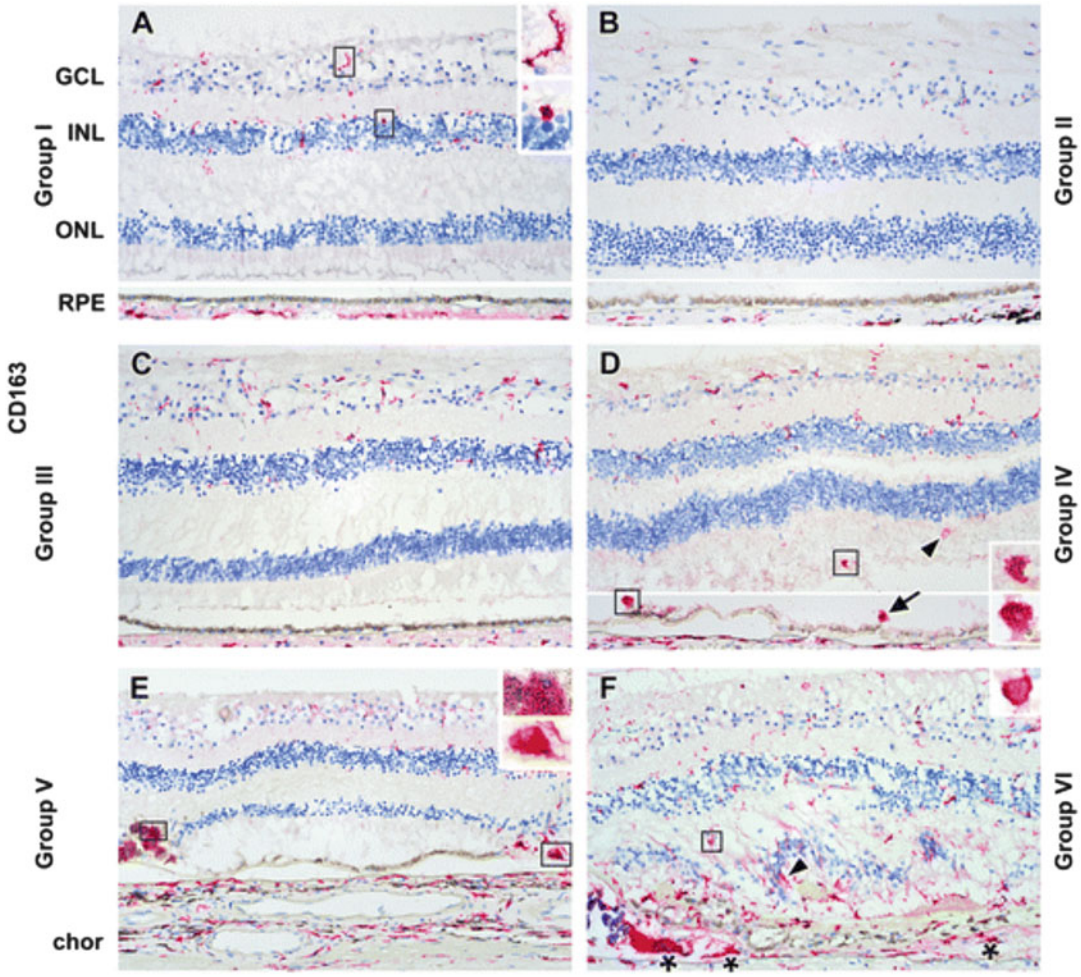

Fig. 6.3 Immunohistochemical localization of CD163+ macrophages in normal, age-matched control eyes and eyes with AMD of various severity grades. CD163+ cells were detected using a red alkaline phosphatase polymer system. The tissue was counterstained with hematoxylin and the nuclei are *blue*. CD163+ cells are present solely in the inner retina in normal, age-matched eyes (**a**) and AMD eyes groups II and III (i.e., early AMD) (**b**, **c**), but were present in the outer retina and subretinal space in eyes with thick subretinal deposits in AMD group IV (**d**), geographic atrophy in group V (**e**), and neovascular AMD with fibrosis in group VI (**f**). *Insets* in **a**: In normal eyes, CD163+ cells in the nerve fiber layer and ganglion cell layer had a dendritic, microglioid phenotype with a small soma and long processes. In the inner plexiform layer and inner nuclear layer, some of the CD163+ cells had a dendritic morphology and others were characterized by a more rounded, epithelioid conformation. *Insets* in **d**, **f**: In eyes from groups IV-VI, CD163+ cells in the outer retina and subretinal space had a rounded morphology with large cell bodies and small processes. *Insets* in **e**: In eyes with geographic atrophy (group V), the CD163+ cells had a larger soma and shorter processes, consistent with an activated macrophage morphology. The edges of geographic atrophy expressed the marker CD163 in cells filled with melanin granules, which most likely represent macrophages that have ingested melanin pigment. Scale bar = 100 μm; *insets* in **a**–**f** are enlarged fourfold. Arrow heads CD163+ macrophages in the outer retina; *arrows*: subretinal CD163+ cells; *: sub-RPE CD163+ cells. The *white line* in panels (**a**, **b**, and **d**) is the break between two separate photomicrographs taken due to postmortem sensory retinal detachment separating photoreceptors from RPE. Reprinted by permission from: Springer Nature, Graefe's Archive for Clinical and Experimental Ophthalmology. Lad EM, et al., Abundance of infiltrating CD163 + cells in the retina of postmortem eyes with dry and neovascular age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 2015; 253:1941-1945. Copyright Springer Nature 2015

nonfunctional haplotypes of the P2RX7 and P2RX4 genes, which give rise to the P2X7 and P2X4 scavenger receptors, respectively, have circulating monocytes with substantial reduction in phagocytic capacity, and this haplotype was over four times more frequent in AMD patients as compared to controls [149]. Thus, monocytes/macrophages with scavenging function may serve a protective role, and loss of this scavenging function or dysfunction of molecular pathways that mediate scavenging and phagocytosis capabilities of macrophages could thus exacerbate the dry AMD phenotype.

(b) *Inflammatory injury at the RPE and retina:* Other evidence suggests that macrophages exhibiting inflammatory effector function may cause nonlethal and lethal injury to RPE cells and photoreceptors and may directly promote AMD disease progression [150, 151]. As noted above, macrophages having an activated morphology or labeling with markers of activation (e.g., HLA-DR) are found within areas of GA and adjacent to areas of GA [145, 147]. Moreover, the frequency and extent of subretinal macrophage infiltration appears to correlate with stage of disease in dry AMD, with abnormally increased frequency of subretinal macrophages in eyes with large drusen and still greater numbers observed in eyes with GA [146, 147, 152]. Another clinical observation linking macrophages to AMD progression has to do with the subretinal and intraretinal hyperreflective foci which are seen by OCT in AMD patients and which have been associated with progression of GA and photoreceptor loss [153, 154]. RPE which are injured in the context of AMD are known to extrude their melanosomes and histology demonstrates that these melanosomes are frequently phagocytosed by macrophages [146, 155]. Interestingly, in vivo flood-illumination adaptive optics imaging (FIAO) in patients with AMD demonstrate that melanin-containing cells corresponding to subretinal and intraretinal hyperreflective foci on OCT and that these cells demonstrate morphologic features of macrophages [156]. Taken together, these observations suggest an association between disease severity and inflammatory macrophage infiltration in eyes with AMD.

Meanwhile, a growing body of evidence from retina mouse models, including for example, the APOE4 transgenic and the CX3CR1-deficient mouse models of subRPE deposit formation and the photo-oxidative stress model, demonstrate that there is increased and persistent infiltration of macrophages in the subretinal space and that these cells mediate RPE cellular injury and photoreceptor death via direct cellular interaction and paracrine release of inflammatory cytokines [147, 152, 155, 157, 158]. Macrophage infiltration occurs as a result of response to injury and as a result of dysregulated immunosuppressive mechanisms that are meant to limit macrophage access to the subretinal space. Following moderate light challenge in CX3CR1-deficient mice or severe light exposure in the photo-oxidative stress model [159], there is rapid infiltration of blood-derived macrophages into the subretinal space, which account for 50% of the macrophages present in the subretinal space [147, 155, 159]. Inhibition of CCR2 substantially reduced this infiltration and reduced the severity of subsequent photoreceptor degeneration in the CX3CR1-deficient mouse model [155]. Under physiologic conditions, the RPE actively promotes an immunosuppressive environment in the subretinal space to limit injury due to inflammation. This includes the expression of Fas ligand (FasL) by the RPE, which interacts with the Fas receptor on macrophages to induce cell death and eliminate them from the subretinal space [152]. The RPE also produces the complex signaling moiety Thrombospondin-1 (TSP-1), which interacts with CD47 on macrophages to reduce phagocytic capacity of macrophages and

sensitize these cells to FasL-mediated cell death and elimination [160–164]. These systems interact to maintain an immunosuppressive environment and prevent macrophage associated cellular injury at the RPE and the outer retina. Loss of FasL expression or TSP-1 production, or aberrations in their respective signaling mechanisms, allows macrophages to persist in the subretinal space and create a pathologic, persistent inflammatory microenvironment. In an analogous fashion, the CX3CL1 ligand is produced by a variety of neuronal cell types and interacts with CX3CR1 expressed by microglia cells to help maintain retinal homeostasis [152, 155, 158]; perturbations in this system may allow or enable infiltration of the neurosensory retina by blood-derived macrophages.

Infiltrating macrophages likely contribute to AMD pathobiology by secretion of pro-inflammatory effector cytokines, which include IL-1β, TNF-α, and IL-6. IL-1β is initially produced as a proform within macrophages following stimulation of TLRs by PAMPs or DAMPs [165, 166]. Macrophage activation by a second stimulus triggers the assembly of the NLRP3 inflammasome to activate caspase-1 to cleave pro-IL-1β to a mature form that is secreted as an active cytokine [167]. IL-1β is toxic to neuronal cells and its secretion by subretinal macrophages has been shown to induce death of photoreceptors, especially cones [168]. Such a mechanism could contribute to loss of photoreceptors that occurs adjacent to GA in spite intact subjacent RPE. Additionally, IL-1β has been shown to contribute to CNV induction and may serve as a pro-inflammatory mediator that mediates NVAMD onset.

Macrophage-derived TNF-α has been implicated in promoting dysfunction of RPE homeostasis, as it has been shown to repress OTX2, a key transcription factor of the RPE [169]. In the adult RPE, OTX2 regulates RPE expression of a number of genes that are essential for the visual cycle, including transthyretin (TTR), a retinol carrier, and retinol dehydrogenase 5 (RDH5) that re-isomerizes all-trans-retinal into 11-cis-retinal [170]. Thus, reduction in OTX2 within RPE by macrophage TNF-α may impair the visual cycle and cause visual dysfunction by limiting the efficiency of photoreceptor function even at the intermediate stage of AMD, since subretinal macrophage infiltration is readily observed in patients with large drusen [146, 152, 155, 171, 172]. Over time, chronic OTX2 repression may induce or contribute to photoreceptor loss; indeed, ablation of OTX2 in adult mice leads to progressive photoreceptor degeneration, suggesting that macrophage TNF-α could promote dry AMD disease progression in this fashion [170]. TNF-α producing macrophages may also mediate visual dysfunction by infiltration into the neurosensory retina where TNF-α may disrupt and injure photoreceptor bipolar synapses and promote Muller cell activation leading to disruption of Muller cell synaptic support and the cone-Muller cell accessory visual cycle [146, 173, 174]. Additionally, patients with the highest levels of TNF-α expression in circulating monocytes have a greater risk of developing NVAMD [175]. TNF-α may directly contribute to new vessel formation or it may also indirectly contribute to CNV induction by activating RPE cells to upregulate production of VEGF, which consequently drives angiogenesis [176, 177] (see Macrophages in NVAMD section).

IL-6 is another cytokine produced by macrophages that may contribute to AMD, as it antagonizes TGF-β and therefore may disrupt the immunosuppressive microenvironment of the retina and subretinal space [178]. IL-6 has been shown to reduce RPE FasL expression, which interferes with the ability of the RPE to eliminate macrophages from the subretinal space [152], and elevated expression of IL-6 in retina models

correlates with persistent of subretinal macrophage infiltration [152]. Moreover, systemic levels of IL-6 correlate with the rate of progression of GA in patients with AMD [179]. Importantly, IL-6 has also been shown to correlate with the presence of macular edema in the setting of NVAMD [180]. Extrapolating to dry AMD biology, since macular edema may reflect more generally Muller cell injury, it is plausible that IL-6 produced by infiltrating macrophages could mediate Muller cell injury in dry AMD [146].

These cytokines are but a subset of macrophage-derived factors that could affect visual function and disease progression in dry AMD; clearly, other cytokines will contribute, and macrophages may also mediate effects through coordinated biology with other immune and nonimmune mechanisms. For instance, subretinal macrophage-produced C1q may mediate photoreceptor degeneration via NLRP3 inflammasome activation and IL-1β production [181].

(c) *Reparative function:* Considerably less evidence is available to understand how macrophages may manifest reparative functions in the setting of dry AMD. The macrophage marker CD163 is frequently considered to be a marker of reparative macrophages since it serves as a scavenger receptor for hemoglobin-haptoglobin complex, it is upregulated by IL-10 [182], and because CD163+ macrophages are frequently found in association with fibrosis and neovascularization (see NVAMD section—neovascular remodeling). As noted above, CD163+ macrophages are prevalent in in postmortem eyes with dry AMD, found in the subretinal space, within areas of GA, at the margins of GA, and within the retina [146] (Fig. 6.3). It is thus possible that reparative macrophages contribute to AMD disease. TGF-β produced by reparative macrophages may serve an immunoregulatory function, downregulating inflammatory activity, limiting tissue injury and cellular loss, and promoting clearance of cellular debris and limited tissue repair. On the other hand, CD163 may not be specific for reparative function and CD163+ macrophages may more broadly label multiple subgroups of macrophages with different effector function. Indeed, some subgroups of CD163+ macrophages have been found to have inflammatory effector function [183], suggesting that the CD163+ macrophages observed in dry AMD may have heterogeneous functions. Additional investigation is needed to identify specific markers of reparative function and understand how perturbations of this macrophage subset might influence dry AMD disease phenotype.

6.3.1.2 Macrophages and NVAMD

Neovascular AMD (NVAMD) is characterized by the onset, formation, and growth of aberrant choroidal neovascularization (CNV) subjacent to the retina. In spite of the efficacy of intravitreal anti-VEGF medications, it still represents the leading cause of vision loss in the elderly [1]. Loss of vision occurs as a result of plasma exudation, hemorrhage, fibrosis, atrophy of overlying RPE, injury and atrophy of overlying photoreceptors, and synaptic dysfunction in the overlying neurosensory retina. There is now considerable evidence from analyses of NVAMD histopathology, preclinical animal models of CNV, and studies of patients with NVAMD that macrophages contribute to various aspects of NVAMD pathobiology, including the onset of NVAMD disease (i.e. triggering onset and development of CNV), CNV formation and growth, extent of disease activity, and synaptic dysfunction within the overlying neurosensory retina. We will explore potential contributions to each aspect of NVAMD disease, with a specific emphasis on understanding the relative contributions of inflammatory (as defined by high expression of inflammatory mediators) and reparative (as defined by high expression of pro-fibrogenic cytokines) macrophage subsets to various stages of disease.

(a) *Onset of NVAMD:* The specific mechanisms that trigger onset of NVAMD, the initial development of incipient CNV, remain unknown. As such, there are no consensus strategies to identify patients who are highest risk for NVAMD conversion, and there are no effective therapies to prevent progression to NVAMD. Numerous histopathologic studies, including Sarks, et al., have demonstrated the presence of macrophages in association with the leading edge of CNV vascular structures, adjacent to or within a thinned and irregular Bruch's membrane, suggesting that macrophages might promote the initial development of neovessels via release of angiogenic factors and/or inflammatory cytokines that mediate initial response to locus of injured or diseased tissue [184] (Fig. 6.4). Inflammatory macrophages have also been observed in close association with endothelial progenitor cells (EPCs) in surgically excised CNV specimens [185], suggesting that macrophages may directly mediate initial steps of neovessel assembly via recruitment and activation of these EPCs at the inner choroid and Bruch's membrane. Using the experimental model of murine laser-induced CNV, several investigators have demonstrated a key role for CCR2+ inflammatory monocytes (which express TNF-α, IL-1β, NOS2, VEGF, and proteases such as MMP-9) in the early development of CNV, as genetic deletion of CCR2 and reduction of this inflammatory monocyte subset substantially reduces CNV induction [186–189]. Intravitreal corticosteroid treatments inhibit incipient CNV development with decreased inflammatory monocyte/macrophage infiltration in the laser induction model [190–192]. In patients, pro-inflammatory monocyte activation state, as reflected by increased expression of TNF-α in peripheral monocytes, is associated with increased risk of NVAMD as compared to patients with dry AMD [175], and other studies have shown that monocytes from NVAMD patients have altered transcriptomes as compared to controls [193]. In addition, inflammatory cytokine receptors CCR1 and CCR2 are co-upregulated on intermediate CD16+ monocytes from NVAMD patients [194] and CD200 upregulated on circulating CD11b + monocytes in NVAMD patients as compared to controls [195]. To further add to this perspective, the PRO-CON clinical trial found that intravitreal anti-VEGF treatment with aflibercept was not efficacious as prophylactic treatment against progression to NVAMD, as compared to sham injection, in high-risk fellow eyes with dry AMD [196]. Taken together, these data collectively support the concept and hypothesis that the specific biologic triggers of NVAMD progression and incipient CNV development extend beyond VEGF, and that pro-inflammatory macrophages may directly trigger NVAMD onset, promoting incipient CNV induction and formation via release of inflammatory meditator such as TNF-α, IL-1β, and MMP-9. These data also highlight the potential of targeted anti-inflammatory drugs as potential therapies to prevent progression to NVAMD.

(b) *CNV Formation and Growth— Angiogenesis:* Following initial onset of disease, the formation and growth of CNV occurs via coordinated assembly of various cell types to form new vessel structures. The prevailing paradigm for new vessel growth, both broadly within vascular biology and specifically for CNV, is *angiogenesis,* wherein endothelial cells resident in the choroid arise from existing choroidal vasculature and proliferate, invade Bruch's membrane, and assemble into a nascent network of capillary tubes, a process that is regulated primarily by VEGF as well as other angiogenic factors [197, 198] (Fig. 6.5) [199]. *Maturation* occurs when the new capillary CNV acquires a pericyte sheath, a process that is mediated largely by PDGF, stabilizing the CNV and allowing it

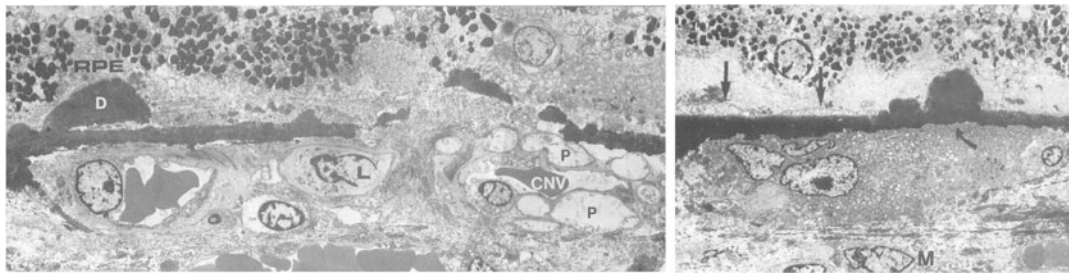

Fig. 6.4 (left) Electron micrograph (×2210) showing activated choroidal neovascularization (CNV) extending into a disrupted Bruch's membrane. Small amorphous hard drusen (D) lie on Bruch's membrane but there is minimal diffuse debris under the retinal pigment epithelium (RPE). (right) Electron micrograph section (×1410) close to CNV, just beyond the vessel leading edge, showing macrophage closely in contact with the outer surface of Bruch's membrane, beneath two hard drusen. The outer collagenous zone appears thinned here (curved arrow). A thin layer of membranous debris lies on the internal surface of Bruch's membrane, external to the basement membrane of the RPE (straight arrows). Adapted with permission from Sarks JP, Sarks SH, and Killingsworth MC. Eye 1997; 11:515-522. Copyright Nature Research 1997

to persist even in the absence of angiogenic factors. The biology of angiogenesis and maturation is fundamental to NVAMD, as the anatomical subtype of capillary CNV comprises approximately 20–25% of all NVAMD cases [200–202]. Moreover, angiogenesis and maturation represent the prevailing biology in the vast majority of studies of experimental laser-induced CNV, since these studies are focusing on the formation and growth of capillary CNV in young (i.e., 2–4 month old) mice. Numerous studies have found that following initial CNV induction, macrophages are recruited to the site of incipient neovessel formation and contribute to capillary angiogenesis and maturation via release of effector growth factors [186, 187]. Inflammatory macrophages are recruited to the incipient CNV beginning at day 2 following induction can promote angiogenesis either directly via release of VEGF or indirectly via release of TNF-α and IL-1β, which stimulate the RPE to produce VEGF and macrophage recruitment factors such as MCP-1 and IL-8 [203]. In young mice, reparative macrophages (frequently identified by expression of cell surface molecules CD163 and/or CD206) begin to infiltrate the lesion at day 4 and express high levels of factors such as VEGF, PDGF, FGF-1, Ang-1, Ang-2, and IL-10, as histopathology with immunostaining of CNV in the laser model demonstrate localization of angiogenic factors with reparative macrophages [203, 204]. Furthermore, multiple studies have demonstrated that depletion of circulating monocytes by systemic clodronate administration or by systemic monocyte or local macrophage depletion using CD11b + -DTR system limits capillary angiogenesis, with reduction in capillary CNV lesion size [205, 206]. Correlative human studies have found that circulating monocytes of NVAMD patients express high levels of VEGF relative to controls [207, 208], suggesting that blood-derived macrophages may directly contribute to angiogenesis in NVAMD, while other data suggest that monocytes from NVAMD patients produce higher levels of MCP-1 and IL-8, both macrophage chemotactic factors, indicating that recruited macrophages may also indirectly amplify this disease process via ongoing recruitment of monocytes to the site of CNV formation [208]. Thus, infiltrating macrophages, predominantly of the reparative subset, may contribute to angiogenesis and maturation of capillary CNV lesions in some patients

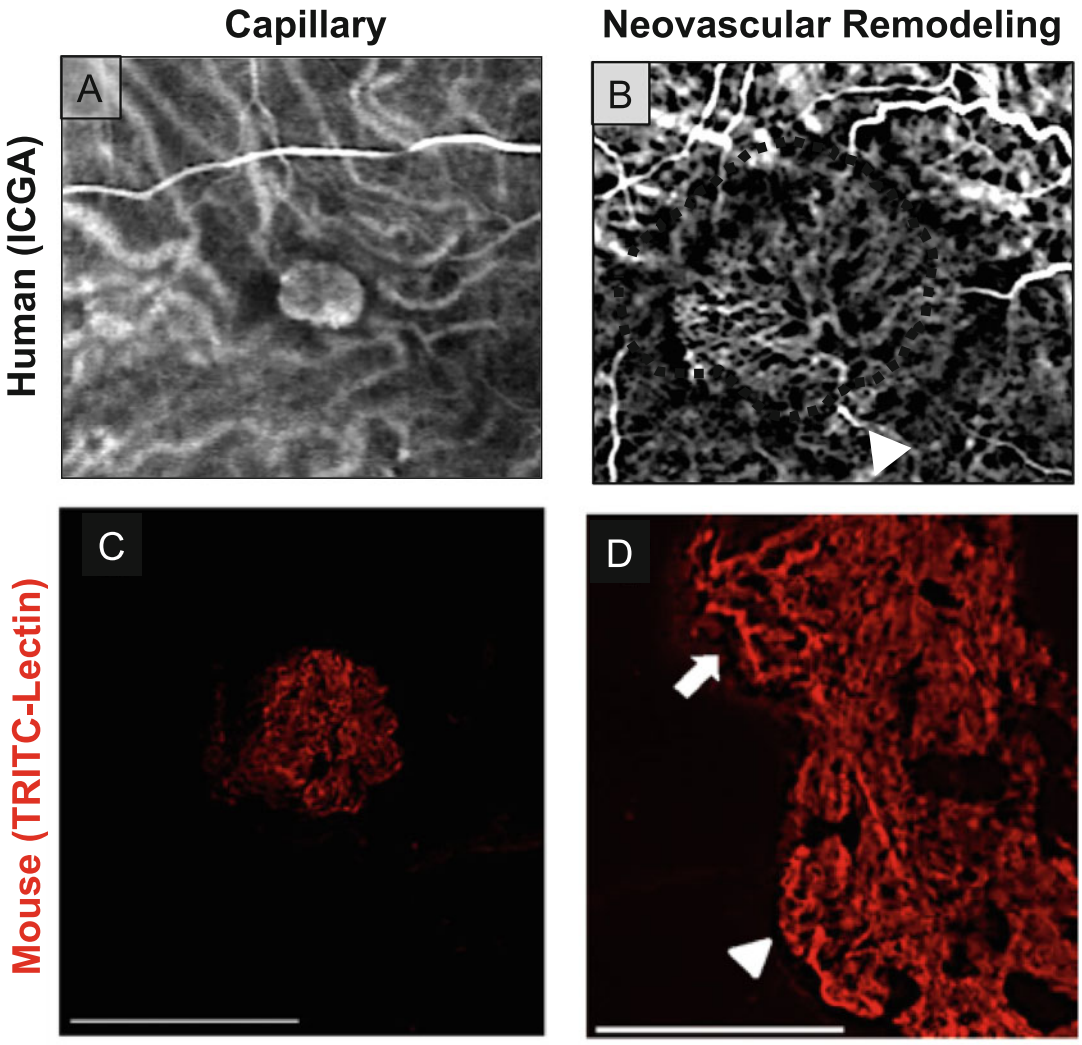

Fig. 6.5 Morphology of choroidal neovascularization (CNV), in human neovascular age-related macular degeneration (AMD) (top, **a**, **b**), and in mouse laser-induced CNV (bottom, **c**, **d**). Representative clinical images of indocyanine green angiography (ICGA) in neovascular AMD demonstrate (**a**) capillary CNV, evident as a small microvascular network; and (**b**) neovascular remodeling (NVR), evident as large caliber feeding artery (arrowhead), many branching arterioles, and minimal capillaries. Representative TRITC-lectin flatmounts of CNV demonstrate analogous morphology features including (**c**) small capillary microvascular network in young (2 month old) mice; and (**d**) branching arterioles (arrow), terminal vascular anastomotic loops (arrowhead) and minimal capillary component (left), all characteristic of NVR. Scale bars = 100 μm. Adapted with permission from Espinosa-Heidmann DG, et al., Invest Ophthalmol Vis Sci. 2013; 54:7439-7449. Copyright Association for Vision and Research in Ophthalmology 2013

via production and secretion of relevant growth factors and cytokines that promote and amplify angiogenesis.

(c) *CNV Formation and Growth—Neovascular Remodeling:* It is increasingly recognized from clinical phenotyping, especially characterization by indocyanine green angiography, that there is a tremendous heterogeneity of CNV morphology among NVAMD patients [200–202, 209, 210]. This

heterogeneity in morphology and anatomical subtypes suggests differential mechanisms of neovascular growth. As noted above, capillary CNV comprise 25% of NVAMD patients. In contrast, patients with arteriolar CNV, characterized by large-caliber feeding artery, many branching arterioles, terminal vascular anastomoses, and minimal capillary component, account for over 50% of NVAMD patients [200–202] (Fig. 6.5) [199]. The arteriolar CNV pattern reflects neovascular remodeling (NVR): the transformation of nascent neovessels into branching arterioles with perivascular fibrosis. As compared to capillary angiogenesis, the pathobiology of NVR is not well understood. Histopathology studies, both from surgically excised CNV and postmortem studies, suggested an increased frequency of macrophages in lesions with arterioles and perivascular fibrosis [145, 211, 212] (Fig. 6.6). Furthermore, CNV with arteriolar morphology are specifically associated with increased infiltration of the CD163+ macrophages [146], which include reparative macrophages, and profibrotic factors produced by macrophages such as TGF-β, FGF, IGF-1, osteopontin, and SPARC localize to macrophages in fibrotic CNV in postmortem studies [203, 213]. Macrophage biology in the setting NVR may share some pathologic overlap with reparative macrophage contributions to atherosclerosis [214] and glomerulosclerosis, where macrophages mediate fibrosis and vascularization of the atheroma [215–218] or mesangial cell proliferation [219–221], respectively. Clinical and pathologic studies demonstrate that macrophages and increased intraocular levels of macrophage-derived cytokines are associated with more active (i.e., exudative or leaking) CNV less frequent in eyes with inactive CNV [145, 180, 222]. Lastly, there is emerging evidence linking biology of reparative macrophages to more severe forms of disease in NVAMD. Patients with NVAMD have increased expression of the transcriptional activator STAT3 in intermediate monocytes, which may contribute to increased arteriolarization and fibrosis in CNV [207]. Additional investigations have observed that the proportion of circulating CD11b+ monocytes directly correlate with the frequency of anti-VEGF injections necessary for disease control [223]. These data collectively suggested that blood-derived macrophages infiltrating the site of CNV may promote NVR (arteriolarization and fibrosis) as well as increased disease activity or exudation, which lead to anti-VEGF resistance in NVAMD.

NVR biology can be modeled in the murine laser-induced CNV model in several different conditions, including mice aged to 16 months of age, mice exposed to cigarette smoke, mice with latent chronic cytomegalovirus (CMV) infection, and mice exposed to low-grade microbial toxins (i.e., PAMPs) [105, 224–226]. In each of these models, CNV lesions exhibit increased size with high-flow, large-caliber arterioles and perivascular fibrosis, along with increased leakage (exudation), as compared to capillary CNV. In the aging model of NVR, depletion of circulating monocytes with clodronate liposomes abrogates the arteriolar phenotype, resulting instead in the formation of capillary CNV, indicating that macrophages drive the development of NVR [227]. Other studies have also demonstrated that increased profibrotic effector function of reparative macrophages in aged mice, and the recruitment and activation of the reparative macrophage subset may be in part mediated by IL-10 signaling, as genetic deletion of IL-10 limits NVR biology, with smaller lesions in association with increased inflammatory macrophage frequency and decreased reparative macrophage infiltration [228, 229]. Furthermore, the NVR-mediating effector function in reparative macrophages may occur via increased STAT3 signaling as well as by

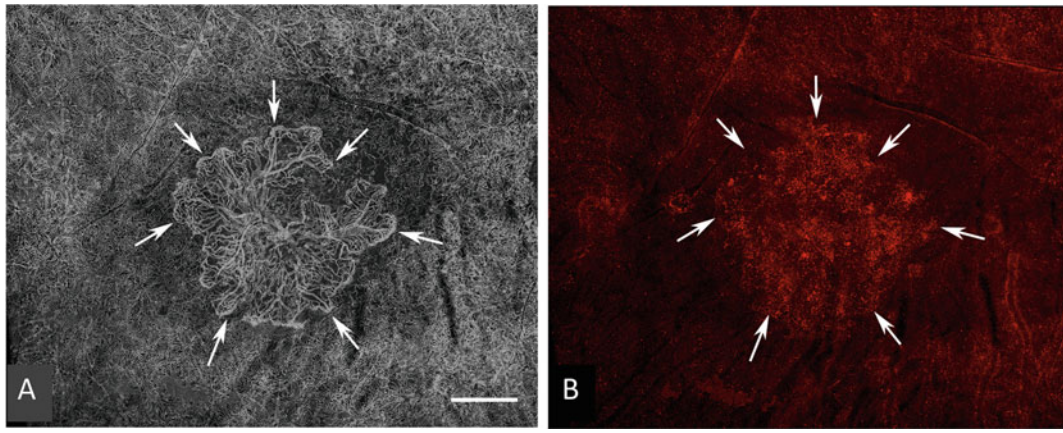

Fig. 6.6 Choroidal flatmount micrograph of a patient with neovascular AMD showing a CNV in the submacular region. (**a**) desaturated Ulex europaeus agglutinin (UEA) lectin stain demonstrates neovascular remodeling (NVR) with central feeder artery, multiple branching arterioles, and terminal vascular loops (arrows). (**b**) Labeling for HLA-DR+ macrophages demonstrates extensive HLA-DR+ macrophage infiltration in association with arteriolarized vessels in NVR CNV lesion. Scale bar = 1 mm. Adapted with permission from McLeod DS, et al., Invest Ophthalmol Vis Sci. 2016; 57:5843-5855. This work is licensed under a Creative Commons Attribution 4.0 International License. Copyright The Authors 2016

alterations in LXR nuclear receptor activity in this macrophage subset, suggesting that these pathways could be targeted to modulate NVR biology [230, 231]. Additionally, CD11b+ reparative macrophages at the site of incipient CNV formation were positive for a variety of factors that mediate fibrovascular growth, including PDGF-β, FGF-1, FGF-2, and TGF-β [204]. Interestingly, local ocular corticosteroids do not prevent perivascular fibrosis and CNV-associated scar formation [232], which is likely due to the fact that corticosteroids have limited effects on the effector function of CD163+ reparative macrophages [233]. The pathobiology of NVR extends beyond macrophages, however, as bone marrow transplantation from aged to young mice transfers age-related susceptibility to NVR independent of macrophage function, as a result of increased frequency of circulating mesenchymal progenitor cells (MPCs) in the bone marrow of aged mice [199]. Collectively, these data support the concept that NVR biology occurs as a result of cross-talk between reparative macrophages and MPCs, wherein macrophages recruit MPCs from the circulation and activate them to become vascular smooth muscle cells (VSMC) and myofibroblasts, establishing the template for arteriolar CNV, early in the process of formation and development [199]. While there is much to be explored and understood about the regulatory mechanisms for NVR, modulation of the cross-talk between reparative macrophages and mesenchymal precursors may represent a novel therapeutic strategy to target NVR and extend benefits of disease control and improved visual function for the large segment of patients with NVAMD and arteriolar CNV.

(d) *Regulation of Macrophage Biology in CNV Formation and Growth:* The specific mechanisms that regulate macrophage biology in the setting of CNV formation and growth are not well understood. In terms of macrophage recruitment and infiltration, as noted above, both MCP-1 and IL-8 [203], as well as IL-6 may mediate the recruitment of monocytes from the circulation to the incipient CNV [234]. There is also evidence to

support a role for the leukotriene B_4 (LTB4)-leukotriene B_4 receptor 1 (BLT1) signaling axis, as blockade of LTB4 reduces macrophage infiltration [235]. Evidence from the laser CNV model indicates that soluble Fas ligand (sFasL) released from injured RPE is a key mediator of monocyte recruitment and infiltration in aged mice [236]. Inhibition of complement factor C3 reduces CNV macrophage infiltration, suggesting that complement components may serve as stimuli of inflammatory or reparative macrophage infiltration and activation [237]. Collectively, these data suggest that there may be multiple pathways, rather than a single master factor, regulating macrophage infiltration to the site of CNV formation. Understanding the regulation of macrophage effector function remains an emerging area of investigation, but several studies have highlighted potential mechanisms. Modulation of macrophage STAT3 signaling [234], inhibition of macrophage Rho-associated kinase (ROCK2) [238], and inhibition of RIP1 kinase [239] may downregulate reparative macrophage effector biology in the setting of CNV formation and growth [228, 229, 234]. Additional investigation into mechanisms of macrophage biology will be essential to develop therapeutic strategies directed against macrophages in NVAMD.

(e) *Macrophage Infiltration in Neurosensory Retina:* While the vast majority of clinical and preclinical studies have centered on the contributions of blood-derived macrophages to CNV induction, formation, and growth, considerably fewer studies have explored the roles and effects of infiltrating blood-derived macrophages to pathology and dysfunction in the overlying neurosensory retina. Histopathology of NVAMD demonstrates significant infiltration of CD163+ macrophages in the neurosensory retina overlying CNV, especially in CNV with arteriolarization and fibrosis [146]. In the murine laser-induced CNV model, using the 810 nm laser that limits injury to the photoreceptors and the overlying retina (as compared to the 532 nm laser), there is substantial infiltration of blood-derived macrophages into the retina overlying CNV, which increases along with the lateral spread of the CNV over time [173] (Fig. 6.7). Retinal macrophage infiltration in this setting is associated with diminished ERG B-wave amplitudes and disruption of photoreceptor-bipolar synapses in the outer plexiform layer and Muller cell injury and activation [174]. Effects on synapses could be mediated by macrophage-derived and secreted TNF-α or IL-6, which may specifically promote Muller injury and activation. Importantly, prevention of retinal macrophage infiltration abrogates the synaptic and visual dysfunction [173, 174], suggesting that specific therapies targeted against retina-infiltrating macrophages in NVAMD could improve visual function independent of leakage control.

(f) *Integrated Hypothesis for Macrophage Biology in NVAMD:* Clearly, there is considerable evidence that macrophages contribute to NVAMD, but much remains to be determined about the specific mechanisms by which macrophages mediate effects on CNV pathobiology. Based on the available clinical, histopathologic, and preclinical evidence to date, we embrace the hypothesis that blood monocytes give rise to distinct macrophage subsets, inflammatory and reparative, and monocytes that give rise to each are preprogrammed for distinct effector biology. We propose that monocytes that are primed to express high levels of TNF-α, IL-1β, NOS2, VEGF, and proteases such as MMP-9 [52], arrive at the locus of disease at the RPE, Bruch's membrane, and choriocapillaris as part of an initial wave of recruitment, where they are transformed into fully activated inflammatory macrophages in situ, secreting effector molecules that contribute to or perhaps directly trigger the induction and initial development of the CNV. Following this initial phase of recruitment, a second

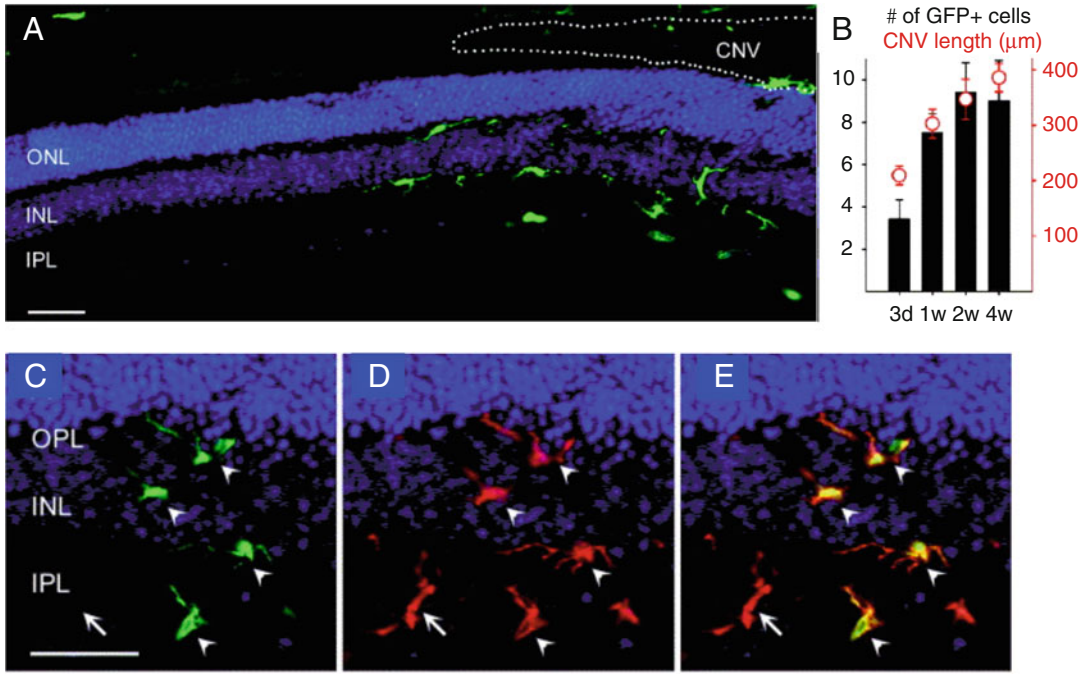

Fig. 6.7 (**a**) Bone marrow-derived cells, labeled green fluorescent protein (GFP), infiltrate the neurosensory retina overlying choroidal neovascularization (CNV) but are absent in adjacent retinal regions outside of the CNV. (**b**) The frequency of GFP+ bone-marrow derived cells directly correlates with the lateral extension of CNV and increases over time following laser induction, plateauing at 2 weeks. (**c–e**) GFP-labeled cells are macrophages. In these representative images (4 weeks after CNV) of (C) GFP-labeling and (**d**) F4/80 macrophage label, all GFP-labeled cells were F4/80 immunoreactive (*arrowheads*), appearing yellow in (**e**). A single resident microglial cell (arrow; not GFP-labeled) could also be seen. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; CH, choroid. Scale Bars = 50 μm. Adapted with permission from Caicedo A, et al., Exp Eye Research 2005; 81:38-47. Copyright Elsevier Ltd. 2005

phase of monocytes that give rise to reparative macrophages are subsequently recruited to the incipient CNV [52–54]. On full transformation, these reparative macrophages secrete either predominantly angiogenic factors (e.g., VEGF, PDGF, and Ang-1) to mediate capillary angiogenesis, the formation and growth of capillary CNV; or profibrotic and vascular factors (e.g., TGF-β, osteopontin, IGF-1, FGF, CTGF) to mediate neovascular remodeling, formation and growth of arteriolarized CNV with perivascular fibrosis via recruitment and activation of mesenchymal precursors to become vascular smooth muscle cells and myofibroblasts. The specific determinants of whether reparative macrophages exhibit either proangiogenic or profibrotic effector function are unknown, but based on available evidence, risk factors such as age, cigarette smoke exposure, low-grade exposures to microbial toxins (e.g., PAMPs), and latent macrophage infection with CMV [105, 108, 224–226] all shift reparative macrophages to profibrotic, neovascular remodeling effector functions [53, 240]. Thus, both subsets contribute to CNV biology, but CNV lesions experience a dynamic, biphasic recruitment of macrophages, first with an early recruitment of inflammatory macrophages promoting induction and initial development of CNV, and subsequently with a second delayed but more sustained recruitment of reparative macrophages that mediate

continued CNV formation and growth. In total, the effects of macrophages on CNV pathobiology is driven not only by this biphasic recruitment of macrophage subsets, but also by the relative numbers of primed monocytes in the blood (giving rise to each subset) at the time of recruitment [241, 242]. Thus, under this paradigm, therapeutic strategies directed against inflammatory macrophages could be effective to prevent progression to NVAMD, while strategies directed against reparative macrophages could be effective to limit CNV growth, and specifically to limit the biology of neovascular remodeling.

6.3.1.3 NLRP3 Inflammasome

The NLRP3 inflammasome is a multimeric complex of cellular cytosolic proteins that assembles in response to specific exogenous and endogenous danger signals, such as pathogen-associated molecular patterns (PAMPs), which include microbial-associated toxins (e.g., LPS, zymosan, viral antigens) and danger associated molecular patterns (DAMPs), which include protein or nucleic acid components (e.g., cytoplasmic DNA, noncoding RNA transcripts) released or exposed following cellular injury [139]. Assembly and activation of the NLRP3 inflammasome thus integrates the cellular response to various injury stimuli, activating caspase-1 via proteolytic cleavage, which in turn cleaves precursors of IL-18 and IL-1β to active cytokines that are secreted and mediate pro-inflammatory signaling [139]. The NLRP3 inflammasome was classically identified and characterized in innate immune cells including macrophages and microglia but has been more recently described and characterized in specialized epithelial cells, including RPE cells. While NLRP3 activation can serve as an important host defense mechanism, dysregulation of inflammasome activation is now thought to contribute to a variety of chronic diseases, including diabetes, neurodegenerative diseases such as Alzheimer's disease, and AMD [139].

Several studies have demonstrated that NLRP3 activation in RPE cells may serve as a final common pathway in response to various injury stimuli linked to AMD and that activation of the NLRP3 inflammasome and production of IL-18 and IL-1β may mediate RPE cellular injury and eventually cell death (by way of pyroptosis, a specialized form of inflammatory programmed cell death), leading to GA [243–247]. Initial work in this area evaluated cytotoxicity associated with the noncoding RNA motif known as Alu repeat and found that deficiency of DICER1 at the RPE led to Alu accumulation and RPE cell death, with corroborative features present in histopathology of human dry AMD [248]. Subsequent investigations found that Alu cytotoxicity and atrophic disease was mediated by activation of the NLRP3 inflammasome and production of IL-18 at the RPE [245]. Moreover, Alu RNA activation of the NLRP3 inflammasome at the RPE was found to be independent of TLR but dependent on P2X7 [249], and P2X7-mediated NLPR3 inflammasome activation and RPE atrophy was readily inhibited by nucleoside reverse transcriptase inhibitors (NRTIs) [250]. Additional work has determined that NLRP3 inflammasome activation is generalizable to other potential triggers of AMD disease, including iron toxicity, complement, reactive oxygen species, and lipid dysregulation [243–247, 251]. Collectively, these findings support inhibition of the NLRP3 inflammasome, IL-18, IL-1β, and potentially downstream effector events as a therapeutic strategy to prevent or slow GA in dry AMD. On the other hand, several other studies suggest that NLRP3 inflammasome expression in the setting of AMD is not found at the RPE but is instead restricted to macrophages and microglia [252–254], and that activation of the NLRP3 inflammasome in macrophages by stimuli such as complement components as well as subsequent IL-18 production actually serve a protective role, limiting severity of experimental CNV [253]. These opposing conclusions highlight that further study is needed to characterize the specific roles of NLRP3 inflammasome activation in different cell types and at various stages

of AMD disease before effective therapeutic strategies can be developed for AMD.

6.3.2 Complement

Histopathological studies have demonstrated that complement, along with other components of the immune system are abundant in drusen, the hallmark of dry AMD [255, 256] (Fig. 6.8) and at the border of GA in advanced dry AMD [257]. In addition, reduced levels of negative regulators of complement have been found in RPE overlying drusen and GA [258, 259]. In addition to histopathological studies, polymorphisms in numerous complement genes have been associated with AMD. The first discovered, and strongest genetic association is the 402H polymorphism in complement factor H (CFH) [4–7]. Subsequently, genome wide association screens (GWAS) have identified multiple complement components including complement component 2 (C2), complement factor B (CFB), complement component 3 (C3), complement component 9 (C9), complement factor I (CFI), and vitronectin (VTX) [260–265]. While these studies have strongly associated complement polymorphisms with AMD, the mechanistic role of complement in the pathogenesis of both dry and neovascular AMD is still being characterized.

Recently, rare and ultra-rare variants in complement proteins have been linked to both development and progression of AMD and have lent support to a functional role for complement dysregulation in AMD pathogenesis. For example, ultra-rare variants of CFH have been linked to autosomal dominant early onset drusen, which is considered to be a severe inherited form of dry AMD [266]. Rare variants of CFH and C3b which are associated with AMD affect the region of contact between CFH and C3b suggesting that in some cases defects in C3b inhibition by CFH result in increased activation of the alternative pathway leading to development of dry AMD [267–269]. Other rare variants in both CFH and CFI have been associated with reduced C3b degradation in serum-based assays [270]. Finally, rare AMD-associated variants in CFI, a major negative regulator of complement, have been associated with reduced serum levels of CFI while rare variants in C9 have been associated with increased serum levels of C9; both of which may predispose to increased complement activity [270]. Taken together, these studies suggest that genetic variants which favor increased complement activity predispose people toward development of AMD. Recently, the increase in clinical trials aimed at prevention of the development of GA or CNV (late AMD) has led to considerable interest in identifying patients at risk for rapid progression as these patients would most benefit from therapy and are better suited for clinical trials. In addition to clinical markers for rapid progression, genetic approaches have been applied. For example, a bivariate GWAS which took into account both AMD status as well as time to progression to advanced AMD defined as CNV or GA identified four previously discovered risk alleles as risk factors for progression to advanced AMD including regions coding for CFH, C3 and C2-CFB-SKIV2L suggesting complement as a risk factor for both development and progression of AMD [271].

Given the suggestion that increased complement activity is a risk factor for AMD, numerous studies on in vitro and in vivo models of AMD have been used to further dissect the molecular mechanisms by which complement may cause or worsen AMD. From these studies, several nonmutually exclusive theories regarding the role of complement in pathogenesis of AMD have emerged. These include direct cellular toxicity to RPE and/or photoreceptors, injury to choroidal endothelium resulting in localized hypoperfusion, upregulation of pro-angiogenic proteins within RPE leading to CNV, and recruitment of macrophages which play important roles in both dry and neovascular AMD (see sections on Macrophages in Dry AMD and Macrophages in NVAMD). In addition, noncanonical roles for CFH have been described which challenge the traditional paradigm of complement biology in AMD. As discussed below, perturbations in a single complement factor can mediate more than one of the above mechanisms and can impact both dry and neovascular AMD.

Effector proteins from the complement cascade are found in drusen and may play a role in

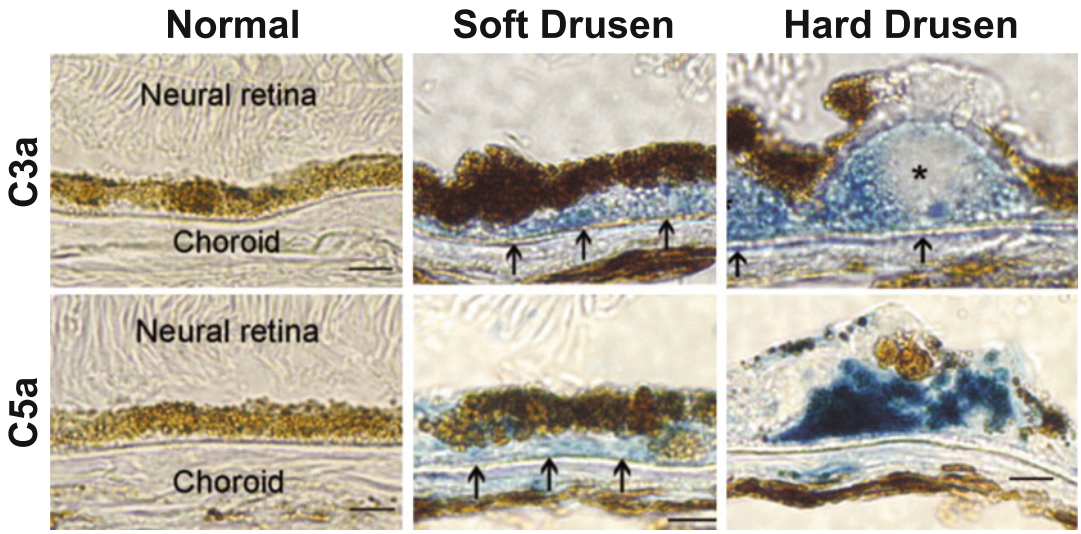

Fig. 6.8 Histopathology of drusen and complement in dry AMD. C3a (top panels) and C5a (bottom panels) are present in soft or hard drusen in histopathological specimens from eyes with dry AMD but not in healthy eyes. C3a and C5a are stained in blue; RPE pigment is seen in brown. Scale bars =10 μm. Adapted with permission from Nozaki, M., et al., Proc Natl Acad Sci USA 2006; 104:2328-33. Copyright National Academy of Sciences, U.S.A. 2006

the development of advanced AMD. For example, both C5a and C3a are found in drusen in human tissues [6, 255, 256] (Fig. 6.8). Both proteins are capable of upregulating VEGF expression in cultured RPE and in mouse RPE-choroid complexes in vivo. C3a and C5a are also upregulated at sites of laser induced CNV in mice. Genetic or pharmacologic blockade of C3a, C5a or their respective receptors results in reduction in experimental CNV lesion size [255]. In addition, both C3 and C5 are localized to photoreceptor outer segments (POS) located at the border of GA in human histological samples. Isolated POS were capable of activating complement in vitro and C5a was found to mediate macrophage recruitment resulting in photoreceptor cell death in a mouse model of toxin induced retinal degeneration. In this model, genetic deletion of C3 and C5 resulted in reduced photoreceptor cell death [257]. Taken together, these data suggest multiple roles for C3 and C5, particularly in the pathogenesis of advanced AMD.

In addition to increased complement effector proteins, reduced levels of endogenous negative regulators of complement have also been noted in histopathological specimens from eyes with AMD. Perturbations of these proteins result in development of RPE dysfunction, thickened Bruch's membrane, macrophage infiltration and more severe CNV in mice. For example, CD59 was found to be decreased in regions of drusen or GA in human pathology specimens [259]. Mice deficient in CD59a (the ortholog to human CD59), a membrane-bound inhibitor of MAC develop subretinal deposits composed of mononuclear phagocytes, with the subretinal myeloid cells appearing comparable to subretinal macrophages observed in human specimens with dry AMD [146, 259]. CD59 is also downregulated at sites of laser-induced CNV in mice and deficiency in CD59 results in increased lesion size which was associated with increased MAC assembly at the lesion site [272]. Interestingly, an RPE-choroid targeted fusion protein of CD59 resulted in decreased lesion size and reduced MAC assembly [272]. CD46, another membrane bound, negative regulator of MAC which is expressed in RPE and neural retina is also reduced in human AMD histopathology

specimens [259]. Mice deficient in CD46 were found to develop a dry AMD phenotype including thickening of Bruch's membrane, RPE vacuolization, multinuclear RPE cells [273]. These mice also demonstrate increased VEGF expression within RPE-choroid and larger lesions in the laser induced CNV model [273]. Finally, pentraxin-3 (PTX3), which can function as a negative regulator of complement activity is decreased in RPE overlying drusen in human samples. PTX3 is upregulated by oxidative stress in cultured RPE and deficiency in PTX3 caused enhanced activation of complement as well as the NLRP3 inflammasome in response to oxidative stress which resulted in enhanced macrophage recruitment to the choroid [258].

Complement-mediated injury to the choriocapillaris endothelial cells resulting in decreased vascular density and subsequent RPE dysfunction has also been proposed as a driver of AMD. Histopathological studies demonstrate that there is a generalized decrease in choriocapillaris vascular density in AMD cases versus controls and further shows reduced choriocapillaris vascular density beneath drusen compared to adjacent regions [274]. This suggests that alterations in the morphology of choriocapillaris are associated with dry AMD. In vitro studies of cultured choriocapillaris endothelium exposed to complement containing human serum demonstrate MAC-mediated cell lysis of some cells and upregulated angiogenesis associated genes including VEGF, MMP-3 and MMP-9 in surviving cells. This observation ties complement to both choriocapillaris loss as well as development of CNV in AMD [275].

Polymorphisms in CFH represent one of the most common genetic associations with AMD. While rare variants which result in defected downregulation of complement activity have been described, the H402 polymorphism has not been found to disrupt these functions. However, noncanonical roles for CFH which are impacted by this polymorphism have been described. CFH was found to be a major serum binding partner for malondialdehyde (MDA) adducts, which are produced by oxidative stress. CFH binding to MDA decreased macrophage phagocytosis of MDA adducts and decreased inflammatory signaling in vivo. However, this function was impaired in H402 CFH [276]. CFH has also been shown to compete with lipoproteins for binding sites within Bruch's membrane; deficiency in CFH results in increased sub-RPE deposits in a mouse model of dry AMD [277].

6.3.3 Adaptive Immunity

Traditionally, adaptive immunity has not been considered as a major contributor to either dry or neovascular AMD. This is in part due to limited histopathologic evidence of lymphocytes in AMD specimens and because the retina and choroid lack an identifiable or characterized lymphatic system for support of a traditional afferent arc of the adaptive immune response. However, as will be discussed, emerging clinical evidence, as well as several preclinical models, suggests plausible albeit limited roles for T cells and for autoimmunity in subset of AMD disease.

6.3.3.1 T Cells and Associations with AMD

While histopathologic evidence of lymphocytes in AMD has been limited, it has not been entirely lacking. Penfold, et al., initially described the presence of lymphocytes in eyes of patients with GA and NVAMD and suggested that lymphocytes may play a role in both diseases by promoting RPE atrophy and alteration in Bruch's membrane, respectively [76, 89]. CD8+ T cells have been observed by fluorescence microscopy in the choroid of frozen sections of eyes of AMD patients with GA [278] and NVAMD [279]. It remains uncertain whether T cells observed in association with GA or within CNV are responding to specific antigens or have been recruited as part of bystander activation. Since MHC class II expression has been found on RPE cells [280] and may be enhanced on microglia or infiltrating macrophages in aging and AMD [281, 282], antigen-presentation to infiltrating T cells is plausible; on the other hand, there is evidence to support antigen-independent activation of cytotoxic CD8+ T

cells in experimental mouse models of RPE injury [283]. In patients, levels of the chemokines CXCL10 and CXCL11, both chemotactic for CXCR3+ T cells, were found at increased levels in the RPE/choroid in patients with NVAMD and GA, respectively, while both chemokines were found to be elevated in the plasma of patients with AMD, as compared to normal controls [284]. In another study, increased percentages of CD56+ and CD28- memory T cells in the blood were associated with increased risk of developing AMD [285], with the risk increasing further for those patients also having at least one CFH H402 allele [285], and it was also found that lower percentages of CD8+ $CXCR3^{high}$ T cells and CD4 + CD69 + CXCR3+ T cells were present in NVAMD as compared to controls [286]. Meanwhile, the percentage of circulating CD4+ T cells was increased in patients with NVAMD with subretinal fibrosis, as compared to patients without subretinal fibrosis [287]. On the other hand, in the murine laser-induced CNV model, IL-4, a T helper cell type 2 (Th2) derived cytokine, conditions resident microglia and recruited macrophages to produce a soluble form of the VEGF Receptor 1 (also known as sFlt1) and subsequently suppress capillary angiogenesis [288]. Collectively, these data suggest the possibility that some T cell subsets may play a role in AMD pathobiology, and that others may play a protective role against the development of various aspects of AMD disease.

6.3.3.2 IL-17 and T_h 17 T cells

IL-17 is a pro-inflammatory cytokine that signals through the IL-17 receptor (IL-17R) system, activating signaling pathways and inducing production of other proinflammatory cytokines (e.g., TNF-α, IL-6) and chemokines (e.g., IL-8, MCP-1) that promote the recruitment of monocytes, neutrophils, and other T cells. IL-17 can also directly mediate effector biology on target cells, including endothelial cells, epithelial cells, and neurons [289]. The predominant source of IL-17 is a subset of CD4+ T cells known as T helper 17 (T_h17) cells, though it can also be produced by other types of immune cells. Increased IL-17 expression has been found in close approximation with loci of GA in the setting of AMD, as compared to normal age-matched controls, and IL-17 localized predominantly to CD3+ cells, a marker of T cells as well as to IBA-1+ macrophages/microglia [290]. IL-17 and another T_h17 cytokine IL-22 have been found to be elevated in the plasma of AMD patients as compared to non-AMD controls [291, 292], and that this elevation may be in part mediated by stimulation of CD4+ T cells by the activated complement component C5a [292]. It has also been demonstrated that hypomethylation of the promoter for IL17RC, a component of the IL-17R complex, in AMD patients as compared to controls, in association with elevated expression of IL-17RC mRNA and protein in peripheral blood as well as in the affected retina and choroid, suggesting a role for both local and systemic alterations in IL-17 signaling in AMD [291]. However, another study analyzed the methylation status of the IL-17RC promoter using multiple analytical methods and found no significant difference in the methylation status between patients with AMD and age-matched controls in two independent cohorts, so the role of altered IL-17R in AMD remains uncertain [293]. There is also debate about the potential role of IL-17 in NVAMD. One study found that IL-17, derived from γδ-T cells and THY1+ innate lymphoid cells (ILCs) and not T_h17 cells, promoted experimental CNV growth in a VEGF independent manner [294]. Other studies offer controverting evidence, indicating that IL-17 alone does not impact vessel growth but can indirectly influence neovascularization by modulating the effects of other angiogenic factors (e.g., VEGF, bFGF, HGF) [295]. Thus, while there is considerable evidence to suggest a potential association of IL-17 with. AMD, its precise role with advanced dry AMD with GA and NVAMD remains to be elucidated [296].

6.3.3.3 Autoimmunity and AMD

Several reports have identified immunoglobulins and immune complexes in association with drusen [256, 297–299], which may reflect one or more aspects of aberrantly activated adaptive immunity including molecular mimicry, antigen-trapping, desequestration, or formation of neo-antigens. There is considerable emerging

evidence that autoimmunity, specifically autoantibody-mediated disease, may play a role in AMD. Penfold and colleagues initially described autoantibodies to retinal astrocytes in association with AMD [300], suggesting a role for antiretinal autoantibodies in a subset of AMD disease. Numerous studies since have demonstrated, by various techniques, especially immunohistochemistry, elevated levels of antiretinal autoantibodies in the peripheral circulation of AMD patients as compared to controls [289]. Multiple different types of autoantibodies have been described, including autoantibodies to glial fibrillary protein (GFAP), which is expressed by Muller cells and astrocytes, recoverin, which is expressed by photoreceptors, and α-enolase, which is found in retinal ganglion cells and inner nuclear layer. Additional autoantibodies have been found to be elevated in the sera of AMD patients, including antibodies to phosphatidyl serine (PS), JO-1, U1-snRNP-68, elastin, cytochrome C, sc-100, and collagen III, all of which were specifically associated with NVAMD/CNV and not dry AMD, while autoantibodies to fibronectins were specifically associated with dry AMD [289]. Autoantibodies to various components of drusen, extracellular matrix, and Bruch's membrane have also been described in AMD patients, indicating that aberrant activation of antibody-mediated adaptive immunity could be mediated by desequestration of RPE and retinal cellular antigens which are aberrantly deposited in the subRPE space in AMD. Alternatively, components of drusen and extracellular deposits, such as oxidized lipoproteins, can become neo-antigens to activate an immune response arc, leading to formation of autoantibodies [289, 301]. As has been found in other diseases such as atherosclerosis, scavenging macrophages may then ingest neo-antigens and become antigen-presenting cells at the site of drusen formation or RPE cell injury, serving to re-stimulate recruited T cells and thereby activating the effector phase of the immune response.

A variation on neo-antigen concept is the development of adjuncts or adducts of RPE and retinal proteins, which are also recognized by autoantibodies. Oxidation of docosahexaenoate-(DHA-) containing lipids generates carboxyethylpyrrole (CEP) protein adducts. CEP-adducted proteins have been found at higher levels in drusen and in blood from AMD patients as compared to normal controls [302–306]. Additionally, mean titers of anti-CEP autoantibody are higher in AMD patients as compared to normal controls [307]. These findings are bolstered by work in mice, where immunization with CEP-adducted mouse serum albumin generated development of autoantibodies directed against CEP and subsequently led to the development of RPE deposits and photoreceptor degeneration [308]. CEP-induced immune response in this model is thought to be via activation of T lymphocytes by inflammatory macrophages, leading to increased expression of interferon-γ (IFN-γ) and IL-17 [308]. Collectively, these findings across human histopathology, studies of AMD sera, and preclinical mouse models provide strong evidence to indicate that factors associated with AMD, specifically oxidative injury, can generate protein adducts that serve as neo-antigens, and the development of autoantibodies against these adducted proteins could potentially contribute to AMD disease progression.

Population-based analyses suggest a wide array of retinal autoantibodies may be present in sera of AMD patients, and antigenic targets include proteins that are implicated in autophagy, immunomodulation, protection from oxidative stress, and apoptosis [309], indicating that aberrant activation of antibody-mediated immune mechanisms could potentially amplify a host of cellular dysfunctions that have been independently associated with AMD, both for disease onset and progression. However, it remains uncertain whether the development of such autoantibodies are isolated to mediating AMD disease or if presence of these autoantibodies reflects a more generalized dysfunction of the immune system with the RPE and retina being an opportunistic target of this more generalized dysfunction [310, 311]. Moreover, the presence of retinal autoantibodies are not specific for disease, as they are frequently found in normal subjects without any sign of AMD or retinal

disease [312]. Presently, there is a lack of direct evidence demonstrating that the presence of retinal autoantibodies is a risk factor either for the onset of early AMD or the development of more advanced AMD disease, such as GA or CNV. However, the current associative data provides a compelling starting point for further investigation into the potential link between autoimmunity and AMD.

6.3.4 Infection and AMD

Interactions between the immune system and infectious pathogens such as bacteria and viruses could also modulate AMD disease pathobiology via alterations in innate and adaptive immune effector mechanisms. Accordingly, several hypotheses have been put forth to suggest an infectious etiology of AMD.

6.3.4.1 Direct infection at AMD locus of disease

One possibility is that the virus or bacteria may directly infiltrate and infect the locus of disease at the RPE, Bruch's membrane, and choriocapillaris. Based on the hypothesis that certain bacterial or viral pathogens may produce chronic infection of vascular endothelial cells or the vascular interstitium leading to vascular disease, several studies have explored such a link. *Chlamydia pneumoniae*, an obligate intracellular bacterium, was identified as a novel risk factor in cardiovascular diseases, and numerous studies identified *C. pneumoniae* as a potential contributor to atherosclerosis. Chronic infection of vascular endothelial cells may upregulate cell surface molecules that recruit macrophages or alter responses to injury. For instance, *C. pneumoniae* endothelial infection can enhance endotoxin binding to LDL particles that might induce various inflammatory cascades at the site of uptake [313]. Additionally, chlamydial heat shock proteins (HSPs) can directly stimulate macrophages and other cellular amplification systems [314]. On this basis, several studies explored whether *C. pneumoniae* may also have relevance to AMD. One study discovered evidence of *C. pneumoniae* in CNV tissue from patients with NVAMD, both by immunohistochemistry (IHC) and polymerase chain reaction (PCR), in contrast to no detectable pathogen in non-AMD tissue specimens [315]. The authors of this study also found that exposure to *C. pneumoniae* induced VEGF production in cultured monocyte-derived macrophages and induced secretion of IL-8 and MCP-1 by cultured RPE cells. Meanwhile, a serological association was found between AMD and presence of antibodies to *C. pneumoniae* [316]. While this study raised the intriguing possibility that direct infection may contribute to AMD pathobiology, there is limited evidence for other pathogens detected within CNV and limited additional evidence that specifically C. pneumoniae infection is a generalizable phenomenon in NVAMD [103, 104]. Another potential mechanism by which pathogens may contribute to disease is by release of PAMPs or microbial toxins, which may directly interact with pattern recognition receptors (PRRs) expressed at the RPE surface. For example, TLR2 is highly localized to the apical surface of the RPE in both human and mouse eyes. Activation of TLR2 signaling, evident as nuclear localization of NF-kB, was present in RPE of human eyes with AMD, while in mice, local ocular inhibition of TLR2 reduced experimental CNV in both laser-induced CNV and spontaneous CNV models [20].

6.3.4.2 Molecular mimicry

Another possibility is molecular mimicry, wherein the foreign pathogen expresses antigens that are closely related to self-antigens present at the RPE and/or retina. For instance, antigen-specific immune responses directed against bacterial heat-shock protein (HSPs) (e.g., *C. pneumoniae* HSPs) may cross-react with host proteins expressed in the retina [317]. Additionally, immune responses to bacterial or viral antigens trapped in tissues after occult infection, may also stimulate antigen-specific immunity, or autoimmunity by cross-reactive molecular mimicry [318]. Alternatively, T cells may be recruited by innate responses and become activated by antigen-independent bystander mechanisms.

6.3.4.3 Total pathogen burden

A distinct concept is total pathogen burden, which hypothesizes that systemic immune alterations observed in association with AMD occur as a result of cumulative infections with multiple pathogens over the course of life [289]. In this hypothesis, immune alterations are not due to one single pathogen or infection, but multiple pathogens carry more and cumulative risk—for example, this idea has been put forth to explain elevated levels of C-reactive protein (CRP) in cardiovascular disease [319]. Accordingly, it has been observed that patients with elevated serum antibody titers against all three of cytomegalovirus (CMV), *C. pneumoniae*, and *Helicobacter pylori* had increased risk of NVAMD (over presence of dry AMD) [108]. The concept of total pathogen burden can be understood through priming effects on the innate immune system. Chronic exposure to microbial components can prime, or partially activate, monocytes in spleen, lymph node, bone marrow, or other sites of exposure or surveillance, altering the expression of certain cytokines and mediators and committing exposed cells to specific effector functions. These functions then become fully manifest upon recruitment to the disease locus (i.e., RPE and retina) and transformation into fully activated macrophages. Indeed, the presence of periodontal disease, which is a known primer of circulating monocytes, has been independently associated with AMD, even after controlling for other more established risk factors [320]. Importantly, immune mechanisms of AMD disease are not mutually exclusive. CEP adducts, which are thought to play a role in autoimmune mechanisms of AMD as noted above, have also been found to potentiate TLR2 and TLR1 signaling in macrophages, and could synergize with total pathogen burden to prime monocyte and macrophage effector function in the setting of AMD [321].

6.3.4.4 Latent infection of immune cells

Cytomegalovirus (CMV) is a common virus that infects people of all ages. While primary infection causes little to no symptoms in most people, it is frequently followed by establishment of persistent or latent infection. In assessing CMV IgG titers among dry AMD patients, NVAMD patients, and controls without AMD, there was a significant association of high CMV titer with presence of NVAMD, as compared to dry AMD and controls, suggesting that CMV could contribute to the development of CNV [108]. Possible mechanisms for this biology include latent infection of monocytes and macrophages, as latent infection occurs in bone marrow hematopoietic progenitor cells destined to develop into monocytes [322]. As these infected cells mature, macrophage pro-inflammatory genes become transactivated by CMV immediate-early gene products that are expressed during latency [322–325]. These activated infected monocytes may produce higher levels of inflammatory mediators upon recruitment to the site of RPE/Bruch's membrane injury, triggering CNV formation. Indeed, in mice, latent infection of macrophages with murine CMV (MCMV) was associated with increased growth and severity of experimental CNV in the laser-induced model [105]. In this model, latent MCMV infection was not detected in the choroid and RPE indicating that the effects of CMV in formation of CNV are not due to local latent infection within ocular tissues. This represents a mechanism distinct from atherosclerosis, where the potential mechanisms for the link between CMV and atherosclerosis center around local infection of the diseased blood vessel center around enhanced scavenging of LDL particles by endothelial cells infected with CMV [326–328].

6.4 Conclusions: Understanding the Role of Immune Mechanisms in AMD

In this chapter, we have reviewed innate immunity, adaptive immunity, and immune amplification especially the complement system, and we have explored how each of these may contribute to AMD.

We embrace the "response to injury" paradigm for AMD pathobiology as a means to understand and integrate how immune mechanisms

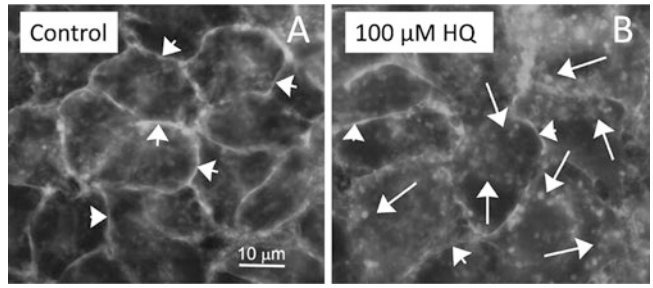

Fig. 6.9 Images of cultured retinal pigment epithelium cells exhibiting extensive cell membrane blebbing following sublethal oxidative injury after exposure to 100 µM hydroquinone (HQ), as compared to control uninjured cells without blebbing (arrows, cell membrane). Scale bar = 10 µm. Adapted with permission from Marin-Castano, ME., et al., Invest Ophthalmol Vis Sci 2006; 47:4098-4112. Copyright Association for Vision and Research in Ophthalmology 2006

contribute to AMD [150]. In this paradigm, various stimuli, including oxidants, lipofuscin cytotoxicity, immune cell-derived mediators, blue light exposure, or systemic factors such as hyperlipidemia, oxidized lipoproteins, and hormonal changes (i.e., increased angiotensin or aldosterone) [150, 329], can mediate nonlethal injury for various cell types, especially, RPE, photoreceptors, and choriocapillaris endothelium. For example, RPE cells can react to nonlethal injury with many responses relevant to deposit formation in AMD, including blebbing of cell membrane (Fig. 6.9) [330], cytosol and organelles (but without activation of programmed cell death or nuclear fragmentation) after oxidant-mediated injury of the cell membrane, which can serve as a starting point for subRPE deposit formation. In this setting, immune mechanisms may serve as a trigger of nonlethal injury. Alternatively, blebbing might activate an immune response by desequestration of intracellular antigens to provide a target for antigen-specific immunity, or blebs might provide a substrate for nonspecific activation of complement, triggering an immune response that can interfere with healthy repair [331, 332]. Importantly, response to injury may be critical not only for disease onset but also for disease progression [150], in the setting of CNV induction and conversion to neovascular AMD or with onset and progression of GA.

In AMD, interaction of various immune mechanisms with nonimmune cells and factors generates exaggerated or abnormal reparative responses to chronic, recurrent injurious stimuli, producing the cardinal pathological features of disease. Developing a precise understanding of how each of these immune mechanisms contributes to AMD pathobiology will be essential to enable the development and validation of clinically useful biomarkers to subtype and stratify AMD patients by specific disease mechanism and to facilitate the development of immune-targeted therapies for patients with AMD.

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AMD Genetics: Methods and Analyses for Association, Progression, and Prediction

7

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Abstract

Age-related macular degeneration (AMD) is a multifactorial neurodegenerative disease, which is a leading cause of vision loss among the elderly in the developed countries. As one of the most successful examples of genome-wide association study (GWAS), a large number of genetic studies have been conducted to explore the genetic basis for AMD and its progression, of which over 30 loci were identified and confirmed. In this chapter, we review the recent development and findings of GWAS for AMD risk and progression. Then, we present emerging methods and models for predicting AMD development or its progression using large-scale genetic data. Finally, we discuss a set of novel statistical and analytical methods that were recently developed to tackle the challenges such as analyzing bilateral correlated eye-level outcomes that are subject to censoring with high-dimensional genetic data. Future directions for analytical studies of AMD genetics are also proposed.

Keywords

AMD genetics · GWAS · Machine learning · Progression · Prediction · Statistical methods

7.1 Introduction

Age-related macular degeneration (AMD) is a heritable neurodegenerative disease and a primary cause of vision loss among the elderly in the developed world. AMD is characterized by the loss of photoreceptor and the reduction of retinal pigment epithelium function in the macula. The disease is progressive and irreversible in affecting central vision. The disease process starts with appearance of drusen and progresses to advanced AMD, which is typically classified into two forms: wet AMD (also called choroidal neovascularization (CNV)) and dry AMD (also

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called geographic atrophy (GA)) [1–3]. Dry AMD, characterized by the presence of drusen and thinning of the macula, is the most common type of advanced AMD and affects 85–90% of the AMD patients. Wet AMD, characterized by bleeding or fluid leaking abnormal blood vessels grown underneath the retina and macula, is considered as the more advanced type of AMD. Although affecting only 10–15% of those who have AMD, wet AMD accounts for 90% of the severe vision damage.

7.2 Case–Control Genetic Association Studies on AMD Risk

In 1990s, twin studies and family aggregation studies had shown that genetics played a role in AMD. In a family aggregation study, the prevalence of AMD was much higher in the first-degree relatives of AMD patients (23.7%) than in relatives of healthy individuals (11.6%) [4]. Twin studies indicated that the heritability of AMD range from 46% to 71%, estimated from comparing AMD concordance rates between monozygotic and dizygotic twins [5]. In the effort to explore AMD genetics in early 2000s, association studies and genetic linkage studies had been conducted to identify candidate susceptibility genes. In 2005, a meta-analysis of linkage scans showed that chromosomes 1q25-31 and 10q26 were the most replicated genomic regions [6]. With advances in technology, in addition to candidate gene studies, genome-wide association studies (GWAS) were able to be conducted to examine the association between AMD status and a genome-wide set of single-nucleotide polymorphisms (SNPs). In the same year of 2005, a landmark GWAS revealed an SNP in an intron of *CFH* gene was strongly associated with AMD; the risk allele at the SNP was in linkage disequilibrium (LD) with a tyrosine–histidine change at amino acid 402 of *CFH* [7]. This region of *CFH* binds heparin and C-reactive protein. This was the first GWAS performed for AMD, showing that the effect size was significantly increased by an odds ratio (OR) of 7.4 (95% confidence interval: 2.9–19) under a recessive model. This study recruited 96 AMD patients and 50 controls, and genotyped 116,204 SNPs. Although both the sample size and number of SNPs were small, this study was the first successful GWAS among complex diseases. With its success, an era for GWAS of complex diseases started. Specifically, for AMD, subsequent GWAS identified several susceptibility loci in complement related genes, including *C2/CFB* [8], *CFI* [9], and *C3* [10].

Genes not in the complement pathway had also been identified to be associated with AMD. Of them, the *ARMS2/HTRA1* locus had a strong AMD association with an odds ratio (OR) of 5.0 and population attributable risk of 57% [11, 12]. Since SNPs in both *ARMS2* and *HTRA1* genes in this locus are in strong LD, variants in both genes could be causally relevant to AMD. This is one of the drawbacks of GWAS that one cannot draw a causal conclusion from GWAS results, but a pure association partly due to the fact of LD among SNPs. Thus, post-GWAS functional analysis is required to help understand the biological process. Among other noncomplement genes associated with AMD, *TGFBR1* and *VEGFA* are related to angiogenesis; *COL10A1* and *COL8A1* are related to extracellular collagen matrix; *APOE*, *CETP*, and *LIPC* are related to high-density lipoprotein cholesterol pathway [13–15].

In early 2010, 18 research groups from multiple countries formed the AMD Gene Consortium in order to facilitate the discovery in AMD genetics, with support from the National Eye Institute (NEI) of the U.S. National Institutes of Health (NIH). In 2013, the consortium published a large GWAS for AMD [13] which included 17,181 cases and 60,074 controls, and 2,442,884 genotyped and imputed SNPs. The study reported 19 loci (Table 7.1) with association of AMD reaching the genome-wide significance level ($P = 5 \times 10^{-8}$), where seven loci reached significance for the first time. The proportion of variability in the risk of AMD that is due to heritability had been estimated at 45–70% [5],

Table 7.1 Results for AMD risk genes reported in the two consortium case–control studies and/or the GWAS progression study

SNP	Chr	Position	Major/minor allele	Gene	Fritsche et al. [15]		Yan et al. [32]	
					OR	*P*-value	HR	*P*-value
rs10922109	1	196,704,632	C/A	*CFH*	0.38	9.6×10^{-618}	0.43	3.5×10^{-37}
rs62247658	3	64,715,155	T/C	*ADAMTS9-AS2*	1.14	1.8×10^{-14}		
rs140647181	3	99,180,668	T/C	*COL8A1*	1.59	1.4×10^{-11}		
rs10033900	4	110,659,067	C/T	*CFI*	1.15	5.4×10^{-17}		
rs62358361	5	39,327,888	G/T	*C9*	1.80	1.3×10^{-14}		
rs116503776	6	31,930,462	G/A	*C2-CFB-SKIV2L*	0.57	1.2×10^{-103}	0.56	8.1×10^{-10}
rs943080	6	43,826,627	T/C	*VEGFA*	0.88	1.1×10^{-14}		
rs79037040	8	23,082,971	T/G	*TNFRSF10A*	0.90	4.5×10^{-11}		
rs1626340	9	101,923,372	G/A	*TGFBR1*	0.88	3.8×10^{-10}		
rs3750846	10	124,215,565	T/C	*ARMS2-HTRA1*	2.81	6.5×10^{-735}	2.04	5.3×10^{-42}
rs9564692	13	31,821,240	C/T	*B3GALTL*	0.89	3.3×10^{-10}		
rs61985136	14	68,769,199	T/C	*RAD51B*	0.90	1.6×10^{-10}		
rs2043085	15	58,680,954	T/C	*LIPC*	0.87	4.3×10^{-15}		
rs5817082	16	56,997,349	C/CA	*CETP*	0.84	3.6×10^{-19}		
rs2230199	19	6,718,387	C/G	*C3*	1.43	3.8×10^{-69}	1.45	1.2×10^{-9}
rs429358	19	45,411,941	T/C	*APOE*	0.70	2.4×10^{-42}		
rs5754227	22	33,105,817	T/C	*SYN3-TIMP3*	0.77	1.1×10^{-24}		
rs8135665	22	38,476,276	C/T	*SLC16A8*	1.14	5.5×10^{-11}		
rs11884770	2	228,086,920	C/T	*COL4A3*	0.90	2.9×10^{-8}		
rs114092250	5	35,494,448	G/A	*PRLR-SPEF2*	0.70	2.1×10^{-8}		
rs7803454	7	99,991,548	C/T	*PILRB-PILRA*	1.13	4.8×10^{-9}		
rs1142	7	104,756,326	C/T	*KMT2E-SRPK2*	1.11	1.4×10^{-9}		
rs71507014	9	73,438,605	GC/G	*TRPM3*	1.10	3.0×10^{-8}		
rs10781182	9	76,617,720	G/T	*MIR6130-RORB*	1.11	2.6×10^{-9}		
rs2740488	9	107,661,742	A/C	*ABCA1*	0.90	1.2×10^{-8}		
rs12357257	10	24,999,593	G/A	*ARHGAP21*	1.11	4.4×10^{-8}		
rs3138141	12	56,115,778	C/A	*RDH5-CD63*	1.16	4.3×10^{-9}		
rs61941274	12	112,132,610	G/A	*ACAD10*	1.51	1.1×10^{-9}		
rs72802342	16	75,234,872	C/A	*CTRB2-CTRB1*	0.79	5.0×10^{-12}		
rs11080055	17	26,649,724	C/A	*TMEM97-VTN*	0.91	1.0×10^{-8}		
rs6565597	17	79,526,821	C/T	*NPLOC4-TSPAN10*	1.13	1.5×10^{-11}		
rs67538026	19	1,031,438	C/T	*CNN2*	0.90	2.6×10^{-8}		
rs142450006	20	44,614,991	TTTTC/T	*MMP9*	0.85	2.4×10^{-10}		
rs201459901	20	56,653,724	T/TA	*C20orf85*	0.76	3.1×10^{-16}		

HR, hazard ratio relative to the minor allele (minor allele/major allele); *OR*, odds ratio

while these 19 loci accounted for 15–65% of the total genetic contribution to AMD (corresponding to 7–46% of the total variability in the risk of AMD). To follow up the candidate AMD genes, Zhan et al. performed a sequencing study in 2335 cases and 789 controls in 10 regions including 57 gene [16]. They identified two rare variants p. Arg1210Cys in *CFH* gene and p.Lys155Gln in *C3* gene. In 2016 [15], the International AMD Genomics Consortium (IAMDGC) systematically examined both common and rare variants of AMD association in >12 million SNPs including 163,714 directly genotyped, mostly rare, protein-altering variants in 16,144 cases and

17,832 controls. This study identified 52 independent AMD-associated SNPs ($P < 5 \times 10^{-8}$) including both common and rare variants across 34 loci (Table 7.1). Rare variants were identified in the complement pathway genes, *CFH* and *CFI*, and noncomplement pathway genes, *TIMP3* and *SLC16A8*. In addition, this study was the first study that examined the genetics of advanced AMD subtypes (wet and dry). It reported that *MMP9* was specific to the risk of wet AMD, but not dry AMD (Table 7.2).

A number of studies implied that the same AMD susceptibility loci have different effects in different ethnic groups. A study showed that the frequency of C allele at *CFH* Y402H variant is ~30% in a group of residents of Northern and Western European ancestry from Utah, but only ~5% in Japanese and Chinese individuals [17]. A study in 2014 examined AMD risk across diverse populations and showed both *rs*1061170 (*CFH* Y402H) and *rs*10490924 (*ARMS2* A69S) were associated with AMD in European Americans but not in other populations, including Mexican Americans, African Americans, or Singaporeans [18]. In addition, another study showed that the common *ARMS2* A69S variant was associated with increased risk of AMD in non-Hispanic whites (OR = 2.1) and Mexican Americans (OR = 2.45), but the direction of the effect was surprisingly reversed in non-Hispanic black individuals (OR = 0.43) [19]. The *T* allele of the *ARMS2* variant was the test allele and its frequency was approximately 13% lower in non-Hispanic black patients compared with non-Hispanic black controls. On the contrary, non-Hispanic white and Mexican American patients have a *T* allele frequency 10% higher than their controls. A recent paper emphasized the importance of protective alleles and their roles in AMD, particularly in the population with low prevalence of AMD (e.g., Timor-Leste) [20].

7.3 Genetic Studies on AMD Progression

To date, most AMD genetic studies focused on cross-sectional studies of advanced AMD (wet or dry). AMD is known to be a progressive disease, particularly in elderly population. It starts with a mild AMD condition with small drusen and no vision loss. It then progresses to intermediate AMD with medium sized drusen and minimal vision loss. Then, the disease progresses to the large drusen stage with pigment changes in the retina and some vision loss. Finally, the condition progresses to the advanced AMD stage with significant vision loss. Some AMD patients maintain a good vision for a long time with little disease progression, while others quickly progress to advanced AMD with significant vision loss. Patients can progress to one or both forms of advanced AMD. The genetic effects of disease progression were largely unexplored until recent years. The NEI-sponsored Age-Related Eye Disease Study (AREDS) was designed to assess risk factors for the development and progression of AMD and to evaluate the effects of different oral supplements of minerals and antioxidants in delaying the AMD progression [1]. Then, a subsequent clinical trial, AREDS2, evaluated some modified formulations of oral supplements on AMD progression on a cohort of population with more severe AMD [21, 22]. Both studies collected DNA samples of consented patients and performed genome-wide genotyping.

Recently, multiple research groups studied the AMD progression using the AREDS and/or AREDS2 data. For example, Seddon et al. [23, 24] and Perlee et al. [25] studied the effects of some known AMD risk variants on progression to advanced AMD using one eye per subject, i.e., the faster-progressed eye. Some other studies analyzed the genetic effects on progression status (e.g., no progression, early progression, or late progression) instead of progression time [26]. Furthermore, some studies analyzed the genetics effects on AMD progression to different stages of the disease. For example, Yu et al. [27] used multistate Markov models to assess the effects of 12 AMD risk loci on the AMD multistate progression from normal to intermediate drusen, then to largen drusen, and eventually to wet AMD or dry AMD. They found those known AMD risk genes were associated with progression within certain but not all stages. For example, genes

Table 7.2 Results for risk loci specific to wet AMD but not dry AMD reported in the consortium case–control study and the progression study

Genes	Case–control, 2013	Case–control, 2016	Progression, 2018
MMP9	Not reported	Reported	Reported
TNR	Not reported	Not reported	Reported
ATF7IP2	Not reported	Not reported	Reported

CFH, C3, CFB, and *ARMS2/HTRA1* were found to be associated with progression from intermediate to large drusen and from large drusen to advanced AMD, but not from normal to intermediate drusen. It is well known that the presence and progression of AMD in one eye is strongly correlated with the disease in its fellow eye. For example, Gangnon et al. [28] used the Beaver Dam Eye study to investigate the effects of the AMD severity in one eye on the incidence and progression of AMD in the fellow eye. They found that more severe AMD in one eye was associated with increased incidence of AMD and accelerated progression in its fellow eye. Therefore, to better analyze the AMD progression, more recently, researchers included the progression times of both eyes with appropriate models to account for the between-eye correlation when analyzing the genetic effects on AMD progression. For example, Sardell et al. [29] analyzed the effects of seven SNPs from four known AMD risk regions on AMD progression. Ding et al. [30] evaluated the effects of the top SNPs from the 34 known AMD risk loci on disease progression. In both papers, the progression time was modeled at eye level and the between-eye correlation was incorporated through a Cox proportional hazards (PH) model with the robust variance covariance.

From all the aforementioned studies that investigate a small set of variants on AMD progression, they found that some, but not all of those AMD risk variants are associated with progression. Most reported risk variants associated AMD progression are in the *CFH* and *ARMS* regions [23, 24, 26, 30, 31]. Additional loci such as *C3, COL8A1, CFB,* and *RAD51B* have also been reported to be associated with AMD progression [24, 30].

In 2018, a first GWAS analysis was conducted using the similar robust Cox PH model to test for association of progression to advanced AMD with ~nine million variants on 2721 Caucasians from the AREDS [32]. Four susceptibility loci showed genome-wide significant association ($P < 5 \times 10^{-8}$) with AMD progression, including *ARMS2-HTRA1*, *CFH*, *C2-CFB-SKIV2L*, and *C3* (Table 7.1 and Fig. 7.1). All four loci were also previously reported in AMD case–control studies. Furthermore, variants near *TNR* and *ATF7IP2* were detected to be associated with progression to wet AMD but not dry AMD (Table 7.2). The variants in these two loci are common variants and these two loci were not reported in any AMD case–control genetics study. Moreover, variants in *MMP9* were associated with progression of wet AMD but not dry AMD (Table 7.2). The same locus was reported to be specific to the risk of wet AMD but not dry AMD in a case–control study as well. In the secondary analysis focusing only on the 34 known AMD risk variants, the previously reported *LIPC* and *CTRB2–CTRB1* were also associated with AMD progression under a less stringent *P* cutoff than the GWAS *P* value cutoff (Table 7.1).

Very recently, Sun et al. [33] proposed a novel copula-based bivariate statistical analysis approach to analyze genetic effects on AMD progression using data from both eyes. They specifically analyzed chromosome 10 using AREDS participants with at least one eye at moderate AMD since study enrollment. Besides the *ARMS2-HTRA1* region, they reported a few other regions on chromosome 10 such as *LOC101928913* and *C10orf11* exhibiting potential association with AMD progression. Those regions have not been reported before in previous case–control or progression studies of AMD. Then, Sun and Ding [34] proposed a more flexible copula approach to account for the interval-censoring and performed a GWAS on analyzing

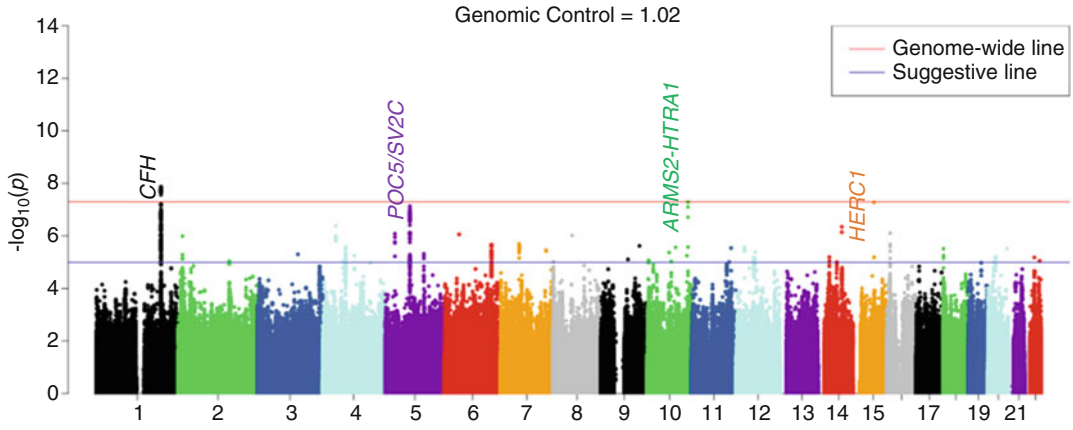

Fig. 7.1 Manhattan plots of GWAS results of AMD progression from Yan et al. [32]. The robust Cox PH model adjusted for baseline AMD severity score (continuous variable), age, smoking status (never, former, and current), and education level (≤high school and >high school). The first two principal components were included to account for population stratification

time-to-late-AMD using AREDS data. Besides confirming the *CFH* and *ARMS2-HTRA1* regions, they also identified the *ATF7IP2* region on chromosome 16 to be associated with AMD progression.

7.4 Prediction Models for AMD Development and Progression

It is known that there are both strong genetic components and important environmental influences on the development and progression of the AMD. Prediction models using demographic, environmental, and genetic factors have been established for AMD prevalence and incidence [35]. Recently, multiple research groups established different prediction models for AMD progression using combination of demographic, environmental, and genetic variables. For example, Seddon et al. [36, 37] established and validated a multivariable prediction model with six variants (in five genes) and other baseline nongenetic variables to predict the progression risk to advanced AMD. Later, the same group expanded their prediction model by adding three new genetic loci and evaluated the effects of those new variants on progression [24]. All these studies used one progression time per subject when developing their prediction models.

Recently, Ding et al. [30] established prediction models with different combinations of nongenetic and genetic factors based on AREDS data and evaluated the model performance using the independent AREDS2 data. Different from the previous approaches, their approach took advantage of all available data by using the progression times from both eyes. They also derived a genetic risk score (GRS) for AMD progression, based on the effects of 34 known AMD risk variants reported from Fritsche et al. [15], and instead of using a set of individual AMD risk variants, they used this composite GRS as a single predictor in the prediction models. They thoroughly evaluated the performance of their prediction models within the AREDS data (using cross-validations) and in an independent cohort from AREDS2 using appropriate measures such as the c-index and Brier score. They found that the prediction model with baseline AMD severity score, age, education level (<= high school or > high school), smoking status (never, former, or current), and the GRS produced satisfactory prediction performance (c-index = 0.89 in AREDS, and = 0.73 in AREDS2). Moreover, adding this GRS to the demographic information alone showed significant improvement in the prediction

performance (c-index increased from 0.62 to 0.75 in AREDS). This work demonstrates the utility and validity of the GRS for AMD prediction.

Fritsche et al. [15] had uploaded ~12 million genetic variants and 35,358 subjects to dbGaP (phs001039.v1.p1) and most of them are Caucasians (32,637). This is by far the largest publicly available AMD genotype dataset, which could be used for predicting AMD risk. Given the large number of sample size and genetic variants, appropriate prediction tools need to be selected. The artificial neural network (NN) method could be a good candidate, since it can learn complex relationship between large number of predictors and outcomes. Several recent developments using NN methods for predicting AMD risks or its progression profiles with large-scale genetics data have seen found in the literature. Furthermore, AMD severity is mainly diagnosed by color fundus images and recent studies have shown the success of machine learning methods in predicting AMD progression using image data [38–45]. Very recently, Yan et al. [46] jointly used large-scale genotypes and fundus images to dynamically predict AMD progression risks with a novel two-stage deep neural network (Fig. 7.2). The results showed that the color fundus photos coupled with genotypes could predict late AMD progression with an averaged area under the curve (AUC) value of 0.85 (95%CI: 0.83–0.86).

7.5 Beyond GWAS

Despite the success of GWAS of AMD, the analysis of other types of omics data beyond DNA has been limited possibly due to the lack of tissue accessibility. Several studies have shown that mitochondrial genetics [47–49], microRNAs [50, 51], and epigenetics [52–54] play roles in AMD pathobiology but they all have small sample size and findings require further investigation. A recent report [55] generated transcriptional profiles of postmortem retinas from 453 controls and AMD cases. The locally expression quantitative trait loci (*cis*-eQTL) analysis revealed 10,474 genetic regulated genes, which include 4541 retina specific eQTLs. They further conducted a transcriptome-wide association study (TWAS) and found three additional AMD-related genes, *RLBP1*, *HIC1,* and *PARP12*. This study indicates that the retina-specific gene expressions could help us understand the genes involved in AMD pathobiology.

7.6 New Statistical Methods Motivated by AMD Data and Research

The wealthy genotype data generated from AMD research, as well as the bilateral nature of the phenotype have motivated comprehensive statistical methodology development in the past few years, which has successfully produced or is producing novel and rigorous statistical methods and software packages for addressing different research objectives.

The newly developed and emerging methods include: (1) Novel copula-based methods and R package ("CopulaCenR") for modeling and testing the bivariate/multivariate data that are subject to right or interval censoring. This is motivated by studying the genetic effects on AMD progression where the outcome data are bivariate time-to-advanced-AMD [33]; (2) Gene-based association tests through functional linear model on (bivariate) time-to-event outcomes [56]; (3) New and robust predictive models for predicting AMD development or progression. In addition to prediction models using genetic risk scores (based on a small group of variants) with traditional logistic model or (robust) Cox PH model [30], new machine-learning-based approaches, such as the random (survival) forest, penalized Lasso regression, and deep neural network using GWAS data are being investigated [46, 57]; (4) Subgroup identification and inference methods for treatment efficacy with time-to-event outcomes. This is highly motivated by the AREDS and AREDS2 studies where the treatment (antioxidant and mineral supplement) showed positive trend in slowing down the AMD progression but did not reach statistical significance level in the entire population. Using various tree-based approaches

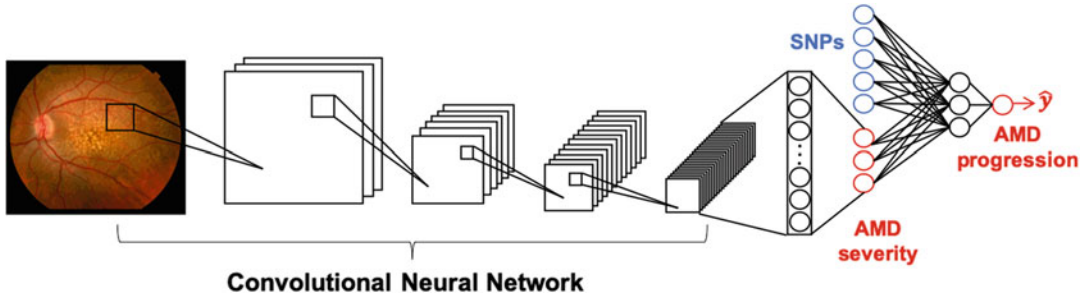

Fig. 7.2 The architecture of the two-stage deep neural network using both fundus image and genetic data for predicting AMD progression risk

and a novel simultaneous inference approach, subgroups (defined by SNPs) with enhanced treatment efficacy have been identified [58]; (5) Other new statistical methods focusing on estimating the association or dependence between two censored variables have been also proposed with motivation from and application on the AMD study [59]. The massive amount and unique features of AMD data become such important assets to statisticians for motivating and applying their novel analytical methods.

7.7 Discussion and Future Direction

Genetic studies of AMD have gained a huge success in the past two decades. Several dozens of AMD-susceptible loci and several pathways have been discovered through GWAS and sequencing studies with international efforts from many countries. However, because classic animal models are not available for AMD and retina tissues are not widely available, the functional roles of discovered loci in AMD biology are still largely unknown. Further collaborations among AMD researches are needed to characterize known AMD variants and to understand the underlying mechanism at transcriptomic or proteomic level. Handa et al. presented a nice perspective to use a system biology approach toward understanding AMD [60]. In addition to the biology research, GWAS of AMD has provided risk factors for disease prediction, which has been shown very accurate in above described studies. To achieve the ultimate goal for personalized medicine, integrative analysis of multilevel data including various omics, environmental, and clinical data with advanced statistical methods is likely to be performed down the road. For example, in the recent two years, several studies have used the AREDS fundus images to perform automated AMD grading by applying convolutional deep learning methods [42, 43, 61]. However, it is more crucial to predict AMD progression profiles over time. In addition to the available genotype data, the AREDS project also includes longitudinal fundus images over 12 years, which allow researchers to collectively use genotypes and fundus images to predict dynamic AMD progression profiles. Besides fundus images, it would be also desirable to have a coherent prediction using multiple types of images (e.g., optical coherence tomography and fundus autofluorescence images). Since late AMD is irreversible, a model that can accurately predict progression profiles over time could urge potential patients to start preventative care early and slow down the disease progression. In the next decade, the genetic studies of AMD will continue growing, likely integrated with many other types of data. With the advance of biological and analytic technology, we anticipate that more genetic variants will be discovered and the functional roles of known loci will be better

understood, leading new therapeutic targets and better diagnosis tools for AMD.

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8 Making Biological Sense of Genetic Studies of Age-Related Macular Degeneration

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Abstract

Age-related macular degeneration (AMD) is a major cause of blindness in older individuals worldwide. The disease is characterized by deposition of drusen between the retinal pigment epithelium (RPE) and Bruch's membrane, RPE atrophy and death of photoreceptors. AMD is a complex disease with multiple genetic and non-genetic risk factors. Genome-wide association studies (GWAS) have identified 52 variants at 34 genetic loci associated with AMD. A majority of the AMD-GWAS variants are present in non-coding region of the genome and could quantitatively impact distinct human traits [called quantitative trait loci (QTLs)] by affecting regulation of gene expression. The integration of different regulatory features, such as open-chromatin regions, histone marks, transcription factor binding sites, with AMD-GWAS can provide meaningful insights into variant's function. However, functional interpretation of variant–gene relationship in AMD is challenging because of inadequate understanding of cell-type specific and context-dependent information in disease-relevant tissues. Here we focus on the role of sequencing-based *omic* studies in assigning biological meaning to disease-associated variants and genes. We also discuss the methods and model systems that can be utilized to unravel molecular mechanisms of a complex disorder like AMD.

Keywords

AMD · GWAS · Exome sequencing · Genome sequencing · QTLs · eQTL · CREs · Aging · AMD-GWAS · Photoreceptors: RPE · Functional genomics

8.1 Introduction

Age-related macular degeneration (AMD) is a major cause of irreversible vision loss resulting from the death of light-sensing photoreceptors, primarily in the macular region of the retina. The manifestations of AMD have been classified based on clinical severity scales [1] and broadly include dysfunction of the retinal pigment epithelium (RPE) and medium-sized insoluble deposits

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(called drusen) (early stage), followed by accumulation of large drusen between the RPE and the Bruch's membrane (intermediate stage), eventually leading to photoreceptor death in advanced stage because of geographic atrophy and/or growth of choroidal capillaries into the neural retina (choroidal neovascularization) [2, 3] (see other chapters in this monograph).

AMD is a complex, late-onset disease with multiple risk factors impacting the eventual clinical outcome; these include aging, environmental factors, and genetic susceptibility (Fig. 8.1). Advanced age is arguably the most significant, non-genetic risk factor for AMD and other common multifactorial neurodegenerative traits [4–7]. Aging is associated with numerous cellular and organismic changes leading to progressive decline of physiological activities. Mitochondrial dysfunction, accumulation of somatic mutations caused by inefficient DNA repair mechanisms, and epigenome modifications are among the mechanisms contributing to aging-associated neurodegeneration [8–10]. AMD progression is also strongly impacted by environmental factors, such as smoking and nutritional status [11, 12], which can alter cellular homeostasis by affecting the redox state and/or epigenome [13, 14]. Influence of epigenomic changes on AMD, in response to advanced age and/or environment, is discussed in another chapter (DeAngelis et al. in this monograph).

Familial aggregation, high concordance among twins, and higher risk of manifesting the symptoms of AMD among first-degree relatives of patients compared to the general population suggested substantial genetic contributions to AMD pathogenesis [15–17]. Candidate gene association and familial linkage studies dominated early investigations in AMD [18]. Surprisingly, the first genome-wide association study (GWAS) using only 50 controls and 96 cases was successful in uncovering the strong association of a coding variant (Y402H) in complement factor H (CFH) with AMD [19]. This association was validated by targeted genotyping and candidate gene approaches [20–23]. Subsequent studies identified many non-coding variants at *CFH*, in other complement genes, and at the *ARMS2/HTRA1* locus [24–28]. A compelling and robust evidence for broader genetic basis of AMD was established by subsequent GWAS using large cohorts of cases and controls [29, 30]. Later, a collaborative GWAS meta-analysis resulted in the identification and validation of common variants at multiple genetic loci associated with AMD [31].

In GWAS, however, the allele frequency of common genetic variants across the genome is compared among large cohorts of affected individuals (cases) and matched controls to determine statistically significant association of variants with the trait [32]. Imputation of variants from 1000 Genome and other large databases [33, 34] for GWAS has greatly facilitated precision mapping and identification of statistically inferred variants at associated genetic loci. GWAS and sequencing-based approaches have permitted delineation of genetic architecture for many complex traits. In this respect, AMD genetics has been at the forefront in achieving rare and unprecedented success by identifying multiple disease-associated variants in independent studies. Here, we will discuss what we have learned from the largest AMD-GWAS conducted by an International Consortium and how to connect genetic variants to cellular function and AMD pathology.

8.2 Current Status of Genetic Susceptibility Loci Identified by AMD-GWAS and Related Studies

GWAS have provided a broader framework for elucidating the contribution of genetic variants to AMD. The most recent AMD-GWAS was performed by International AMD Genomics Consortium and used a customized chip that included genome-wide common variants and rare variants from the protein-coding regions to evaluate 16,144 patients with advanced AMD, and 17,832 controls [35]. This study identified 52 independent common and rare variants at 34 loci that exhibited an association with AMD (Table 8.1). Notably, rare variants in the *CFH*, *TIMP3*, *CFI*, and *SLC16A8* genes provided further evidence in support of their role in AMD

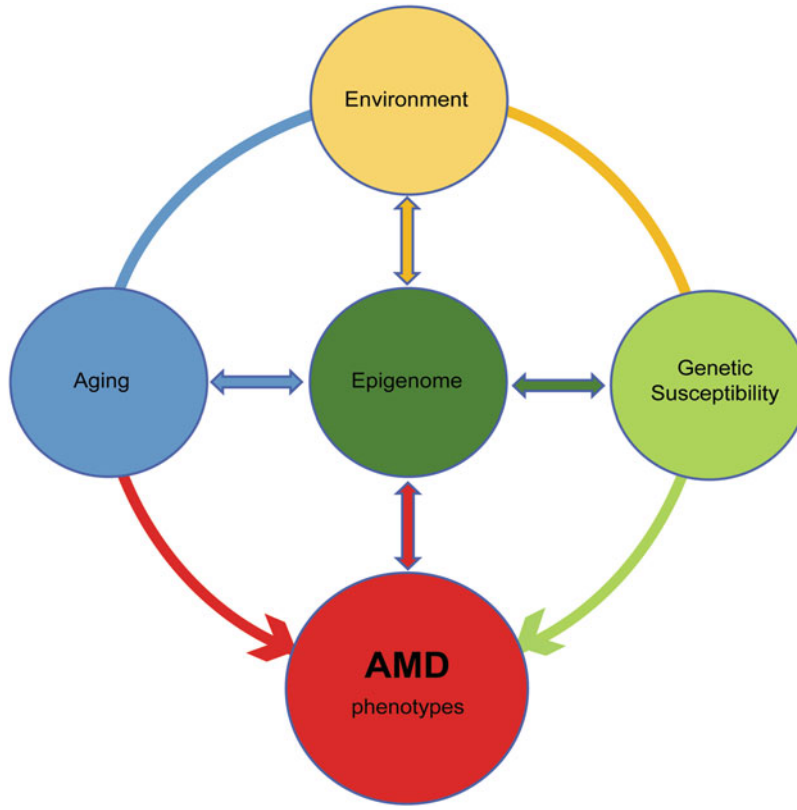

Fig. 8.1 Age-related macular degeneration (AMD), a complex multifactorial disorder. The major risk factors for AMD include genetic susceptibility and aging. Environmental factors such as smoking, and high-fat diet are also associated with AMD. Increasing evidences support changes in epigenome of the individuals affected with AMD

pathology. However, the study design was limited to genotyped variants on the chip and did not discover novel, rare protein-altering variants at most genetic loci. Concurrent genetic studies that were based on sequencing the variants directly have identified rare variants in genes including *CFH*, *C3*, *C9*, and *CFI* [36–40], validating their direct contribution to AMD pathogenesis.

Rare variants in the coding region can help in pointing to a target gene at an associated locus, facilitating their potential pharmacological significance. However, a large sample size is critical for rare variant studies to reach statistical significance. Targeted and whole exome sequencing are hybridization-based and relatively inexpensive methods for analyzing a large number of samples [41, 42]. However, inefficient capture and inability to examine large structural variants such as translocations and inversions represent some of the limitations associated with hybridization methods. Whole genome sequencing is expected to replace such capture-based technologies.

GWAS-identified AMD susceptibility variants have provided a wealth of clues for biological experimentation [35, 43]. Dysregulation of the complement pathway is now established in AMD pathology, in large part because of the identification of rare variants in or around several genes of the alternative complement pathway. Other AMD-associated pathways include lipid metabolism/cholesterol transport, angiogenesis, and extracellular matrix reorganization [28, 43]. However, further genetic and biological evidences are needed to unambiguously demonstrate their role in disease pathology.

8.3 Challenges: From Associated Genetic Loci to Causal Susceptibility Variants

Functional interpretation of GWAS findings has been difficult since the association of a locus with the disease does not clearly specify which variant or the target gene at that particular locus is causal.

Table 8.1 Summary of genes/loci identified in Caucasian population by AMD genetic studies

AMD locus	Lead GWAS SNP	GWAS *p*-value	Major/minor allele	Chromosome: Position	AMD target genes
ABCA1	rs2740488	1.2×10^{-8}	A/C	9: 107,661,742	
ACAD10	rs61941274	1.1×10^{-9}	G/A	12: 112,132,610	*SH2B3*
ADAMTS9-AS2	rs62247658	1.8×10^{-14}	T/C	3: 64,715,155	
APOE	rs429358	2.4×10^{-42}	T/C	19: 45,411,941	
ARHGAP21	rs12357257	4.4×10^{-8}	G/A	10: 24,999,593	
ARMS2/HTRA1	rs3750846	6.5×10^{-735}	T/C	10: 124,215,565	
B3GALTL	rs9564692	3.3×10^{-10}	C/T	13: 31,821,240	*B3GLCT*
CETP	rs5817082	3.6×10^{-19}	C/CA	16: 56,997,349	
CFH	rs10922109	9.6×10^{-618}	C/A	1: 196,704,632	
CFI	rs10033900	5.4×10^{-17}	C/T	4: 110,659,067	*CFI, PLA2G12A*
CNN2	rs67538026	2.6×10^{-8}	C/T	19: 1,031,438	
COL4A3	rs11884770	2.9×10^{-8}	C/T	2: 228,086,920	
COL8A1	rs140647181	1.4×10^{-11}	T/C	3: 99,180,668	
CTRB2/CTRB1	rs72802342	5.0×10^{-12}	C/A	16: 75,234,872	
C2/CFB/SKIV2L	rs116503776	1.2×10^{-103}	G/A	6: 31,930,462	
C3	rs2230199	3.8×10^{-69}	C/G	19: 6,718,387	
C9	rs62358361	1.3×10^{-14}	G/T	5: 39,327,888	
C20orf85	rs201459901	3.1×10^{-16}	T/TA	20: 56,653,724	
KMT2E/SRPK2	rs1142	1.4×10^{-9}	C/T	7: 104,756,326	
LIPC	rs2043085	4.3×10^{-15}	T/C	15: 58,680,954	
MIR6130/RORB	rs10781182	2.6×10^{-9}	G/T	9: 76,617,720	
MMP9	rs142450006	2.4×10^{-10}	TTTTC/T	20: 44,614,991	
NPLOC4/TSPAN10	rs6565597	1.5×10^{-11}	C/T	17: 79,526,821	
PILRB/PILRA	rs7803454	4.8×10^{-9}	C/T	7: 99,991,548	*PILRB/PILRA*, *ZCWPW1*, *TSC22D4, MEPCE*
PRLR/SPEF2	rs114092250	2.1×10^{-8}	G/A	5: 35,494,448	
RAD51B	rs61985136	1.6×10^{-10}	T/C	14: 68,769,199	
RDH5/CD63	rs3138141	4.3×10^{-9}	C/A	12: 56,115,778	*BLOC1S1*
SLC16A8	rs8135665	5.5×10^{-11}	C/T	22: 38,476,276	
SYN3/TIMP3	rs5754227	1.1×10^{-24}	T/C	22: 33,105,817	
TGFBR1	rs1626340	3.8×10^{-10}	G/A	9: 101,923,372	
TMEM97/VTN	rs11080055	1.0×10^{-8}	C/A	17: 26,649,724	*POLDIP2*, *SLC13A2*, *TMEM199*
TNFRSF10A	rs79037040	4.5×10^{-11}	T/G	8: 23,082,971	
TRPM3	rs71507014	3.0×10^{-8}	GC/G	9: 73,438,605	
VEGFA	rs943080	1.1×10^{-14}	T/C	6: 43,826,627	

Identification of causal variant(s) is challenging as most variants at a locus are likely in linkage disequilibrium with the lead variant [44]. In addition, more than one causal variant may be disease-causing within a locus. Furthermore, a majority of the GWAS signals reside in the non-coding or intergenic region of the genome, and many associated variants may be far from an annotated gene [45].

8.4 Regulation of Genetic Information by Susceptibility Variants

The advent of sequencing-based "*omic*" analyses has made it possible to study multiple features of the genome (Fig. 8.2), providing an opportunity to address some of the challenges pertaining to complex diseases. Non-coding, disease-associated variants can influence quantitative differences in human traits [called quantitative trait loci (QTLs)] at many different levels. Reduced cost of sequencing has paved ways to exploit a variety of methods for constructing distinct types of QTL maps. QTLs can exert their regulatory effect by affecting the expression of a gene (eQTL) [46], splicing (sQTL) [47], chromatin accessibility (dsQTL) [48], DNA methylation (mQTL) [49], transcription factor binding (bQTL) [50], and/or protein translation (pQTL) [51] (Fig. 8.3). Genetic variants affecting multiple molecular traits are called molecular QTLs (molQTL). The regulatory variants can also act as modifiers of coding variant penetrance and contribute to shaping the genetic architecture of human traits. For example, the missense SNP rs199643834 (p.Lys508Arg) identified in the tumor suppressor gene *FLCN* can cause autosomal-dominant Birt–Hogg–Dubé Syndrome [52], and its penetrance is modified by an eQTL rs1708629 in the 5′-untranslated region of *FLCN* [53]. In this scenario, the eQTL in *cis* with the coding variant resulted in increased penetrance of the coding variant and decreased expression of the functional variant [53]. We will briefly discuss different types of QTLs here and elaborate on eQTLs in Sect. 8.6.

Splicing is generally tissue and developmental stage specific and a highly regulated process, which results in protein diversity. Extensive alternate splicing has been reported in the retina [54], and especially in the photoreceptors [55]. Identification of splice site mutations in retinal degenerative diseases further highlights the importance of splicing in retina [56, 57]. sQTLs can be analyzed from the RNA seq data by counting the percentage of exons over total gene read counts and treating SNP genotype as an independent variable [58]. sQTLs are present throughout the genome, with enrichment in the coding regions [59]; however, their relationship with splicing events has not been reported in the retina.

Gene expression and regulation require chromatin modifications and binding of specific transcription factors (TFs) in promoter and enhancer regions. TF binding is determined in part by open chromatin regions in the genome. Widespread decrease in chromatin accessibility has been reported in AMD [60]. Thus, it is important to understand the role of variants in modifying chromatin accessibility and TF binding (dsQTLs). DNase I footprinting is performed to identify regions that are bound to TFs [61]. Once the DNase hypersensitive regions are characterized

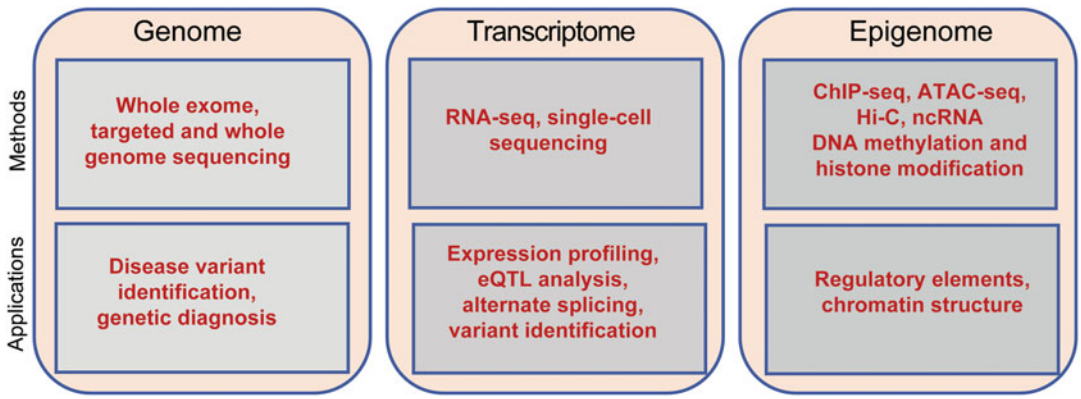

Fig. 8.2 Different approaches for unraveling AMD complexity. The sequence-based omics tools to address the disease complexity in the genome, transcriptome, and epigenome of individuals with AMD

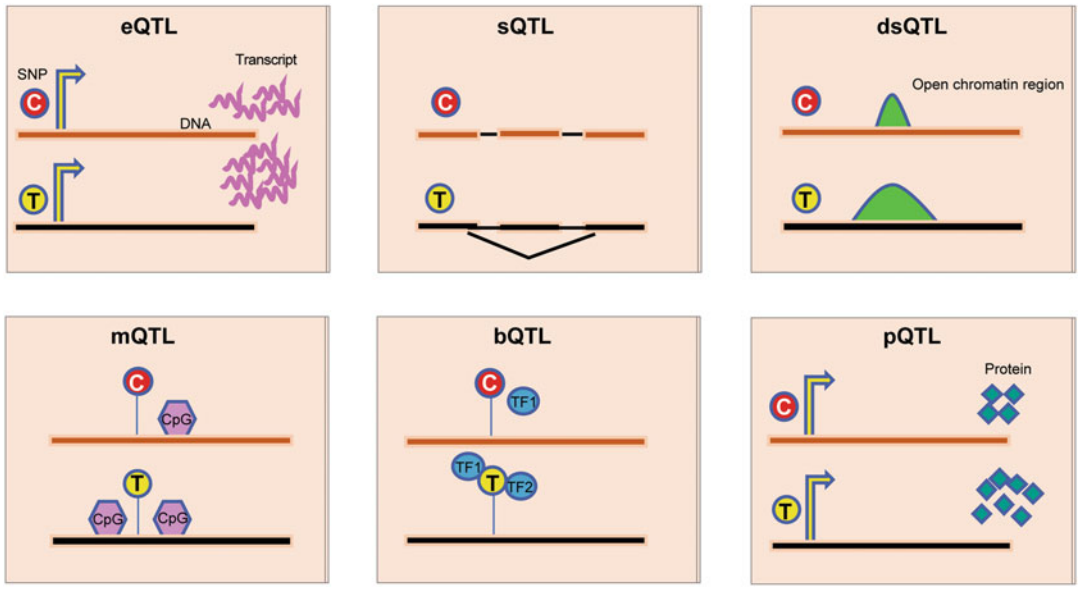

Fig. 8.3 The regulatory roles of quantitative trait loci (QTLs). The non-coding variant associated with a phenotype can regulate number of cellular processes such as gene expression (eQTL), splicing (sQTL), open chromatin regions (dsQTL), methylation (mQTL), transcription factor binding (bQTL), and protein synthesis (pQTL)

in the genome, one can test the association between these regions and SNP genotypes [48].

The epigenetic modifications, such as DNA methylation, at specific loci can be influenced by genetic variants. These sites, referred to as methylation QTLs (mQTLs), are identified by integrating genotyping data with methylation profiles obtained from chip or bisulfite sequencing. The variants can also exert their effect by preferred binding of the alleles of different TFs [62]. ChIP-seq in tissue of interest, combined with genotypes, can provide such variants, which when associated more frequently with a specific TF are called binding QTLs (bQTLs).

Given that proteins are effective targets for drug discovery, it is important to understand key features associated with efficient protein translation. Genetic variants that directly influence protein levels in a quantitative manner are called pQTLs [63]. Additionally, differences in the length of the 3'UTRs can have functional consequences, such as affecting the binding of RNA binding proteins, miRNAs, localization, and translation.

8.5 Functional Characterization of the Variants

The number of trait–variant relationships in GWAS has increased over the years. However, functional characterization of these associations is still limited. A majority of the identified GWAS variants are present in non-coding regions of the genome that are enriched non-coding RNAs and transcriptional regulatory regions called "cis-regulatory elements" (CREs) such as promoter, enhancer, and silencer elements [64, 65]. Large-scale public datasets generated by ENCODE [66] and the FANTOM consortium [67], and the NIH Roadmap epigenome project [68] now provide a huge resource for characterization of putative CREs. Integration of genome-wide profiles of open chromatin regions (using DNase-seq or ATAC-seq), H3K27Ac, H3K4me1 (active/poised enhancers or promoters), H3K36me3 (actively transcribed regions), H3K9me3 (heterochromatin) marks, and specific TF binding sites can provide insights into the prioritization of probable causal variants predicted in CREs [69].

Interaction among regulatory regions is another critical component in modulating gene expression patterns. GWAS-risk variants can be far away from the genes they control (from a few to millions of nucleotides), making it difficult to study their contribution to gene regulation. The regulatory landscapes in the genome are defined by architectural chromatin units, called topological associated domains (TADs), which are enriched in distal QTLs [70, 71]. With the advent of chromosome conformation capture techniques (e.g., 3C, 4C, and HiC), one can map the long-range interactions in the genome [72] to gain mechanistic insights into candidate variants.

Single-cell/nucleus sequencing can also provide better insights in diverse cellular architecture and function [73, 74], especially in a neuronal tissue like the retina with many different kinds of cells. Given that most of the variants function in cell-type specific manner [75], investigating the occurrence and relationship of variants to a trait in a particular cell type will help in better understanding of their role in normal and disease condition(s).

8.6 Regulation of Gene Expression by Genomic Variants

Genotype-Tissue Expression (GTEx) project was initiated to establish a large resource for studying the relationship between genetic variations and gene expression (eQTLs) across multiple tissues and cell types [76]. Established in 2010, the most recent data release (v8) includes 17,382 samples from 948 donors across 53 tissues types. The Encyclopedia of DNA Elements (ENCODE) is actively building a comprehensive resource for functional elements in the genome, employing a variety of assays and methods [77]. The overlap between eQTLs and identified GWAS loci has been reported for a number of different diseases [78, 79], and their identification and functional characterization in AMD could reveal potential mechanisms. However, retina and other ocular tissues have little or no representation in GTEx and ENCODE, which created an unmet need in vision research and partly delayed the functional understanding of GWAS findings despite its enormous success in AMD genetic studies.

As described earlier, eQTLs are the variants that can affect gene expression by altering transcription or mRNA stability. The variants can affect the expression of genes nearby (called *cis*-eQTLs) or far away (called *trans*-eQTLs) (Fig. 8.4). Earlier in 2019, we reported a comprehensive catalogue of *cis*-eQTLs in the retina from over 400 human donors. *Cis*-eQTL mapping was performed on 17,389 genes and ~9 million genotyped and imputed variants that identified 14,856 independent *cis*-eQTLs, consisting of 14,565 genetic variants (eVariants), which control expression of 10,474 genes (eGenes) in the retina at false-discovery rate (FDR) of 5% [80]. Several additional features of the *cis*-eQTLs were noteworthy in this study. A large proportion of the expressed genes (~70%) were found to be under genetic control, and a majority of the genes had a single eQTL. The strength of association was contingent upon the eVariant's distance from the transcription start site (TSS) of its corresponding eGene. These features were similar to what had been observed across multiple other tissues [76]. A comparison of retinal *cis*-eQTL with other tissues eQTL from GTEx project revealed that almost 70% of the *cis*-eQTLs identified in the retina were also regulating gene expression in one or more tissues. As predicted, *cis*-eQTLs in GTEx tissues with smaller sample size [76] exhibited a larger proportion shared with the retinal eQTLs and consistent with patterns of sharing reported among GTEx tissues. However, when compared at the gene level, most of the genes were regulated across tissues.

An active area of research has been to find methods to leverage the information from eQTLs to gain insights into reported GWAS loci (colocalization) as well as identification of novel loci using transcriptome-wide association studies (TWAS). Colocalization methods primarily examine the variants that are significant at

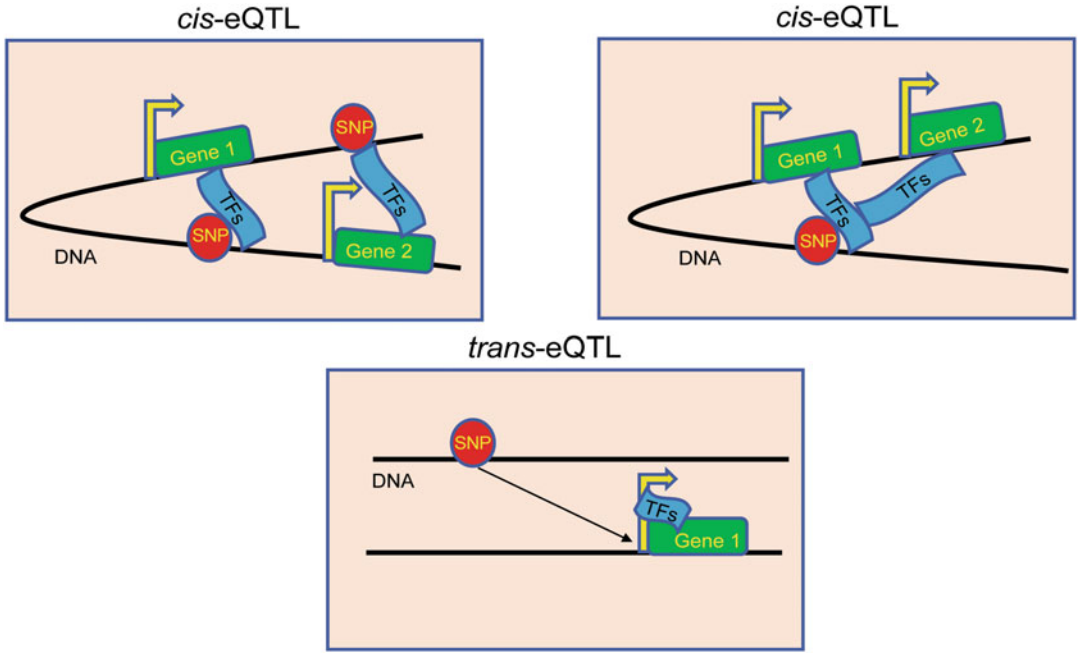

Fig. 8.4 Effect of variant on gene expression (eQTL). The variants that affect the expression of a nearby gene are *cis*-eQTL. The variants that affect the expression of genes from large distance (>1 Mb) or from a different chromosome are *trans*-eQTL. One variant can affect expression of one or more than one target gene

known GWAS loci and also enriched for eQTLs. Statistical methods, including PrediXscan [81], Sherlock [82], eCAVIAR [83], and TWAS [84], have been developed for identifying candidate causal variants and target gene prioritization. These methods are much likely to succeed when expression data are derived from a trait-related tissue [85], further emphasizing the critical role of profiling diverse cells and tissues in humans.

Our analyses indicated the regulation of several reported AMD-GWAS loci by eQTLs and identified target genes at six of these loci [80]. Retinal TWAS analysis also uncovered three candidate genes and suggested several others [80]. Similar analyses with additional tissue samples and using other AMD-relevant tissues/cell types (such as macula and RPE) are expected to provide validation and valuable functional insights in genes and pathways contributing to AMD pathology.

8.7 From Association to Function

We have overcome a major limitation in genomic data generation with the advent of next-generation sequencing technologies, and with rapid and accurate profiling of genomic features that can be integrated to identify functional elements of the non-coding genome. However, methods for assaying and functionally characterizing these regulatory variants on a large scale still represent a major bottleneck. Reporter gene expression assays are becoming a tool of choice for testing the function of non-coding variants (Fig. 8.5). Massively parallel reporter assays (MPRAs) are being developed to validate enhancer/regulatory variant functions; in such assays, candidate regulatory elements are introduced in a plasmid with an easily quantifiable reporter (often luciferase) [86]. MPRAs can be scaled up to test thousands of variants in a single experiment [87]. However, a limitation on

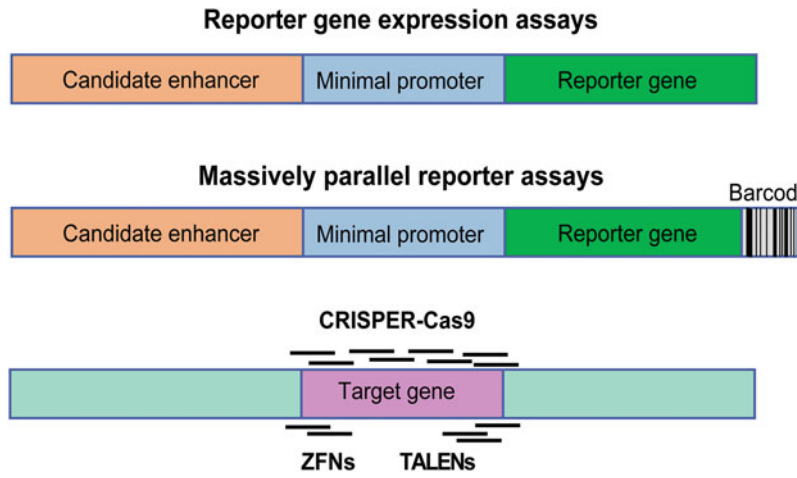

Fig. 8.5 Gene expression assays to validate the regulatory effect of non-coding variants. Gene expression assays like reporter gene, massively parallel reporter assay, CRISPER-Cas 9, ZFNs, and TALENs have been developed to test the enhancer/repressor effect of the non-coding variants

size of the oligonucleotide, technical variations within experiments, and the lack of a chromosomal context represent some of the challenges associated with these methods. CREs can work in *cis*- or in *trans*, but these reporter assays fail to accommodate for the *trans* regulation. Additionally, most of these experiments are performed in cell lines, providing little optimal biological context. More recently, the genome editing tools such as zinc finger nucleases (ZFNs), transcription activators such as effector nucleases (TALENs), and CRISPR-Cas9 system have begun to be utilized to directly test the effect of regulatory variants (Fig. 8.5) [88].

8.8 Existing AMD Disease Models for Functional Characterizations

Mouse models are widely used for modeling human retinal diseases, including AMD [89, 90]. These models are cost-effective and easy to manipulate. Several pathological features of AMD such as development of drusen, thickening of Bruch's membrane, complement activation, and accumulation of macrophages can be recapitulated in mouse models of AMD [91, 92]. The CFH-knockout mouse shows retinal abnormalities accompanied by decreased visual acuity and reduced ERG responses with age [93]. Mouse models of other genes including complement factor C3, C3a, and C5a receptors, chemokines like CCL2, CCR2, and CX3CL1 are reported to show AMD-like phenotypes [94, 95]. Mice on high-fat diet when exposed to blue light exhibit thickening of the Bruch's membrane [96]. Similarly, mice exposed to cigarette smoke also demonstrate thickening of the Bruch's membrane and basal laminar deposits [97]. Thus, mouse models of AMD have provided some meaningful insights in understanding the contribution of genetic and environmental factors in AMD progression. However, the lack of fovea and surrounding macula and limited biological age represent major limitations of mouse studies for AMD modeling.

Patient-derived induced pluripotent stem cells (iPSCs) have permitted disease modeling in a dish, with new tools for gene manipulation and high throughput screening methods. Self-organizing neural retina-like structures with proper polarity, lamination, and distinct cell types can be generated from iPSCs in three-dimensional (3-D) organoid cultures [98, 99]. Retinal organoids from patient iPSCs are closer to "in-vivo" system and provide a better biological context. Multiple groups are investigating the potential of stem cell-derived RPE cells for therapeutic interventions in AMD (see Chapter by Banin and colleagues in this issue).

8.9 Future Directions

We are witnessing a rapid change in clinical research and patient management, accelerated by advances in genomics, computational medicine, and stem cell-based technologies. Diversity of human genetic variations greatly impacts individual-specific healthy and disease phenotypes, including the response to treatment. Identification and incorporation of genetic findings that can have predictive outcomes in healthcare have been a major goal of precision medicine. Along these lines, how such an extensive array of regulatory genetic variants affect gene or protein expression in a specific tissue or cell remains a mystery. Integration of genotyping and whole genome datasets to distinct QTLs and functional validation of disease-relevant genetic variations would significantly augment the current clinical paradigms. AMD is an ideal complex disease to lead such efforts.

A multifactorial disease like AMD is responsive to additional factors, such as advanced age and environmental cues. Involvement of immune response genes in AMD further strengthens this view [100, 101]. Identification of the cellular context in which disease variants manifest is an essential component in annotating their functional relevance. It would be imperative to identify how distinct environmental factors, such as smoking, and nutritional components impact specific changes in gene/protein expression and in what cellular context. Indeed, elucidation of epigenomic changes in aging and in relevant environmental context would be highly desirable for comprehensive understanding of AMD and development of appropriate treatment modalities. Pioneering advances in computational tools have also made it possible to integrate large patient databases with clinical phenotypes to genome-wide genetic and epigenetic information. We are living in exciting times for biomedical research and expect that basic research advances in genetics of AMD will provide meaningful translations into clinical applications, including personalized management of this devastating blinding disease.

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Age-Related Macular Degeneration: From Epigenetics to Therapeutic Implications

9

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Abstract

Aberrant regulation of epigenetic mechanisms, including the two most common types; DNA methylation and histone modification have been implicated in common chronic progressive conditions, including Alzheimer disease, cardiovascular disease, and age-related macular degeneration (AMD). All these conditions are complex, meaning that environmental factors, genetic factors, and their interactions play a role in disease pathophysiology. Although genome wide association studies (GWAS), and studies on twins demonstrate the genetic/hereditary component to these complex diseases, including AMD, this contribution is much less than 100%. Moreover, the contribution of the hereditary component decreases in the advanced, later onset forms of these chronic diseases including AMD. This underscores the need to elucidate how the genetic and environmental factors function to exert their influence on disease pathophysiology. By teasing out epigenetic mechanisms and how they exert their influence on AMD, therapeutic targets can be tailored to prevent and/or slow down disease progression. Epigenetic studies that incorporate well-characterized patient tissue samples (including affected tissues and peripheral blood), similar to those relevant to gene expression studies, along with genetic and epidemiological information, can be the first step in developing appropriate functional assays to validate findings and identify potential therapies.

Keywords

Age-related macular degeneration · Epigenetics · Methylation · Histone modification · Chromatin · Induced pluripotent stem cells · Functional assays · Therapies · Genetics

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9.1 Introduction

Since the publication of the genome wide association study (GWAS) on 40,633 individuals which

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included, both end stage and intermediate subtypes, demonstrated that 52 variants located in 34 loci, were associated with age-related macular degeneration (AMD) (for review please see DeAngelis et al. (2017) as well as the excellent reviews published as part of the special issue) much work has gone into understanding the function of these loci in an effort to develop appropriate therapeutics to treat disease with limited success [1, 2]. At present, most of the therapies (Bayer/Regeneron's Eylea [aflibercept], Roche/Genentech's Lucentis [ranibizumab], and Roche's anti-neoplastic agent Avastin [bevacizumab]), are directed toward treatment of the end stage neovascular AMD subtype, target established abnormal blood vessel growth through antibody-based inhibition of vascular endothelial growth factor (VEGF), and demonstrate a range of efficacy. For a small subset of patients, anti-VEGF treatment results in stable to improved visual acuity without the need for ongoing treatment. However, the majority of patients require indefinite treatment, do not regain vision, or demonstrate progression of disease despite therapies. Currently, there are no approved drug therapies for the early/intermediate phenotype or geographic atrophic subtype. AMD therapies that target genes statistically associated with AMD risk, in phase 3 clinical trials, include the C5 inhibitor, avacincaptad pegol (Zimura, IvericBio, New York, New York, USA), which is directed toward reducing lesion size in geographic atrophy and the C3 inhibitor, Pegcetacoplan (APL-2, Apellis Pharmaceuticals, Waltham, Massachusetts, USA) which demonstrated slowed lesion growth for geographic atrophy. To the best of our knowledge no gene-based therapies have made it to Phase III for the early or intermediated subtypes of AMD (https://clinicaltrials.gov; also please see Ammar MJ et al. (2020), Current opinion in Ophthalmology and reviewed in this special issue) [3]. While additional genes (that have been discovered or yet to be discovered) may only be minor players in terms of their contribution to the total genetic variance of AMD, effect size does not always correlate with the importance to pathogenesis and hence treatment of AMD. In other words, it is important to consider that the proportion of cases attributable to certain genetic variants does not necessarily reflect the role of these genes in pathophysiology of disease and therefore it is important to continue to search for other mechanisms that may influence the expression of these genes in order to develop targeted AMD therapeutics [4–7]. Studies of the concordance or discordance of a disease between identical and fraternal twins, largely the gold standard for calculating heritability of a complex disease, demonstrate the heritability for AMD is greater in concordance for monozygotic (37%) than dizygotic (19%) twins [8–10]. Another study demonstrated that there is 18% concordance for the late stage forms AMD in monozygotic twins versus 6% for dizygotic twins [11]. These two studies importantly underscore the genetic contribution to all AMD phenotypes. However, given that these percentages for AMD concordance in identical twins are significantly less than 100% and extremely discordant siblings (one with neovascular AMD and one without) exist that have the same epidemiological and genetic risk factors; emphasizes the need to go beyond the nucleotide sequence level to identify other factors that influence AMD phenotype and pathophysiology [12–14].

Expression is the first step in ascribing function of a gene. Control of gene expression is fundamental to the viability of a cell and hence an organ. Therefore, gene expression is integral to maintain homeostasis in response to environmental changes. Mechanisms that underlie control of gene expression change by modifying chromatin without changing the nucleotide sequence, this is known as Epigenetics. Epigenetics refers to functionally relevant changes in gene expression caused by mechanisms other than changes in the DNA nucleotide sequence. Histone proteins are the primary protein components of chromatin and are responsible for condensing the DNA into nucleosomes that form a "beads-on-a-string conformation." Chemical modifications of chromatin can influence which genes can be expressed and in which particular tissues. Epigenetics enables an organism or cell to respond to changing influences in the environment during

development and throughout one's lifetime. These epigenetic changes to the genome can be inherited if these changes occur in cells giving rise to gametes. In contrast to epigenetics, the definition of the genetic code, which is the nucleotide sequence instructing a cell on how to build a protein, this nucleotide sequence is the same in all tissues and is set for life. These biochemical changes of the nucleotide sequence can be due to methylation and/or histone modifications (reviewed in [14–17]). This in turn could induce or repress gene transcription. All cytosines (CpGs) in the genome are subject to methylation. Methylation of cytosines is a reversible modification of DNA, so there is the potential to reverse disease causality. Generally, in DNA methylation, methyl marks are added to CpG sites to convert cytosine to 5-methylcyotosine causing the repression of gene transcription. Thus, hypomethylation of the gene promoter generally induces transcription and hypermethylation of the gene promoter generally suppresses gene expression. The majority of genes in the mammalian genome are methylated. Histone modifications also serve as markers recognized by specific transcription factors involved in activation or repression of mammalian gene expression. Acetylation marks are associated with active promoters and are among the most well-studied histone modifications. Histone deacetylases (HDACs) remove (erase) acetyl groups from histone lysine residues. Histone phosphorylation is also associated with active transcription. This type of modification alters the activity of the DNA that is wrapped around them. In this review we will focus on DNA methylation, and histone deacetylase modifications and the combination thereof, as potential therapeutic interventions for age-related macular degeneration.

AMD phenotype is heterogeneous yet the same set of nucleotide sequences can be associated with different AMD phenotypes, especially variants in the two loci (CFH [1q] and ARMS2/HTRA1 [10q]) that explain 50% of the genetic component of AMD [1]. Although significant advances have been made in the area of AMD diagnostics, treatment of AMD is not at a level whereby we can slow disease progression or restore vision in a significant manner, particularly for patients with atrophic AMD. Ideally studying the affected tissues of a disease beyond the nucleotide sequence level, in the case of AMD; neural retina, retinal pigment epithelium (RPE), and vitreous, is inherently more translational for disease pathophysiology given the limitations of animal models [18, 19]. As part of a systems biology based approach, data from donor tissue can then be used to compare back to serums, plasma, primary cell lines, and induced pluripotent stem cells (iPSCs) at the gene expression and epigenetic level from patients with and without AMD [20–22]. This would enable the appropriate development of functional assays that can be used in the identification of disease mechanisms and therapeutics [18]. Similar to reports of postmortem time affecting gene expression in retinal tissues, reports also demonstrate that longer postmortem intervals can skew results of methylation studies and increase methylation variation, particularly in neural tissues [23–26]. Moreover, utilizing bisulfite sequencing in brain tissue demonstrated that longer postmortem times correlated with variance of methylation [27]. Longer postmortem intervals were also found to give spurious results for 5mC (DNA methylation) and 5hmC (DNA hydroxymethylation) in adult rat brains compared to neonate rat brains that were not attributable to disease mechanism [26]. However, postmortem interval time, as a confounder, has yet to be assessed in epigenetic studies of human RPE and neural retina.

9.2 DNA Methylation as a Biomarker

Hypermethylation which causes the silencing of genes is recognized as a crucial factor in cancer onset and progression [28, 29]. Highly sensitive assays have been developed to assess gene-promoter methylation in tissues and biological fluids for diagnosis and progression of systemic diseases [30–35]. The detection of methylated genes in sputum could lead to the development of a screening test to non-invasively identify early AMD in high-risk individuals. Given that

methylation is closely tied to gene expression, and gene expression differs from cell to cell and hence tissue to tissue, methylation would be expected to differ as well, this will need to be further explored for AMD. A role for methylation in the developing and aging mammalian retina has been well established [16, 36]. CpG sites are subject to methylation and there are approximately 23 million sites found throughout the human genome. Bisulfite sequencing either at the whole genome level or exome level (which currently offers greater depth of coverage than whole genome bisulfite sequencing) are still considered the gold standard for determining single base pair methylation status. Unfortunately, at this time whole genome methylation sequencing is not yet ready for high throughput. Utilizing a variety of methodological approaches on peripheral blood and donor eye tissue on patients with and without AMD, studies have demonstrated the contribution of methylation to AMD pathophysiology. Similarly, epidemiological risk factors associated with AMD (reviewed in the current special issue), including smoking, diet, hyper lipidemia, and obesity have also been found to be associated with changes in DNA methylation [37–45]. Table 9.1 summarizes all methylation studies, that included human samples with AMD reported to date [46, 50, 51, 53]. Most of these studies have employed a targeted approach however, there are two studies that used a chip-based approach to interrogate the whole methylome. One study conducted a bioinformatics analysis on already generated gene expression datasets which contained methylation profiles for 118 samples from extramacular neural retina (Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/ geo) [50]. Sixty-three samples had preclinical AMD and/or AMD. Using DNA immunoprecipitation (MeDIP)-qPCR on peripheral blood from AMD patients from the top hits of the bioinformatic analysis, revealed that the DNA methylation levels of HSP90AA1, HSPE1, HSP90B1, CDKN1C, EZR, IGF2, SLC2A1 were hypomethylated in AMD patients (neural retina) compared to controls, whereas NOP56 and PI3KR were hypermethylated significantly in AMD peripheral blood samples compared to controls. It was unclear how the diagnosis of AMD stage was made, number of samples used, and age of samples in the validation experiment. Utilizing, a targeted pyrosequencing-based methylation analysis of three CpG islands in LINE1 (a surrogate marker of global methylation) DNA methyltransferase (DNMT1, DNMT3B) activity was found to be higher in the peripheral blood of ten neovascular AMD patients compared to 40 controls [46]. Using short-end injection capillary zone electrophoresis, Pinna et al. (2016) demonstrated that whole blood methylation was likely not a factor in AMD mechanism after analyzing data from 27 neovascular patients, 39 early AMD patients, and 132 controls [53]. Using whole blood of identical twins discordant for AMD phenotype from the US Twin Registry, Hutchison et al. (2014) found large genomic regions containing known GWAS AMD-associated variants are differentially methylated between the different types of AMD phenotype [55]. Another study by Hunter et al. (2012) using the Illumina 27 K platform to assess methylation in an AMD case-control study of retina pigment epithelium/choroid samples showed no differences in the CpG sites upstream from CFH or CFB [48]. In contrast to the results reported by Hutchison, Hunter et al. did not report methylation changes upstream of either CFH or CFB (two well established AMD genetic loci). A study on three pairs of twins (one monozygotic and two dizygotic) with discordant AMD phenotypes from the Australia Twin registry, using an MeDIP chip (a DNA human promoter array), found significant methylation in the IL17RC promoter [52]. The differential methylation of the IL17RC promoter in AMD correlated inversely with gene expression of patients with AMD compared with their unaffected twins in peripheral blood, which corresponded to increased expression of *IL17RC* in the retina and RPA/choroid from formalin-fixed, paraffin-embedded ocular sections of donor eyes. Oliver et al. (2015), performed genome-wide wide DNA methylation profiling using the Illumina 450 k BeadChip array on neural retina (nine controls vs. nine AMD patients) and separately on peripheral blood from 100 case-control trios

Table 9.1 Studies investigating epigenetics in AMD-affected individuals

Method	Tissue[a]	AMD type	Post-mortem time (hours)	Phenotyping method (grading scale)	No. of biological replicates from macular region (N/E/I/L)[b]	% female	Age (years)	Findings	References
ATAC-Seq	Neural retina, RPE	Dry	<14	Medical records/ dissecting microscope (grading scale not reported)	Neural retina: 11/5/not reported/9 RPE: 3/2/not applicable/6	70	Neural retina: 79–94 RPE: 84–94 please spell out NR to not reported	Alteration of chromatin accessibility in AMD samples	[32]
Targeted bisulfite pyrosequencing	Peripheral blood	Wet	Not specified	Retinal exam, OCT, fluorescein angiography (grading scale not reported)	10/40 of unreported stage	50	68.9	Increased DNA Methyltransferase activity in AMD samples	[46]
Illumina 450 K, bisulfite pyrosequencing	Peripheral blood, retina	Dry/wet	Blood: Not specified Neural retina: Not reported	Clinical exam, histology (AREDS)	Blood: 99 normal/100 GA/99 NV Retina: 6–10 normal/9–13 AMD	63	Blood: 79.3 Neural retina: 83.6–88.3	Differential methylation in *ARMS2* locus	[47]
Targeted bisulfite pyrosequencing	Neural retina, RPE	Not reported	5.2	Not reported (grading scale not reported)	10 normal/11 AMD	Not reported	Not reported	*GSTM1* hypermethylation in AMD samples	[48]
Targeted bisulfite pyrosequencing	Peripheral blood, neural retina, RPE	Dry/wet	Blood: Not specified Neural retina/ RPE: Not reported	Blood: OCT, fundus photography Neural retina/ RPE: Macula photography (AREDS)	Blood: 120 normal/100 AMD Neural retina: 6 normal/9 AMD RPE: 3 normal/3 AMD	Blood: 46 (normal)/61 (AMD) Neural retina: 83 (normal)/33 (AMD) RPE: 0	Blood: 71.0–72.1 Neural retina: 87.8–91.7 RPE: 85.3–86.3	*IL17RC* hypomethylation is not an AMD biomarker	[49]
Bioinformatic analysis on gene expression	Initial data derived from peripheral	Dry/wet	Retina tissue, not specified	AREDS scale for neural retina; not specified for	Peripheral retina: 118 (55 normal and 63 donors	Not reported	Not reported	Different genes reported to be hypomethylated and	[50]

(continued)

Table 9.1 (continued)

Method	Tissue[a]	AMD type	Post-mortem time (hours)	Phenotyping method (grading scale)	No. of biological replicates from macular region (N/E/I/L)[b]	% female	Age (years)	Findings	References
omnibus (GEO; http://www.ncbi.nlm.nih.gov/ GEO	retina; validation on peripheral retina		blood not specified	peripheral blood samples	with preclinical AMD or AMD; peripheral blood collected from affiliated eye Hospital of Nanchang University for AMD cases and normal; phenotyping not specified			hypermethylated in peripheral blood of patients compared to hyper methylation compared to controls	
llumina450k BeadChip array and pyrosequencing	RPE cells from eyes of human donors (Bruch's membrane, whole RPE for validation	Early dry, intermediate/ geographic atrophy	DNA was extracted between 24 and 36 h post-mortem for RPE cells; RPE for validation at 6 h	Age-related eye disease study (AREDS) scale and Minnesota grading system	450 K: 25 AMD donors and 19 controls; Discovery cohort: 44 RPE cells (25 AMD and 19 normal) validation: Combined cohort ($n = 55$) included 26 AREDS2 (81.25%) and 6 AREDS3 (18.75%).	39	>50 years	Genome wide methylation in the RPE is not a contributing factor to AMD contributing factor to AMD; reduced methylated loci found at cg18934822 *SKI* in AMD patients and hyper methylation of cg22508626 *GTF2H4* in females with AMD	[51]
MeDIP -chip	Whole blood and paraffin embedded	Discordant for phenotyped MZ:CNV;	Not specified	AREDS	Three pairs of twins (1 monozygotic and	MZ = $n = 1$ DZ, $n = 1$; case control; AMD 45% controls;	Twins: 78, 79 and 88; case-control from Australia: AMD, 85, controls,	Hypomethylated IL17RC promoter in late AMD versus controls for	[52]

	(FFPE) archived slides	DZ; intermediate AMD and GA;FFPE slides CNV and unrelated case-controls: GA and CNV			2 dizygotic); FFPE: 26 AMD eyes GA = 5; CNV = 21; normal $n = 6$ Collected; whole peripheral blood from genomic DNA, 95 CNV, 107 GA and 96 controls	53%; NEI case control; 50%; controls 50% AMD	70.2 years; NEI: AMD:64 years, controls, 79.6 years	MZ and DZ twins as well as case controls; IL17RC was highly expressed in the late AMD macular tissues versus controls; immunoreactivity against IL-17RC higher in macula of AMD patients then in macula of controls; overall, no statistical differences found between CNV and GA	
Capillary electrophoresis and percentage of methylated to total cytosine (mC/tC) was calculated	Whole blood	Early AMD and CNV	Not specified	Ophthalmic exam, including fundus photography; CNV also included fluorescein angiography and OCT	Early AMD = 39; CNV = 27; normal = 132	Early AMD =69%; CNV =55.5%; controls = 69%	Early AMD = 77.8 years CNV = 79.1 years; controls = 77 years	Whole blood DNA methylation is not a marker for AMD	[53]
HDAC isoforms were predicted and were compiled into three QconCAT proteins: QconCATs were determined	Brain tissue (frontal cortex), neural retina	Frontal cortex with AD and without; normal retina; AMD retina	Not specified	Not specified	Normal retina from patients without AD = 4; normal retina from patients with AD $n = 3$ and AMD retina and AD patients n = 3	Not specified	Not specified	HDACs 1 through 6 express differently in retina versus frontal cortex and disease state	[54]

(continued)

Table 9.1 (continued)

Method	Tissue[a]	AMD type	Post-mortem time (hours)	Phenotyping method (grading scale)	No. of biological replicates from macular region (N/E/I/L)[b]	% female	Age (years)	Findings	References
experimentally using an Agilent 6550 QTOF and mass deconvolution with MagTran 1.0 software.									

[a]RPE = retinal pigmented epithelium
[b]N/E/I/L = normal, early AMD, Intermediate AMD, Late AMD

that included late stage AMD (GA and NV), and found differential methylation sites in the ARMS2 gene promoter in both peripheral blood and neural retina [47]. They also identified a gene previously not associated with AMD risk at the nucleotide sequence level, PRSS50, in both blood and retina. It is not clear what if any differences in methylation were significant in blood only or unique to neural retina. In the second whole genome methylation study done to date, Porter et al. (2019), utilizing the Illumina 450 k BeadChip array on RPE cells from 44 human donor eyes (25 AMD and 19 normal controls), followed by validation with bisulfite pyrosequencing in 55 RPE samples (30 AMD and 25 normal controls) found, SKI and GTF2H4, to be differentially methylated in AMD patients versus controls [51]. Both of these genes, SKI and GTF2H4 are novel and not previously associated with AMD at the nucleotide sequence level. Moreover, they ruled out genome wide methylation in the RPE as a contributing factor to AMD. It is clear from the methylation studies described above and in Table 9.1 that findings from the RPE, the neural retina, and peripheral blood from patients do not overlap between studies. Also unclear is if the macula and/or the peripheral tissue of the diseased donor eyes was utilized—as difference in gene expression have been reported between the macula and periphery for both RPE/Choroid and neural retina [56]. Methylation represents a possible biomarker for AMD, utilization of the Epic Chip from Illumina (850 k sites as opposed to 450 k sites) can process a large number of samples, minimizing technical variation, however these chips only capture a portion of the epigenome, and are unable to differentiate between cytosine methylation and hydroxymethylation. The challenge of studying methylation as a biomarker for AMD include, tissue and cell specificity and obtaining primary tissue from diseased and healthy control individuals. It is still unclear what the role of epigenetics is in the AMD phenotype, it may be a mediator between genes and the environment or a modifier. It is also unclear what role confounders, including age, gender, and ethnicity, play in methylation for AMD.

9.2.1 Histone Modifications as Biomarkers

DNA methylation is intertwined with histone acetylation status. Histone Deacetylases (HDACs) are also post-transcriptional modifiers that regulate the protein acetylation implicated in several pathophysiologic states. Abnormal histone homeostasis has been implicated in various neuropathologies [57–62]. Histone acetyltransferases add, and HDACs remove acetyl groups to and from histone lysine residues, respectively. Histone marks require the capture (Chromatin immunoprecipitation; ChIP-Seq) or sequencing (Assay for transposase-accessible chromatin using sequencing; ATAC-Seq) of chromatin fragments. The use of HDACs as a therapeutic target in other neurodegenerative disorders has been under investigation for at least the past 10 years. The functional response to treatment primarily in cancers with broad-spectrum inhibitors such as HDACs has been mixed; protective in some instances, and contraindicated in others [63–67]. This is likely because HDAC inhibitors target many HDAC isoforms, and these isoforms are known to have different and specialized functions. It is likely that these histone deacetylases will differ between tissue types and hence ocular tissue types (e.g., neural retina, RPE, and peripheral blood) [54, 68–72]. Utilizing donor neural retina and donor human frontal cortex, both from patients with and without Alzheimer disease (AD) one group showed that HDAC isoforms from the frontal lobe had different changes in controls compared to AD patients-specifically, demonstrating a decrease in HDAC1 and 2, with HDAC5 increasing [54]. This group also compared to AMD donor retina and found that HDAC5 and HDAC6 showed a decrease in the AMD retina and an even greater decrease in retina from AD

donors. Moreover, HDAC1, 2, and 5 had similar levels of concentration in both normal frontal cortex and retina tissues, while HDAC6 in retina was about five-fold than in normal frontal cortex. Additionally, while HDAC7 and HDA5 were detected in retina they were not detected in frontal cortex utilizing donor eyes from patients with AMD. This study indicates that HDACs likely function differently between disease states and tissue. Utilizing donor RPE and neural retina from patients with and without AMD (eight normal eyes from five donors, and three early stage and five geographic atrophic eyes from a total of five AMD donors), global decreases in chromatin accessibility were found to occur in the RPE of patients with early AMD, and in the neural retina of late stage AMD, geographic atrophy. Further cigarette smoke treatment of RPE cells was demonstrated to recapitulate the chromatin accessibility changes seen in AMD, providing an epigenetic link between a known risk factor for AMD (smoking) and AMD pathology. Finally, it was demonstrated that HDAC10, HDAC11, and SIRT1 were significantly differentially expressed between AMD and controls with overexpression of HDAC11 in the RPE of early stage AMD [32]. Sirtuins are a class of HDACs implicated in lifespan regulation and the promotion of healthy aging through various epigenetic and non-epigenetic cellular roles, including telomere maintenance, DNA repair, metabolism, stress tolerance, cellular differentiation, apoptosis, and inflammation [73, 74]. Sirtuins have also been implicated in age-related diseases, such as diabetes, cardiovascular disease (CVD), neurodegenerative diseases, including AMD as indicated above. SIRT1 in particular is believed to exhibit neuroprotective effects in the retina at least partially through its antioxidant, energy balancing, and antiapoptotic functions. Seventy-eight abnormal SIRT1 localization is also thought to promote the apoptosis of photoreceptor cells and the accelerated aging in the rd10 mouse model of retinal degeneration [75, 76]. SIRT6, which is regulated by SIRT1, has also been implicated in retinal aging, and SIRT6 deficiency in mice results in increased levels of retinal cell apoptosis [77, 78].

9.2.2 The Role of Induced Pluripotent Stem Cells (iPSCs) in the Development of Epigenetic Therapies

One must think about the next steps in epigenetic studies for designing appropriate functional assays to test findings and to manipulate such assays, therapeutically to identify potential targets for disease onset and progression. In addition to primary cells from the affected tissues which are limited in use by the number of passages, iPSCs are increasingly an avenue to create such assays. Induced pluripotent stem cells can be derived from nearly any somatic tissue, and can be differentiated to a myriad of cell types and tissues [79]. This fact has established high expectations for the iPSCs to serve as models for the development of therapeutics, as well as differentiated iPSCs themselves serving as a therapeutic. Indeed, iPSC research is addressing both of these roles, each with challenges to overcome [79–84]. In the eye, the RPE can be readily differentiated from iPSCs (iPSC-RPE). Clinical trials using iPSC-RPE, however, have yielded limited success for treating visual dystrophies, such as AMD [85–87].

Using differentiated iPSCs to model disease and develop therapeutics, currently holds more promise, particularly for visual dystrophies. While animal models exist for many forms of visual dystrophies, they are often insufficient for truly modeling human disease, due to a number of factors. For instance, mouse AMD models exist, but mice lack a macula, and have a different ratio of rod and cone photoreceptors, relative to humans. On the other hand, iPSC-RPE can be derived from affected individuals, which will contain the full complement of genetic factors contributing to disease, and recapitulate the disease hallmarks associated with aging, in general, and AMD specifically, namely drusen. This provides an opportunity to not only investigate the underlying mechanism of pathogenesis, but also design and test potential therapeutics. Drug screening is one area where iPSC-derived tissues are playing an important role, to overcome the insufficiency of animal testing. iPSC-derived

cardiomyocytes are being used to screen drugs for risk of arrhythmic risk, cardiotoxicity, and myopathic effects [88–90]. While the potential for iPSC-derived tissues to either act as a therapeutic agent, or serve to develop one, is high, there are significant hurdles to overcome before they become mainstream.

Tissue derived from iPSC is often assumed to be an exact replica of the somatic target tissue. From a morphological standpoint, the derived tissue looks like the native tissue, and it may have similar functions. The iPSC-derived tissue will express the native tissue signature genes, but these are relatively superficial aspects. An aspect often overlooked of iPSC differentiation is the retention of an epigenetic memory. An epigenetic memory is the maintenance of epigenetic features that are carried from one cell type to the next. For example, methylation of genes necessary for the development and homeostasis of the somatic tissue can be carried through iPSC reprogramming and differentiation to the target cell type or tissue. While it is believed that a majority of the epigenome is erased during reprogramming, it is known that enough is maintained for the differentiated tissue to de-differentiate back to the somatic cell type [91–94]. The extent of epigenetic memory maintained throughout the reprogramming and differentiation process is not yet known, but the issues this may cause for modeling or therapeutic cell transplantation are apparent. For example, as discussed in the Introduction, the genetic contribution to AMD pathogenesis is only 37%, so other factors in addition to environmental, such as epigenetics, may play a role [12]. It can be reasoned that if epigenetic mechanisms underlie AMD pathogenesis, at least partially, then it will be necessary to use a mulitpronged/systems based biology approach to elucidate disease mechanism.

9.2.3 Analysis Tools

For disease modeling and elucidating the epigenetic mechanism of AMD pathogenesis, much work remains to be done. In the case of iPSC reprogramming and differentiation, the epigenetic landscape, ideally both DNA methylation and histone marks, need to be mapped in the parent somatic tissue, the reprogrammed iPSCs, and the differentiated iPSCs. The latter of which needs to also be compared to the native differentiated target tissue, as well. To tackle these issues, resources have been, and are continuing to be developed. Table 9.2 lists databases that aggregate epigenetic data to ensure rigorous analysis of new studies. One database in particular, Epigenie, maintains an extensive list of various epigenetic tools for analyzing epigenetic data from differential DNA methylation to histone modifications. This is an important consideration as some tools may be developed to a specific platform (e.g., DNA methylation array vs. whole genome bisulfite sequencing), while others can analyze data from a multitude of platforms. Moreover, analysis tools that perform statistical analysis, often use differing statistical models. This is an important consideration when designing experiments, and as it is unlikely that any one tool can address all questions, so it may be beneficial to use more than one tool and integrate the results post-analysis.

9.3 Future Directions: Epigenetics as Therapeutics

We have described the two common classes of epigenetic changes; methylation and histone modification, DNA methylation has been the most commonly described. The study of DNA methylation as a therapeutic target and/or intervention has likely been the most developed in oncology, in terms of translation to the clinic and not so much in neuropathologies or cardiovascular disease [95–97]. There also been studies of HDACs and inhibition of cancer. Initially HDACs were described as repressors, because of their functional effects on histones they have also been shown to activate transcription. The studies described above suggest the potential for methylation and histone modifications underlying AMD mechanism and hence therapeutics. Regardless, disease onset and progression in AMD is a process governed by several factors including epidemiology, genetic, and epigenetic

Table 9.2 Epigenetic databases

Database	Website	Tissue dataset	Source
dbEM	http://crdd.osdd.net/raghava/dbem/	Cancer and normal tissues and cell lines	Varied
EpiFactors	http://epifactors.autosome.ru	Varied	Varied
Roadmap Epigenomics Project	http://www.roadmapepigenomics.org/	Normal primary tissues and stem cell lines	NIH roadmap Epigenomics mapping consortium
FeatSNP	http://featsnp.org/	Brain	NIH roadmap Epigenomics mapping consortium
UCSC Genome Browser	https://genome.ucsc.edu/	Varied	Varied
WashU EpiGenome browser	http://epigenomegateway.wustl.edu/browser/	Varied	NIH roadmap Epigenomics mapping consortium; ENCODE
NCBI GEO Repository	https://www.ncbi.nlm.nih.gov/gds?term=%22epigenomics%22%5BFilter%5D	Varied	Varied
Ensembl	http://useast.ensembl.org/info/website/tutorials/encode.html	Varied cell lines	Varied
NGSMethDB	https://bioinfo2.ugr.es/NGSmethDB/	Varied publically-available bisulfite data	Varied
Canadian Epigenomics, Environment and Health Research Consortium Platform (CEEHRC)	http://www.epigenomes.ca//	Varied	CEEHRC
Epigenie	https://epigenie.com/epigenetic-tools-and-databases/	List of epigenetic resources	Varied

changes. Studies that employ a systems biology based approach that include well characterized collections of tissue, which account for tissue integrity and represent each stage of the disease, may help us to understand the molecular changes taking place in the disease process. These studies should not only include eye tissue but whole blood as well, to precisely define which factors contribute to disease pathophysiology at a systemic or localized level to better tailor therapies to individuals. Genome-wide studies which can provide an agnostic approach and are informed by epigenetic, whole transcriptome expression, miRNA sequencing and genetic data from the different tissues of the same donor, for each stage of the disease should provide targets for functional assays. However, whether these factors are causes or effects of disease progression will have to be elucidated with further studies that include functional assays such as iPSC cells to determine the mechanistic role of each of these factors individually or in combination to ensure meaningful translation to patients.

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Mitochondria: The Retina's Achilles' Heel in AMD

10

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Abstract

Strong experimental evidence from studies in human donor retinas and animal models supports the idea that the retinal pathology associated with age-related macular degeneration (AMD) involves mitochondrial dysfunction and consequent altered retinal metabolism. This chapter provides a brief overview of mitochondrial structure and function, summarizes evidence for mitochondrial defects in AMD, and highlights the potential ramifications of these defects on retinal health and function. Discussion of mitochondrial haplogroups and their association with AMD brings to light how mitochondrial genetics can influence disease outcome. As one of the most metabolically active tissues in the human body, there is strong evidence that disruption in key metabolic pathways contributes to AMD pathology. The section on retinal metabolism reviews cell-specific metabolic differences and how the metabolic interdependence of each retinal cell type creates a unique ecosystem that is disrupted in the diseased retina. The final discussion includes strategies for therapeutic interventions that target key mitochondrial pathways as a treatment for AMD.

Keywords

Mitochondria · Age-related macular degeneration · Metabolism · Ecosystem model · Proteomics · mtDNA

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10.1 Introduction to Mitochondria

10.1.1 Mitochondrial Origins

Every cell contains two types of DNA, the nuclear DNA, which is inherited from both mother and father, along with the small circular mitochondrial DNA (mtDNA) that is inherited only from the mother. The "Endosymbiotic Theory" explains the existence of these very different types of DNA co-existing within cells. In early evolution, free-living, aerobic bacteria were

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incorporated into the early, ancestral eukaryotic cells. Over time, this symbiotic relationship resulted in increased energy production that allowed progression from single-cell eukaryotes to multicellular organisms, tissues, and the diversity of species found worldwide. Evidence supporting this theory includes similarities between bacterial and mitochondrial (MT) patterns of gene arrangements, small subunit ribosomal RNAs (rRNA), and protein data [1–3].

10.1.2 MT Distribution and Content

The retina is one of the most metabolically active tissues in the body due, in part, to the high concentration of MT present in nearly all cells. The MT within different retinal cell types are localized toward the sources of oxygen, which in the human retina are the choriocapillaris and the inner retinal vasculature. In the retinal pigment epithelium (RPE), MT are clustered along the basal border of the cell, in close proximity to the choriocapillaris (Fig. 10.1). Photoreceptors are nourished by both the choriocapillaris as well as the inner retinal vasculature. Reflecting the multiple sources of oxygen, photoreceptor MT are located at two sites—densely packed within the inner segments and at the synaptic terminals. Müller cells, which span the entire length of the neural retina and thus also derive oxygen from both sources, have MT that are

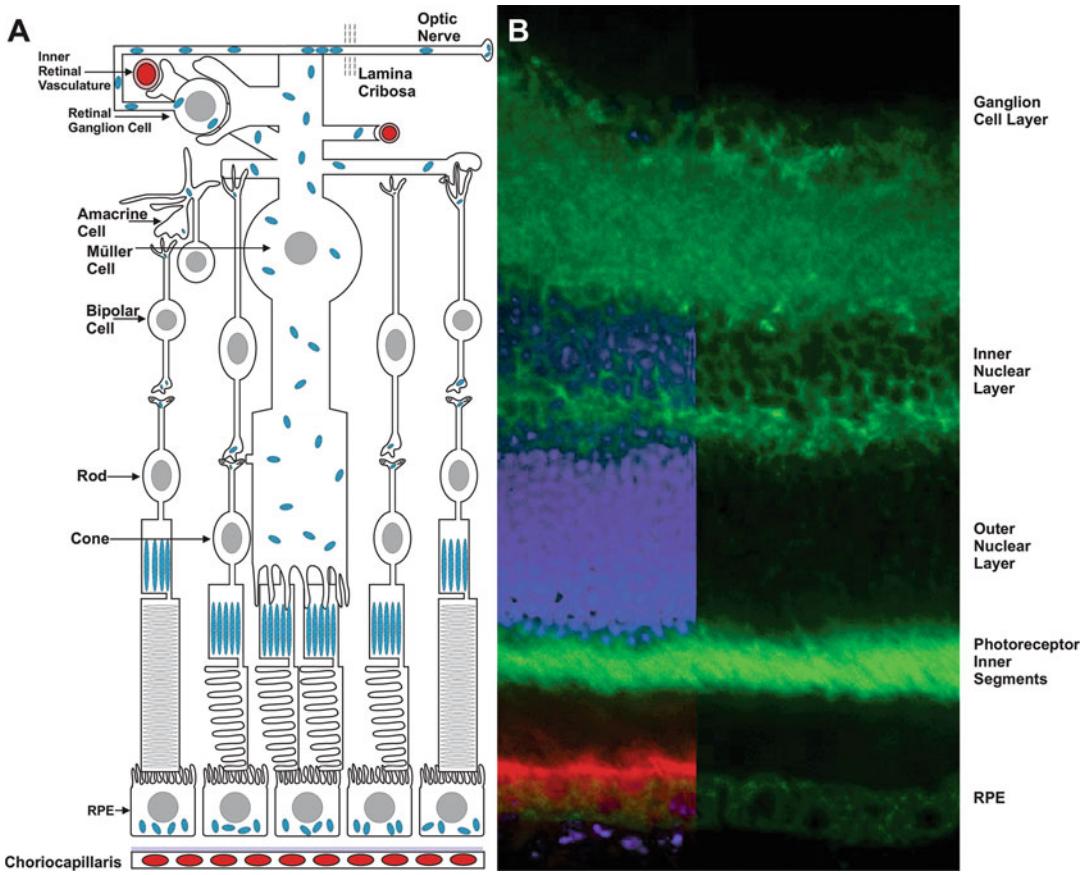

Fig. 10.1 Mitochondrial localization. (**a**) Schematic of retinal cells showing location of mitochondria (labeled blue) within each cell type. Position of the two major blood supplies (choriocapillaris and the inner retinal vasculature) is indicated in red. (**b**) Mouse retinal section stained with anti-TOMM20 (green) to indicate position of mitochondria, anti-Ezrin (red) to indicate the RPE apical membrane, and DAPI (blue) for staining nuclei (Illustration by S. Atilano; Micrograph provided by John Ash and Emily Brown)

evenly distributed throughout the cell [4]. MT in the retinal ganglion cells (RGC) are present in the soma where mtDNA replication occurs and are abundant along the unmyelinated axons where they move bidirectionally along microtubule tracks [5]. MT accumulate just anterior to the lamina cribrosa, but are drastically reduced once the axons exit the eye and form the myelinated optic nerve.

In addition to the cell-specific distribution of MT, the number of MT within a cell can vary from 100 to several thousand depending upon the cell's energy requirement. Additionally, MT content is very dynamic and can be adjusted when energy demands change by either making more MT or eliminating MT via the processes of biogenesis and mitophagy, respectively. The master regulator of MT biogenesis is PGC-1α, a co-activator of transcription factors (NRF-1, PPARα, mtTFAM) that upregulate genes involved in MT biogenesis. Mitophagy is a specialized version of autophagy involving the selective degradation of MT that are either not needed for the current cellular energy requirements or are damaged. MT degradation involves multiple steps starting with the segregation of unwanted or damaged MT from healthy segments. The healthy MT can then fuse with other healthy MT thereby mixing contents and forming ever-changing MT networks (Fig. 10.2). This dynamic process of fission to remove damaged MT segments and fusion of healthy MT is essential for maintaining a population of functional MT. The damaged MT are surrounded by a double membrane structure called an autophagosome, which then fuses with lysosomes. Lysosomal enzymes then digest the contents of the phagosome. Thus, in the healthy cell, there is continual MT turnover involving coordination and balance between both biogenesis and mitophagy.

There is good evidence suggesting the processes involved in MT turnover are adversely affected with AMD. Analysis of RPE MT in human donor eyes using electron microscopy showed there was a significant decrease in MT number and size in donors with AMD compared with nondiseased age-matched controls [6]. Lower MT numbers are consistent with defects in MT biogenesis and the smaller MT suggests defects in MT fusion. Autophagy defects, which will have a negative impact on mitophagy, have also been reported in human donors with AMD. In one study, immunohistochemical staining of human retinal sections showed p62 accumulated in the macula of donors with AMD [7]. Elevated p62, which is normally degraded via autophagy, is an indicator of decreased autophagic flux. Data from a study in primary cultures of RPE directly showed autophagic flux is reduced in RPE from donors with AMD [8].

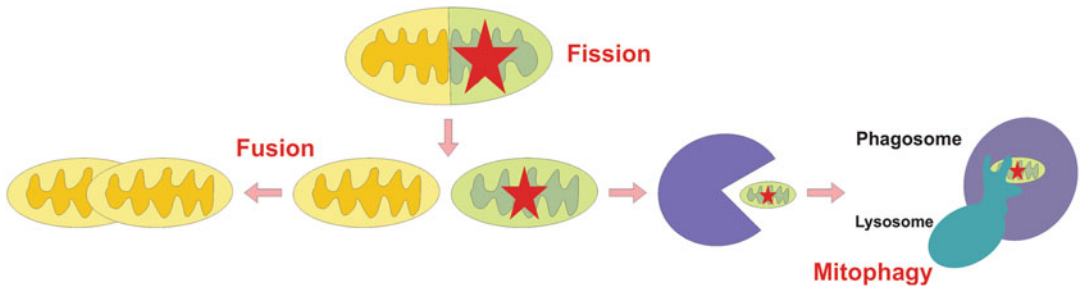

Fig. 10.2 Mitophagy eliminates damaged MT. Fission of MT networks allows for segregation of damaged MT (star, green) from healthy MT (yellow). Healthy MT can then fuse with other healthy MT to replenish the dynamic MT network. The damaged MT are encapsulated by a double-walled membrane, forming the phagosome, which fuses with lysosomes. The lysosomal enzymes digest mitochondria in the phagosome (Illustration by S. Atilano)

10.1.3 MT Structure and Function

Each MT consists of two separate membranes (outer and inner) that divide discrete compartments (Intermembrane Space and Matrix), each containing different proteins with specific functions (Fig. 10.3). The outer membrane is permeable to small molecules (e.g., oxygen, calcium, sugars) that pass through the lipid bilayer. Channels within the outer mitochondrial membrane contain porin proteins (also known as voltage-dependent anion channels, VDAC) that allow passage of ions and molecules less than 5000 Daltons, such as ADP and ATP. The vast majority of proteins that reside in the MT (~1500 proteins) are encoded by the nuclear genome, produced in the cytosol, and imported through the outer MT membrane via the "translocase of the outer membrane" (TOM) complex into the Intermembrane Space [3, 9]. Also embedded in the outer membrane are the proteins involved in MT fission (Fis1/2) and fusion (Mitofusin).

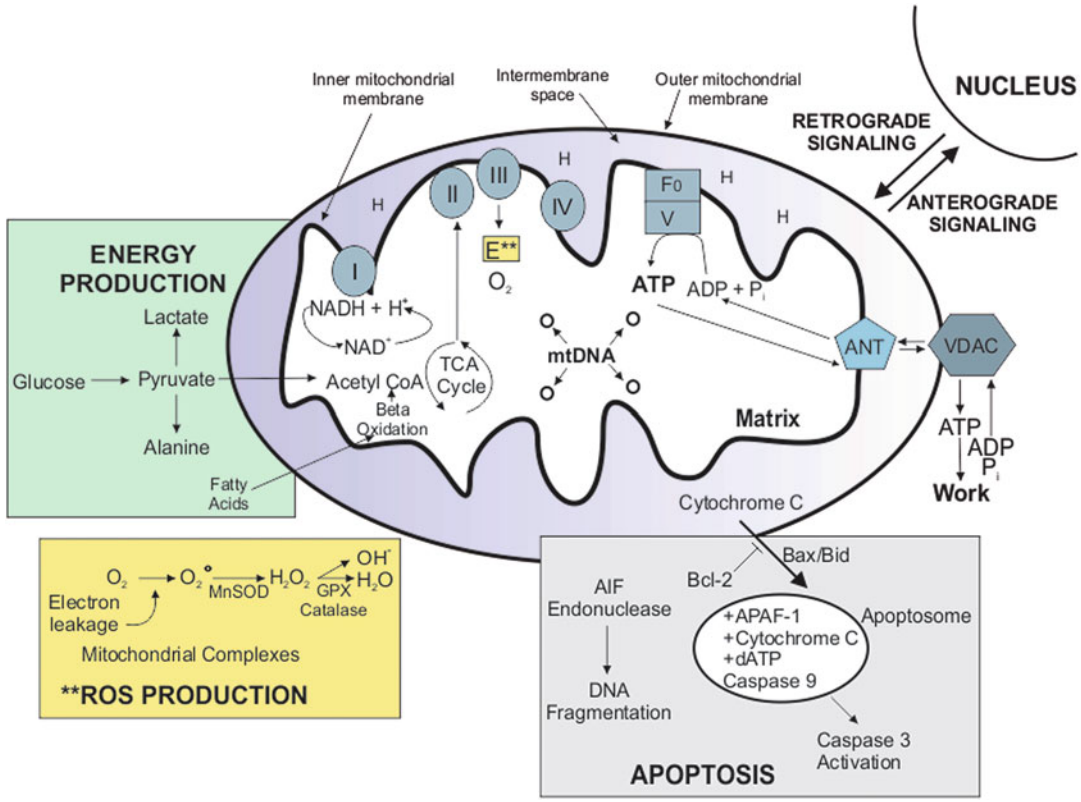

Fig. 10.3 Mitochondrial structure and function. Schematic shows gross mitochondrial structures, including the Inner and Outer membranes that separate the Intermembrane Space and the Matrix. Also depicted are the Complexes (I–V) that are part of the electron transport chain and the energy pathways that feed into these Complexes (Acetyl CoA, TCA cycle, NADH). Hydrogen ions (designated as H) that accumulate in the intermembrane space flow back into the matrix through Complex V, driving the phosphorylation of ADP to form ATP. Boxes outside the MT summarize key functions of energy production, regulation of apoptosis, ROS production and signaling to and from the nucleus via anterograde/retrograde signaling. E = reactive oxygen species; mtDNA = mitochondrial DNA; ROS = reactive oxygen species; VDAC = voltage dependent anion channel; ANT = adenine nucleotide translocase; MnSOD = manganese superoxide dismutase; GPX = glutathione peroxides; AIF Endonuclease = apoptosis-inducing factor; APAf-1 = apoptotic protease activating factor. (Modified from www.mitomap.org, and Nicholis and Minczuk, Exp Gerontol. 2014. 56:175-81. Illustration by S. Atilano)

Proteins involved in regulating apoptosis (Bcl-2, Bax/Bak) bind to the outer membrane but are not integrated into the membrane.

The Intermembrane Space is the site of the "ion motive force," a term that refers to the accumulation of hydrogen ions into this compartment as a consequence of these ions being pumped from the matrix by protein Complexes (I, III, IV) of the electron transport chain (ETC). The exit of these hydrogen ions through the ATP synthase, also known as Complex V of the ETC, back into the matrix is what generates ATP. The Intermembrane Space is also the site of multiple proteins involved in initiating apoptosis, such as cytochrome c, which is sequestered in this compartment by its interaction with cardiolipin, a MT-specific lipid of the inner membrane. Other regulators of apoptosis include apoptosis inducing factor, high-temperature requirement protein/Omi, and Smac/diablo. Release of these proteins from the Intermembrane Space into the cytosol initiates a cascade of events that culminates in apoptosis and cell death. Other proteins present in the Intermembrane Space are involved in the import and folding of proteins that reside in this compartment, as well as the antioxidant, superoxide dismutase, which helps to detoxify reactive oxygen species generated by the ETC.

The inner membrane, with similar lipid content as bacterial membranes, has high cardiolipin content and is selectively permeable, allowing specific molecules (e.g., oxygen, carbon dioxide, and water) into the matrix [10]. Cardiolipin is a negatively charged lipid that drives transport of positively-charged molecules across the outer membrane and is required for maintaining function of the ETC proteins. The surface area of the inner membrane is increased significantly by numerous invaginations called cristae that contain embedded protein respiratory complexes associated with the ETC (Complexes I, III, IV) and production of ATP (Complex V). Multiple proteins are involved in maintaining cristae integrity, including mitofilin, optic atrophy 1 (OPA1) and ChChd3. Maintenance of cristae integrity is essential for optimal energy production, which requires that the ETC proteins are localized in close proximity for effective transfer of electrons.

Data from human donor eyes support the idea that MT architecture is disrupted with AMD. In a study using electron microscopy to analyze RPE MT, Feher and colleagues reported significant disruptions in MT membranes and cristae in donors with AMD compared to age-matched controls [6]. In a proteomic analysis of RPE, increased mitofilin was reported in donors at more advanced stages of AMD [11]. Since mitofilin helps to maintain cristae integrity, the authors suggested the increase could be a compensatory response to help rescue and stabilize the degenerating cristae.

Also embedded in the inner membrane is the "translocase of the inner membrane" (TIM) complex that, working in conjunction with TOM, is responsible for importing nuclear-encoded proteins into the matrix. Multiple heat shock proteins (mtHSP60, mtHSP70, HSP90) assist with the import and folding of resident MT proteins and are localized both in the Intermembrane Space and Matrix. Other inner membrane proteins that work in concert with outer membrane proteins include the adenine nucleotide translocator (ANT), which associates with VDAC in the outer membrane and forms the MT permeability transition pore that opens to allow escape of cytochrome c and other proteins involved in initiating apoptosis. ANT is also involved in moving ADP and ATP into and out of the MT. The inner membrane protein OPA1 interacts with mitofusion in the outer membrane to initiate MT fusion. Mutations in the OPA1 gene have been associated with optic atrophy type 1, which is a dominantly inherited optic neuropathy resulting in progressive loss of visual acuity, often leading to legal blindness [12].

Proteomic analysis of RPE from human donors with AMD showed decreased content of several heat shock proteins that are critical for the import of MT resident proteins. Identified proteins include mtHSP60 and in two separate proteomic studies of the RPE and isolated MT, a decrease in mtHSP70 was observed [11, 13]. These two heat shock proteins assist TOM and TIM with the import and refolding of nuclear encoded proteins and therefore, a reduction in these two proteins in AMD suggests

potential defects in this critical process in diseased RPE.

Recent analysis of the MT matrix has identified 495 proteins that reside in this central compartment [14]. The processes ongoing in the matrix and some of the associated proteins include: mtDNA replication and transcription (TFAM, TFB2M, POLRmt), protein translation (RNA polymerase, ribosomes, Tufm, Rnase P), protein quality control (Lon and ClpXP proteases, chaperones, methionine sulfoxide reductase), and energy production (ETC Complexes). In addition to proteins, the matrix also contains mtDNA (*discussed in greater detail in* Sect. 10.2) and all the machinery required to produce the 13 proteins that are encoded by the MT genome. These 13 proteins, which are part of the ~100 subunits that make up the multisubunit Complexes of the ETC, are absolutely required for a functional ETC. Many MT diseases caused by mutations in either mtDNA or in the nuclear-encoded genes of ETC subunits have ophthalmic involvement [12]. For example, Leber Hereditary Optic Neuropathy (LHON) is maternally-inherited and caused by specific point mutations in subunits of Complex I that are encoded by the MT genome.

The MT have been referred to as the "powerhouse of the cell" because all mechanisms for generating energy (ATP), except for glycolysis, are conducted within the MT matrix. The citric acid cycle (TCA) and β-oxidation, two energy-producing pathways that use glucose and fatty acids as substrates, produce acetyl-CoA (Fig. 10.3). This product is used to generate the energy substrates NADH and FADH2, which are utilized by the ETC in a process known as oxidative phosphorylation. The sequential transfer of electrons between each ETC Complex (I, III, IV) culminating in the reduction of oxygen to water provides the energy to pump hydrogen ions from the matrix into the Intermembrane Space. Release of hydrogen ions through Complex V (ATP synthase) into the matrix drives the phosphorylation of ADP to form ATP. Complex II, also known as succinate dehydrogenase, is the only MT enzyme that participates in both the TCA and oxidative phosphorylation. Complex II transfers electrons that are produced by the oxidation of succinate to fumarate (TCA) to Complex III (oxidative phosphorylation).

Strong experimental evidence supports the hypothesis that RPE MT function is detrimentally altered with AMD. Direct measurement of MT function in primary RPE cultures showed that RPE from human donors with AMD exhibited reduced MT respiration [15] and ATP production [8, 15]. Data from a proteomic analysis of isolated RPE MT from human donors with AMD reported decreased content for several protein subunits of the ETC [11]. Three of the subunits with lower content are part of Complex V, which could result in decreased ATP production. Additionally, Complex V is involved in stabilizing MT morphology and maintaining membrane potential. A loss of these functions would be detrimental to cell viability. *Further discussion of how failures in retinal metabolism contribute to AMD pathology will be included in Sect. 10.3.*

While the most familiar role of the MT is in energy production, this organelle performs a number of additional functions that are essential for cell viability. As previously mentioned, MT regulate apoptosis and subsequent cell death through the release of various proteins (cytochrome c, AIF endonuclease) that are normally sequestered in the MT. They are the site of heme and steroid biosynthesis, and provide calcium buffering for the cell when cytosolic calcium levels increase over normal levels. Calcium is freely permeable through the outer membrane, but requires the aid of the calcium uniporter and Na/Ca exchanger in order to be shuttled into and out of the matrix. Not only does the MT provide buffering from excess calcium, its influx into the MT matrix plays an important role in regulating MT energy production, for example, through the activation of isocitrate dehydrogenase, one of the key regulatory enzymes of the TCA. In neurons, concurrent calcium increases in the cytosol and MT act to synchronize neuronal activity with MT bioenergetics [16].

MT also serve as a signaling platform, communicating changes in the MT environment and cellular energy demands to and from the nucleus in a process referred to anterograde/retrograde signaling (Fig. 10.3). Reactive oxygen species

(ROS), which are produced as a normal by-product of respiration, serve as signaling molecules between the MT and the nucleus. Communication between the MT and nucleus is also induced by changes in the MT electrochemical gradient, the unfolded protein response, and fluctuating levels of ATP or calcium. These varied signals initiate changes in gene expression in both the nucleus and MT to accommodate changing energy demands and cellular conditions. For example, changes in cellular ROS content can initiate signaling through redox-sensitive transcription factors, such as Nrf2 and NfKB, which are proteins that utilize the oxidation of cysteine residues as the signal for activation and respond by translocating into the nucleus and upregulating gene expression to counteract the oxidative stress. Examples of redox-regulated transcription factors include AP-1, p53, estrogen and glucocorticoid receptors, Hif-1, and Nrf-2.

Under normal conditions, these multiple signaling mechanisms play an essential role in maintaining cell health. Abnormal conditions, for example, excessive amounts of MT calcium or high ROS, can disrupt signaling and cell homeostasis. In particular, excessive ROS can cause oxidative damage to lipids, proteins, and DNA. Oxidation of lipids can disrupt membrane integrity and increase membrane permeability to previously excluded molecules. Lipid oxidation can also change the fluidity of membranes in a way that could adversely affect the function of transmembrane proteins, like ETC Complexes, whose function requires a specific lipid environment. Oxidation of the MT-specific lipid cardiolipin in the MT inner membrane disrupts its interaction with cytochrome c, which is tethered to the membrane by cardiolipin. Once cytochrome c is released into the cytosol, it activates apoptosis and causes cell death. Oxidative cleavage of lipids can also generate 4-hydroxynonenal (HNE) and carboxyethylpyrrole (CEP), which can form adducts on DNA and covalently modify lysine residues on proteins. CEP is a specific oxidative fragment of docosahexaenoic acid, which is found in neural tissue and is particularly abundant in the outer retina. Increased CEP-adducted protein has been found in drusen, AMD patient plasma, and AMD donor photoreceptors [17, 18]. AMD-like lesions were observed in mice after immunization with CEP-adducted albumin, suggesting that this specific oxidation product may be toxic to retinal tissue [19]. Abundant HNE adducts have also been reported in retinal proteins, although HNE was not significantly increased with AMD [20]. However, the enzymes used for detoxifying HNE were significantly elevated in retinas from AMD donors suggesting that the retina had mounted a compensatory response to this specific type of lipid oxidation product.

In addition to the generation of lipid-derived adducts, ROS can also directly damage MT proteins, causing protein modifications such as carbonyls, deamidation, and modification of tyrosine to nitrotyrosine. This damage can impair the ability of MT to generate energy by affecting proteins of the ETC, TCA cycle, beta oxidation, or affect calcium buffering capacity by inactivating the calcium transporters located in the inner membrane. ROS-induced damage to mtDNA includes double- and single-strand breaks and formation of DNA adducts, which can interrupt translation and replication of the MT genome. mtDNA damage (*discussed in detail in Sect. 10.2*) can ultimately cause mutations or deletions in key MT-encoded proteins or tRNA that are required for synthesis of MT proteins.

10.2 Influence of mtDNA on Retinal Disease

To date, the nuclear DNA, with approximately 20,000 protein-coding genes, has been studied extensively for its relationship to development and diseases. In contrast, significantly less is known about the role that mtDNA plays in human pathologies. For the past 50–60 years, it has been thought that the main cellular contribution of mtDNA was energy production. However, more recent studies have demonstrated that mtDNA plays important roles in retrograde signaling (from mitochondria to nucleus), modulating nuclear genes, and encoding for

MT-Derived Peptides (MDPs) that are cyto-protective against aging and diseases [21–24]. This section will provide an overview of mtDNA structure and discuss how mtDNA influences retinal diseases, and in particular AMD.

10.2.1 mtDNA Structure

MT are unique in that they have their own DNA that is inherited through the maternal lineage. The human mtDNA forms a closed circle of double stranded DNA, with 16,569 nucleotide pairs, comprised of two strands (Heavy and Light Strands) that are differentiated by their nucleotide content (Fig. 10.4). The heavy strand is guanine rich and encodes for 28 genes while the light strand is cytosine rich and encodes for 9 genes. Unlike the nuclear genome, mtDNA lacks introns and has a major Non-Coding Region (NCR) that includes the O_H (the Heavy-strand origin) for replication, Heavy-Strand promoter (HSP) and Light-Strand promoter (LSP) for transcription, along with the Displacement-loop (D-loop), which is actually a stable, third short DNA strand (also referred to as 7S DNA) [25]. The hypervariable regions -1 (HVR1) and -2 (HVR2) are also located within the NCR and are frequent sites of mutations and polymorphisms. The coding region of mtDNA codes for 37 genes including 13 protein subunits essential for oxidative phosphorylation, 2 ribosomal RNAs and 22 transfer RNAs [26–28].

Within a cell there is a single DNA copy of the nuclear genome but multiple copies of mtDNA because there can be thousands of MT per cell, and within each mitochondrion, 1 to 10 copies of mtDNA. With aging and exposure to oxidative stress, mtDNA molecules can be damaged, which

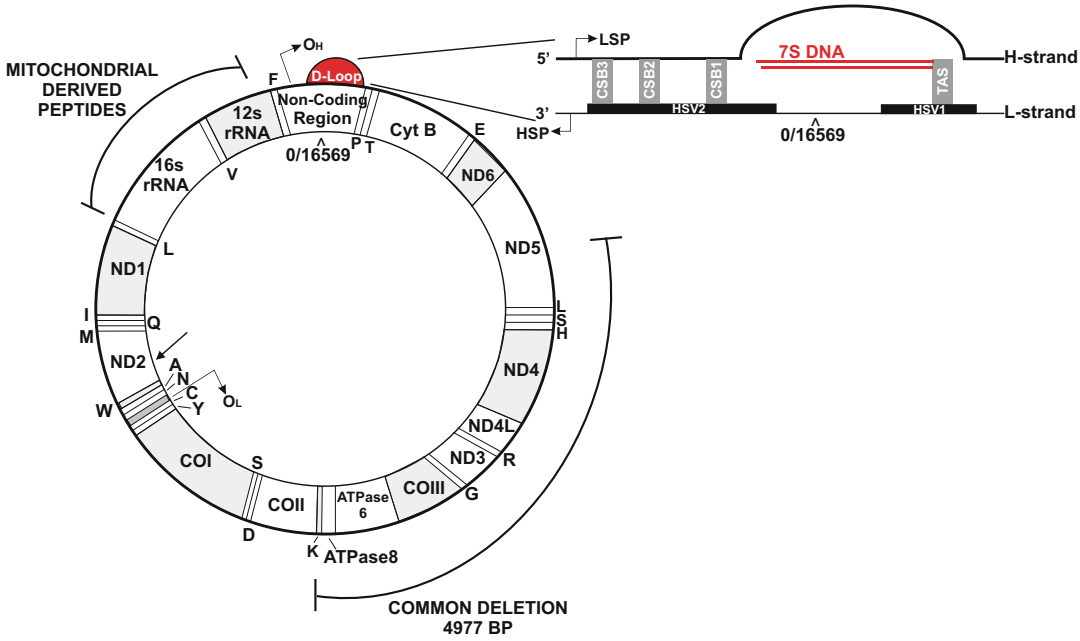

Fig. 10.4 Map of human mtDNA showing non-coding region (NCR) and coding genome. The displacement loop (D-loop) is found within the NCR and has a short, third strand of DNA (7S DNA). The Mitochondrial Derived Peptides are encoded from the 16s RNA and 12s RNA regions of the mtDNA. The region encompassing the "Common Deletion" includes 4977 base pairs. OH, Heavy strand origin; OL Light strand origin; HSP, Heavy strand promoter; LSP, Light strand promoter; CSB, Conserved sequence block; TAS, Termination associated sequences; HVS, Hypervariable region (Modified from www.mitomap.org and Modified/Adapted with Permission from ScienceDirect, Nicholls and Minczuk 2014. Illustration by S. Atilano)

results in a mixture of nonmutated (wildtype) and mutant mtDNA within the same cell. This mixture of damaged and undamaged mtDNA is termed heteroplasmy. When cells with heteroplasmic mitochondria divide, the two types of mtDNA are randomly or in some instances nonrandomly distributed into the daughter cells [29–33]. Cells can function with relatively low levels of heteroplasmy but once this threshold is breached, abnormal functions, cell death and diseases can occur.

10.2.2 Mechanisms That Influence Disease Processes

The contribution of the mtDNA to disease and pathology can be the result of inheritable recent mutations, the ancient adaptive polymorphism changes (haplogroups) or the somatic changes associated with aging. Each mechanism will be discussed below.

10.2.2.1 Maternally Inherited mtDNA Disease-Causing Mutations

There are well described categories of diseases caused by specific mutations within the mtDNA genome, such as MELAS (Mitochondrial encephalopathy lactic acidosis and stroke-like episodes) and MERRF (Myoclonic epilepsy and ragged red fibers) [34, 35]. Individuals born with these mtDNA mutations have deficiencies in the various respiratory complexes of the ETC, resulting in decreased ATP production. They often have numerous systemic abnormalities including deafness, diabetes, myopathies, and neuropathies. The common ocular signs of these mtDNA mutations are retinal pigmentary degenerations and optic nerve atrophy. To date, there are no reports of these types of maternally inherited mtDNA mutations associated with AMD.

10.2.2.2 Maternally Inherited mtDNA Haplogroups and Their Association with AMD

Haplogroups are defined as an accumulation of mtDNA single nucleotide polymorphisms (SNP) that can be traced through maternal lineages and represent populations of different geographic origins (Fig. 10.5). These SNP variations have occurred over more than 150,000 years in response to migration patterns and climate adaptations [3]. The oldest haplogroups are located in Africa (L0, L1, L2, L4-6). The L3 population migrated north and gave rise to founder haplogroups M, N and R. From these founder haplogroups, the European (H, T, U, V, W, X, I, J, K haplogroups), Asian (A, B, C, D, F, G haplogroups) and the most recent Native American haplogroups (A, B, C, D) have evolved. Therefore, no matter the present-day location of an individual, mtDNA analyses can identify their maternal-origin lineage. This is significant because haplogroup-defining SNP variants within the mtDNA coding region can be nonsynonymous (amino acid changing), which can alter efficiencies of energy production, causing increased ROS formation, apoptosis and cell death. If the haplogroup-defining SNP variants are found in the mtDNA Non-Coding Region, then the mtDNA replication and transcription rates can be affected. This means that each haplogroup, with its different set of SNPs, can produce unique bioenergetic properties, which may play a role in developing diseases and responding to medications.

Since AMD is found most commonly in Caucasian populations, the European haplogroups have been analyzed and it has been reported that J, T and U haplogroups are associated with AMD [36–41] while the H haplogroup has a protective effect [39]. Large, soft drusen and retinal pigment abnormalities, which are characteristics of AMD retinas, have been associated with J and U haplogroups [37]. The haplogroup T-associated SNP (A4917G), located in the NADH dehydrogenase subunit 2 of complex I (MT-ND2), is an independent predictor of AMD [36]. Two variants of the T2 haplogroup, A11812G of MT-ND4 and A14233G of MT-ND6 located in respiratory complex I, are 2.5 times more likely to be associated with advanced AMD than the age-matched control subjects [40]. Additionally, Udar and coworkers

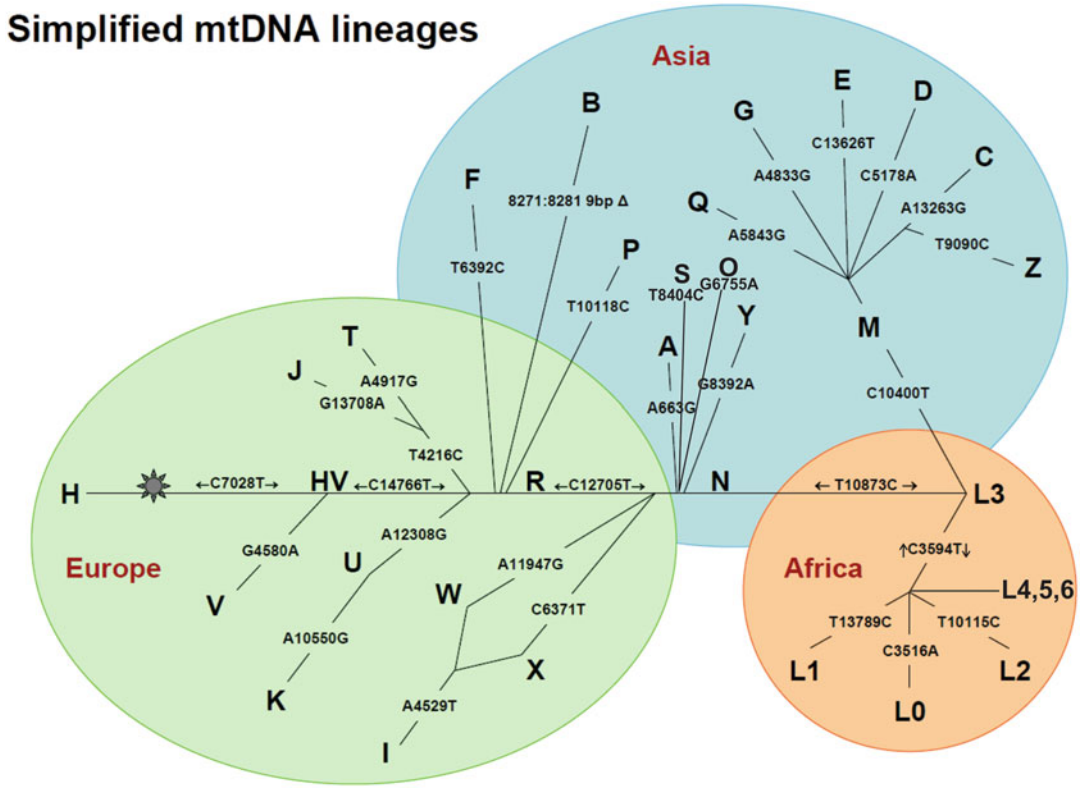

Fig. 10.5 Schematic of the major, high branching haplogroup-defining mitochondrial SNPs. The SNPs on the left side of the position number is the rCRS nucleotide (GeneBank NC_012920) and defines that haplogroup. The diverging haplogroup is represented by the SNP on the right of the number (Modified from www.mitomap.org, Illustration by S. Atilano)

found that patients with late AMD showed strong association with noncoding mtDNA D-loop SNPs in the neural retina [41].

Our knowledge and understanding of the effects of mtDNA upon cellular homeostasis has been advanced through the use of the transmitochondrial cybrid model, which are cell lines with identical nuclei, but contain MT from different subjects. Using human retinal pigment epithelial cell cybrids, it has been demonstrated that mtDNA can greatly affect growth rates of cells, bioenergetics and expression levels of nuclear genes for complement, inflammation and angiogenesis, pathways which are important in human retinal diseases [23, 42–45]. In addition, cybrids with H (protective against AMD) versus J (high risk for AMD) mtDNA haplogroups have different responses to heat stress, hydrogen peroxide, and ultraviolet radiation [42, 46–48]. These studies demonstrate that an individual's mtDNA background sets up baseline cellular homeostasis, making the cells differentially susceptible to identical stressors.

The mechanism(s) by which mtDNA haplogroups influence AMD risk is unclear. It is known that different SNP variants in MT can alter cellular bioenergetics causing partial uncoupling of oxidative phosphorylation and decreased efficiency of ATP production [49, 50]. If MT-generated energy levels decline below a specific bioenergetic threshold, the MT permeability transition pore (mtPTP) can be opened and the apoptosis pathway activated. Models of conplastic strains of mice (where the nuclear genome from one strain has been crossed onto the cytoplasm of another, designated as Nuclear

genome-mtCytoplasmic genome) have also demonstrated that the mtDNA affects pathways related to behavior, immunity and cognition [51].

There are a number of ocular diseases associated with specific haplogroups [52–57] but the literature lacks reports examining thousands of patients. The analyses for mtDNA haplogroups and variants are often difficult because the mitochondrial protocols are more limited than those available for nuclear gene analyses. The common approaches to characterize mtDNA patterns are to isolate the total DNA from blood or saliva, then characterize the lineages by identifying the haplogroup defining SNPs (Fig. 10.5) using PCR/RFLP (polymerase chain reaction/restriction fragment length polymorphism), custom designed TaqMan probes and/or whole mitochondrial DNA sequencing. At present, the platforms/chips used for GWAS (genome wide association studies) do not have enough SNPs on them to determine haplogroups accurately, making it very difficult to use that format for large scale mtDNA classification. In addition, the mtDNA SNP numbering on the commercial arrays is variable, making analyses of the data inconsistent and unreliable. Until this technical problem is resolved, it is unlikely that analyses for large numbers of AMD populations will be available.

10.2.2.3 Somatic Age-Related mtDNA Damage Associated with AMD

The third mechanism by which mtDNA can affect disease processes is through the cumulative mtDNA damage associated with aging. Human and animal studies show, the levels of mtDNA fragmentation/degradation/deletion is increased significantly with aging [58, 59]. Environmental factors, such as oxidative stress and ultraviolet radiation, can cause strand breaks, deletions and new SNP variants. In addition, one of the errors of double-strand break repair is the deletion of a 4977 base pair region of the MT genome called the "Common Deletion" (Fig. 10.4). This deletion accumulates with age in postmitotic tissue, such as brain, skeletal muscle, heart and RPE [60, 61]. Several factors make mtDNA more susceptible to damage, including the lack of protective histones and its location within the MT matrix, where it is in close proximity to sites of ROS formation. Importantly, MT have poor DNA repair processes, thereby allowing the damage to accumulate with aging.

There are numerous reports of MT abnormalities, including mtDNA damage, in both the neural retina and RPE with aging and AMD [6, 11, 58, 60, 62–65]. It was reported that sequence analyses of the mtDNA D-loop from the neural retina found a significantly greater number of SNPs per person in the AMD population compared to either older or younger normal groups [62]. Increased oxidative DNA damage in retinas from donors with AMD was also shown using immune-histochemical staining with antibodies that recognize 8-OH-dG, a specific DNA adduct generated by ROS that serves as a biomarker of oxidative stress [41]. In the RPE from donors with AMD, an accumulation of mtDNA oxidative damage was found compared to RPE from age-matched normal donors [60, 65]. These findings were supported by analysis of primary cultures of RPE cells where increased DNA damage and fivefold increased somatic mutations were observed in RPE from AMD donors compared with age-matched controls [66]. Interestingly, the increased mtDNA damage associated with AMD progression occurs in the RPE but not the neural retina, and is localized to specific MT genome regions, including the regulatory D-loop [65]. Deletions occurring in coding regions for subunits of Complex I (ND3, ND4, ND4L, ND5), Complex IV (Cytochrome Oxidase III), or Complex V (ATPase 6, ATPase 8), could have tremendous impact on cellular bioenergetics and functions. Furthermore, this type of damage can increase heteroplasmy (mixture of damaged and undamaged mtDNA) and decrease copy numbers.

Using a model of AMD cybrid cells (RPE cell lines with identical nuclei but MT from individual AMD patients) the destructive impact that damaged MT have on retinal cells has been demonstrated [24, 67]. When MT from AMD subjects are placed into human retinal cell lines, there was a significant loss of viability, impaired MT functions, upregulation of pro-apoptosis, pro-autophagy, pro-ER stress genes, and enhanced vulnerability to oxidative stress.

Importantly, Nashine and coworkers showed that although damaged, these AMD MT can be rescued by treatment with Humanin-G, a cytoprotective peptide that increased the cellular longevity [24]. Humanin is one of several MT-derived peptides that are encoded by the MT genome within the 16S ribosomal RNA gene. The role of these MT-derived peptides in MT metabolic control and cytoprotection is currently being explored [68]. Initial results are encouraging researchers to pursue the MT-targeting therapies to prevent cell death associated with AMD. It is highly likely that future investigations will yield multiprong approaches to further characterize the damaged AMD MT and identify novel therapeutic agents to restore the defective MT to healthier status.

10.3 Introduction to the Metabolic Ecosystem Concept and Its Link to AMD Pathology

Section 10.3 will discuss the concept of a retina/RPE ecosystem by first providing an overview of the pertinent metabolic features and pathways within individual retinal cells. The final section will consider the idea that a bioenergetic crisis in the RPE is the primary event that tips the metabolic balance toward AMD [69] and addresses the critical question of why the macula is preferentially and most severely affected with AMD.

The idea of a metabolic ecosystem within the eye makes sense when one considers the many important and diverse metabolic interactions that occur between organs and tissues in a whole organism. All living cells convert fuels into metabolic energy. Each type of cell adapts its biochemical machinery to make the most effective use of the types of fuels available to it and to the timing and magnitude of its energy needs. Cells within an organism generally have metabolic features that allow them to support each other. For example, when muscles are highly active, they can produce pyruvate at a rate faster than it can be oxidized by the cell's MT. Glycolysis requires a continuous supply of NAD+ to oxidize glucose. The muscles are able to sustain energy production through glycolysis by regenerating NAD+ via reduction of pyruvate to lactate. Remarkably, the liver can recycle the lactate. Lactate is exported from muscles to the blood and imported into liver cells. Energy from fatty acid oxidation in the liver cells can then be used to reduce the lactate to glucose. The glucose is exported from the liver and then taken up by the muscles to support the continuing energy demands of the active muscle. The metabolic interdependence in this classic biochemistry example highlights how distinct cells with specialized metabolic features can support each other and how they function as a metabolic ecosystem.

10.3.1 Metabolic Features of the Retina

10.3.1.1 Aerobic Glycolysis in the Retina

Cells within the vertebrate eye also have specialized metabolic features. This was discovered in the early 1920s by Krebs and Warburg when they were exploring glycolytic and oxidative metabolism in a variety of tissues [70, 71]. They noted that tumors and retinas are especially glycolytic, meaning that the ratio of lactate production to O_2 consumption is substantially higher in these tissues compared to other tissues they had examined. They observed that lactate was produced even when plenty of O_2 was available. Since then that finding has been confirmed many times over [72–77]. Production of lactate caused by a shortage of O_2 is common and is referred to as "anaerobic glycolysis." Production of lactate by retinas or tumors is referred to as "aerobic glycolysis" because it occurs even when O_2 is readily available [78]. In recognition of its discovery by Warburg, this type of metabolism is referred to as the "Warburg Effect."

The site of aerobic glycolysis in retinas hasn't been identified directly, but several observations indicate it occurs in photoreceptors. Most of the lactate produced by an eye comes from the outer retina, which is made up primarily of photoreceptors [76]. Hexokinase, which catalyzes the first step in glycolysis is enriched in the outer

retina [79–81] and specialized isoforms of glycolytic enzymes like hexokinase II [80, 81] and pyruvate kinase M2 [72, 82–84] are specifically expressed in photoreceptors. Lactate dehydrogenase A is expressed at high levels in photoreceptors [72, 82, 85]. Inactivating pyruvate kinase, lactate dehydrogenase [72] or hexokinase II [80] expression in rods alters their morphology in ways that appear to be cell-autonomous [72]. A fluorescent glucose derivative gavaged into the stomachs of either mice or zebrafish [86] or injected into the tail veins of mice [87] accumulates in the retinas, primarily in photoreceptors.

10.3.1.2 Mitochondria in Photoreceptors

Retinas can consume other fuels including glutamine, lactate, pyruvate [88], fatty acids and ketone bodies [89, 90]. Those fuels require MT to oxidize them and the retinas are very capable of actively consuming O_2. Linsenmeier's and Cringle's in vivo measurements [91, 92] of the distributions of O_2 in retinas have shown that nearly all of the O_2 available from the choroid circulation can be consumed by the outer retina, most likely by the photoreceptors.

MT are abundant in rods and cones [93]. They are in a region of the photoreceptor just below the base of the outer segment known as the ellipsoid. When there is inner retinal vascularization, as is the case for humans, mitochondria also accumulate at photoreceptor synaptic terminals [94]. In animals such as frogs, rabbits, guinea pigs, horses and birds, the inner retinas are mostly avascular [95]. Those retinas do not have a substantial source of O_2 from the inner retina. Photoreceptor synapses in avascular retinas can obtain ATP made at the ellipsoid and transferred to the synapse via a phosphocreatine shuttle [96]. An isoform of creatine kinase that localizes to mitochondria can make phosphocreatine from ATP at the ellipsoid, which then can diffuse from the ellipsoid to the synaptic terminal where the phosphocreatine is used by another isoform of creatine kinase to make ATP. Measurements of metabolic flux through mitochondrial intermediates revealed that some aspects of mitochondrial function are more active in darkness than in light [74]. This could be a consequence of the higher cytosolic free Ca^{2+} levels in darkness. Higher cytosolic Ca^{2+} can alter the concentration of free Ca^{2+} in the mitochondrial matrix [97]. Flux measurements suggest oxidation of α-ketoglutarate to succinyl CoA, a step in the TCA cycle, is stimulated in darkness compared to in light [74], consistent with findings that Ca^{2+} can stimulate α-ketoglutarate dehydrogenase activity in vitro [98]. Mitochondria in retinas appear relatively uncoupled [74] and consumption of O_2 does not appear to be limited by ATP demand [74, 99, 100].

MT morphology in photoreceptors is striking and diverse [86, 96, 101]. For example, MT in mammalian rods is highly elongated and line up close to the plasma membrane in parallel with the major axis of the rod cell. In contrast, MT in cones of zebrafish retinas coalesces into a tightly packed cluster just below the outer segment. The tight cluster of MT in the zebrafish cones can maintain distinct pools of Ca^{2+} in the outer vs. inner segments of a cone cell [97]. There also is evidence that MT can influence the optical properties of photoreceptors [93]. Any advantages that these unique and diverse structures provide to photoreceptors still need to be identified. MT are more abundant and may be more active in cones [101]. In primate retinas the sizes and volumes of MT vary throughout the fovea, perifovea and peripheral regions of the retina [93]. The morphologies of these MT change when photoreceptors become affected by AMD and the changes can be followed by Optical Coherence Tomography (OCT) [102].

10.3.1.3 Müller Cells

Müller glia span the retina radially with their apical processes extending around the inner segments of the photoreceptors and their end feet at the inner surface of the retina. Just below the apical processes the junctions between Müller cells create a visible structure referred to as the outer limiting membrane. Within the retina the Müller cells extend horizontal processes of diverse morphology that infiltrate between neurons.

Immunohistochemical studies suggest that Müller cells may not have some of the enzymes needed for glycolysis. Pyruvate kinase and hexokinase, which are detected readily in photoreceptors, have not yet been detected in Müller cells [83, 85]. Immunocytochemistry indicates that Müller cells also lack a key enzyme required for the malate-aspartate shuttle that shuttles electrons from glycolysis into MT [103]. It is important to know whether the portion of Müller cells in the outer retina has glucose transporters, which are required for direct uptake of glucose. However, the overlap of Müller cell apical processes with the ellipsoid region of rods, which contain the GLUT-1 glucose transporter, makes it difficult to resolve unambiguously the localization in the apical processes of enzymes and transporters. Co-labeling with several rod and Müller cell-specific antibodies suggest that GLUT-1 in Müller cells is abundant in the end-feet but not in the apical processes [86]. However, more precise and higher-resolution studies will be needed to resolve this definitively. Müller cell MT are small and abundant just underneath the apical processes [86]. Glial cells are the only cells in the retina with the enzyme, glutamine synthetase, needed to synthesize glutamine. The ability of retinas to synthesize glutamine from lactate and aspartate indicates that Müller glia are capable of taking up and metabolizing lactate [83].

10.3.1.4 Glycogen in Retinas

Glycogen is present in retinas. It is most abundant in the end-feet of Müller cells. Glycogen also can be detected in cone photoreceptors. The amount of glycogen stored in Müller cell end feet can vary depending on the amount of glucose available to the retina [104]. There is a report that Müller cells express the enzymes needed for gluconeogenesis [105]. This is an important observation, because gluconeogenesis from lactate could produce glucose for glycogen storage to support the activity of inner retinal neurons. However, that line of investigation has not been pursued beyond the initial reports 30 years ago and needs further confirmation. Glycogen and gluconeogenesis in Müller cells could be fundamentally important for retinal survival and function.

10.3.2 Metabolic Features of the RPE

RPE cells form a cellular monolayer between the choriocapillaris and retina [106]. They are sealed together by tight junctions and function as a blood retina barrier for the outer retina. To reach the retina, fuels and metabolites from blood in the choriocapillaris must pass through membrane transporters in both the basolateral and apical plasma surfaces of RPE cells. Glucose from the blood must be able to reach the retina to support the high glycolytic activity of the retina. The glucose transporter, GLUT-1, is exceptionally abundant on both surfaces of the RPE [107, 108]. Monocarboxylate transporters are differentially distributed to the two surfaces of the RPE. MCT1 is on the apical side whereas MCT3 is on the basolateral side [109].

RPE cells perform vital functions that support the retina [106]. Once a day they phagocytose the tips of the rod outer segments and either metabolize their contents or recycle them back to the retina. RPE cells also have the capacity to esterify and isomerize all-trans retinol, a product of photobleaching visual pigments [110]. This is an essential process in the pathway for regeneration of rhodopsin after it has undergone photobleaching in rod outer segments.

10.3.2.1 Glycolysis in the RPE

The exceptional abundance of glucose transporters on both sides of the RPE [107, 108] highlights how RPE cells are adapted to facilitate transport of as much glucose as possible from the choriocapillaris to the retina. Consistent with this important metabolic function, RPE cells consume glucose much more slowly than retina. Like all cells, RPE cells need fuel for energy and anabolic activities. Compared to retinas, RPE cells have a limited ability to use glucose. However, they can oxidize lactic acid, glutamine, and fatty acids as alternative fuels [74, 86, 90, 111]. RPE cells also actively consume proline and they can export a

variety of metabolites involved in energy production and anabolic activities [111]. RPE cell metabolism is more enriched than the retina in two other important metabolic pathways, reductive carboxylation of α-ketoglutarate to produce citrate [112], and carboxylation of pyruvate to produce oxaloacetate [86]. All of these metabolic features appear to be adaptations that can sustain metabolic requirements of RPE cells while minimizing their dependence on glucose.

10.3.2.2 Glycogen in the RPE

RPE cells store glycogen. The amount of stored glycogen in cultured human retinal epithelial cells increases when there is abundant glucose in the medium in which they grow and decreases when glucose levels in the medium have been depleted [113]. These findings suggest that glycogen stored in RPE cells can ensure a steady supply of glucose to the retina, but this potentially critically important activity of RPE cells has not yet been investigated directly.

10.3.3 Metabolic Synergism in the Eye: A Retina/RPE Ecosystem

As described in the preceding sections RPE cells and the neurons and glia of the retina have diverse and specialized metabolic features. The distinct metabolic functions of cells in the eye appear generally analogous to the distinct and diverse metabolic roles of various types of plants and animals in the ecosystem of a forest or ocean.

Based on currently available data it is likely that the retina/RPE metabolic ecosystem functions as portrayed in the schematic model in Fig. 10.6 [86]. Glucose from the choriocapillaris enters and leaves RPE cells through abundant GLUT-1 glucose transporters on the basolateral and apical surfaces of the RPE cells. When glucose reaches photoreceptors most of it is oxidized rapidly by glycolysis and then reduced to lactate. The lactate, with metabolic energy still remaining within its chemical bonds, is exported from the photoreceptors to both the RPE and to Müller cells where it can be oxidized by MT to drive synthesis of ATP.

A significant advantage of this type of metabolic strategy is that oxidation of lactate imported into RPE cells can deplete NAD^+ in the RPE cells. Since NAD^+ is needed for glycolysis, depletion of NAD^+ by lactate further suppresses glycolysis in RPE cells so that more glucose can pass through the RPE and reach the retina.

10.3.3.1 Findings from In Vivo Experiments that Support the Concept of an Essential Retina/RPE Ecosystem

This concept of metabolic interdependence is generally supported by the results of several recent experiments. Genetic manipulations that affect a specific type of cell in the retina or RPE affect the viability not only of that specific type of cell, but also of other types of cells that were not affected directly by the mutation. Some of these examples are described briefly in the following paragraphs.

Example 1 *Cone viability depends on the presence of rods*. Rods are more abundant than cones in human and mouse retinas. Even within the human macula there are 9 times more rods than cones [114]. Although rods and cones carry out similar functions in the eye, many of the phototransduction genes that have specific roles in rods are not needed and are not expressed in cones because other genes fulfill those functions in cones [115]. For example, PDE6β, PDE6γ and rhodopsin are expressed only in rods, not in cones. A mutation in any of these genes can directly disrupt rod viability. However, after the rods degenerate, cones then also begin to degenerate, even though the mutation did not influence cones directly. Recent findings provide clues about the nature of this "bystander effect." As rods die, cones show evidence of nutrient deprivation [116]. The evidence is a change in the phosphorylation state of mTOR, a protein that senses the availability of nutrients. Recent findings have identified two biochemical mechanisms that may be responsible for depriving cones of essential nutrients. First, rods in a normal retina produce and release a polypeptide, rod-derived cone viability factor (RdCVF)

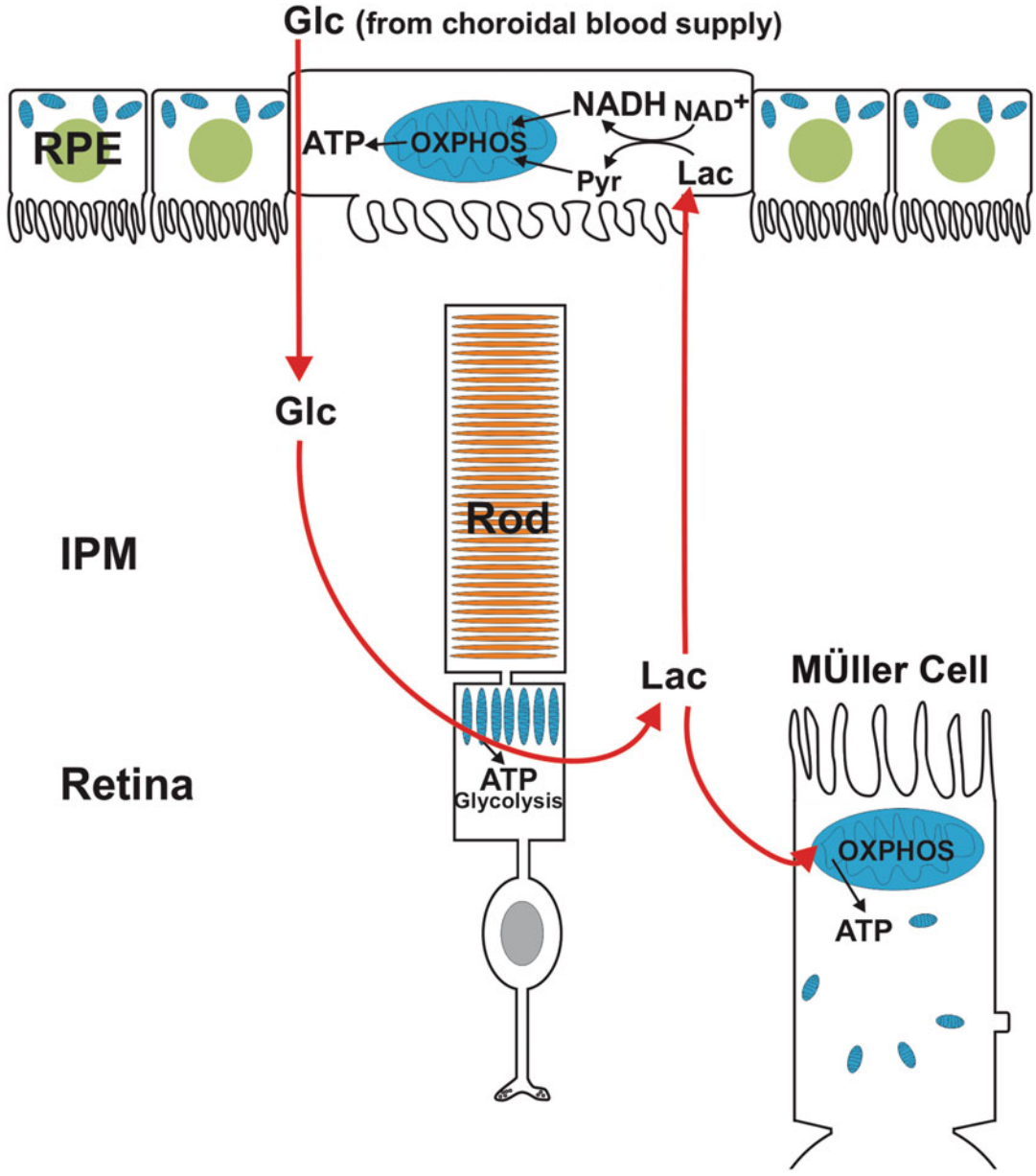

Fig. 10.6 Summary of the metabolic interactions between RPE, photoreceptors and MÜller cells. Glucose from the choroidal blood supply passes through glucose transporters on the basolateral and apical surfaces of the RPE to reach the retina. The glucose that reaches the Interphotoreceptor Matrix (IPM) is taken up by glucose transporters on the plasma membrane of the rod inner segments. Glucose in the rod cell is converted rapidly to lactate by aerobic glycolysis and then released from the photoreceptor. The lactate released can be used either by Müller cells or by the RPE. Studies have shown that lactate can suppress glycolysis in the RPE to minimize consumption of glucose by the RPE so that more glucose can reach the retina

[117, 118]. RdCVF interacts with a protein, basigin, on cones, that stabilizes or stimulates the activity of glucose transporters on the cone plasma membrane. When rods degenerate, RdCVF no longer is released by rods, glucose transporters on cones are not stabilized and

cones starve. The absence of rods also can cause cones to starve because glucose becomes trapped within the RPE. Normally, when a fluorescent glucose derivative is injected into the tail veins of mice it passes through the RPE and accumulates in photoreceptors in the retina [87]. However, as rods degenerate the fluorescent glucose becomes trapped within the RPE making it inaccessible to the cones [117, 118]. Part of this glucose trapping may be caused by failure of the ecosystem (Fig. 10.6). Less lactate will be made by the retina in the absence of rods. The RPE will rely on glucose instead of lactate for fuel so less glucose reaches the retina. Other effects of rod loss on RPE function also may contribute to glucose trapping.

Example 2 *The viability of rods relies on mitochondrial activity in RPE cells*. MT require a specific transcription factor, TFAM, for expression of genes encoded by mitochondrial DNA. When TFAM is inactivated specifically in RPE cells, the metabolism of the RPE cells become less specialized and consumption of glucose by glycolysis increases [119]. This causes rod photoreceptors in the retina to degenerate. This observation is consistent with two ideas: (i) RPE minimizes its consumption of glucose so sufficient glucose is available to fuel the retina and (ii) maintenance of ATP production and other MT functions in the RPE is essential for retinal health.

Example 3 *The viability of rods relies on restraint of glycolytic activity in RPE cells*. Unrestrained glycolytic activity in RPE cells could cause the RPE to consume so much glucose that not enough glucose passes through the RPE to fuel the retina. Hypoxia-Inducible factor (HIF1α) can stimulate transcription of genes that encode the enzymes that catalyze glycolysis. Von Hippel-Lindau Factor (VHF) is a protein that suppresses glycolysis by helping to degrade HIF. When VHF expression is blocked specifically in the RPE, HIF1α is stabilized. That makes the RPE more glycolytic and it causes photoreceptors to degenerate [120], consistent with the model in Fig. 10.6.

Example 4 *Robustness is enhanced when glycolytic activity is increased in rods*. Photoreceptor degeneration can be induced by deleterious mutations in genes that encode subunits of a rod cGMP phosphodiesterase. Normal retinas are highly glycolytic. However, expression of glycolysis genes normally is limited by expression of a protein, SIRT6. When SIRT6 expression is blocked in photoreceptors, the rate of glycolysis in retinas increases several folds above its already high level [121]. Degeneration caused by a PDE6 mutation is slowed substantially when glycolysis is enhanced by SIRT6 inactivation. This is consistent with the model in Fig. 10.6 because enhanced lactate production from the mutant rods can suppress RPE glycolysis so more glucose can reach the retina.

Taken together, all of these findings support the idea that diverse metabolic features of cells in the retina and RPE contribute specific metabolic roles that sustain the viability and function of cells in a metabolic ecosystem.

10.3.4 Relationship Between AMD and the Metabolic Ecosystem of the RPE and Retina

The observations that enhanced glycolysis in RPE cells induces rod degeneration and that enhanced glycolysis in photoreceptors makes them more robust suggest that the state of the retina/RPE metabolic ecosystem can influence degenerative diseases, such as AMD. The distribution of rods and cones and the distributions and volumes of ellipsoid MT in human retinas are not homogeneous [93, 122–125]. It is likely that the efficiency of the RPE/retina metabolic ecosystem depends heavily on RPE MT being able to use lactate, glutamine, fatty acids and proline as fuels to minimize consumption of glucose. Human RPE MT accumulate DNA damage with advancing age [60, 66] and cumulative damage compromises their metabolic activity [8, 15]. Since the RPE MT have a central role in supporting the metabolic ecosystem sketched in

Fig. 10.6, the regions of the nonhomogeneous retina that are most dependent on the metabolic ecosystem ought to be the first regions where photoreceptor stress and damage become evident. Since cones in the central fovea are more enriched in MT than rods [93] they may be less dependent on the metabolic ecosystem, that is, they may be more capable of efficiently using limited amounts of glucose than other regions of the retina. Just outside the central fovea, rods become more abundant, but since they are more sparse in this region their net output of lactate would be limited. This could make the rods in the perifoveal region the most reliant on RPE MT and therefore most sensitive to damage of RPE MT. This hypothesis may help explain why AMD correlates with RPE MT damage and why it initially affects perifoveal rods. Further studies in which the metabolic capabilities of the central fovea, perifovea, and peripheral retinas are compared in nonhuman primate retinas and in human donor retinas will be required to evaluate the validity of this hypothesis.

10.4 Enhancing MT Function and Bioenergetics As a Therapeutic Strategy for Treating AMD

Studies suggest that metabolic dysregulation may be a major contributing factor to disease pathogenesis in AMD [15, 65, 69, 86]. Mutations in over 200 genes have been associated with retinal degenerations (RetNet, [126]), and diseases such as AMD are multifactorial, with genes and environment contributing to disease. Due to the large number of mutations and factors that can lead to retinal degeneration, gene independent strategies that focus on targeting the biological pathways that lead to retinal degeneration or retinal neuroprotection are crucial. Based on strong evidence that dysfunction of the metabolic ecosystem leads to the retinal degeneration associated with AMD, pathways related to energy metabolism, MT biogenesis, and oxidative stress may be ideal targets to treat AMD. This section will discuss several studies that have examined the role of these pathways in AMD and how targeting these pathways may be neuroprotective.

10.4.1 Energy Metabolism

10.4.1.1 AMPK

A viable strategy for treating AMD is to target pathways that stimulate or shift the metabolic ecosystem back to its homeostatic state. An ideal target for enhancing metabolic function is a key pathway involved in regulating energy levels in the cell. One such protein involved in regulating cellular energy metabolism in a variety of tissues is 5′ adenosine monophosphate protein kinase (AMPK). AMPK is activated by cellular metabolic stress and is expressed ubiquitously all eukaryotic cells. AMPK directly binds AMP, ADP, or ATP allowing it to detect energy levels in the cell. AMPK is activated by upstream kinases, including liver kinase B1 (LKB1) and Ca/calmodulin-activated protein kinase (CaMMKKβ), as well as pharmacological activators, such as AICAR and metformin [127]. Activation of AMPK promotes downstream energy producing pathways, including glucose metabolism, MT function, and autophagy, and inhibits downstream energy consuming pathways, including protein synthesis and fatty acid metabolism. This makes AMPK an ideal target for diseases such as AMD, where MT and metabolic dysfunction likely plays an important role in disease pathogenesis.

Several studies have examined the role of AMPK and upstream kinase LKB1 in the retina. Interestingly, LKB1 expression in the retina decreases with age in mice [128]. Studies examining conditional deletion of LKB1 in retinal progenitor cells found that deletion of LBK1 results in loss of ONL thickness, shifts in synaptic positioning, a reduction in the total number of synapses, and loss of rod and cone function. Knocking down AMPK, produced a similar phenotype, suggesting that AMPK plays an important role in this process. Interestingly, the authors were able to reverse the phenotype by 20% using metformin to activate AMPK and by 50% using caloric restriction to activate AMPK. Conversely,

feeding the animals a high fat diet resulted in a 70% increase in synaptic mislocalization. The authors found a reduction of phosphorylated, or active, AMPK in older mice, suggesting that levels of activated AMPK decrease in the retina with age [128].

Other studies have investigated metformin-mediated activation of AMPK as a neuroprotective therapy. Studies have shown that systemic metformin treatment is able to activate AMPK in the retina [129]. Metformin treatment preserved retinal function and morphology in mouse models of light-induced retinal degeneration, the Rd10 model of inherited retinal degeneration, and was protective to the RPE in a model of RPE injury using sodium iodate [129]. Metformin protection was dose-dependent, and was associated with increased mitochondrial DNA copy number, increased ATP levels, and reduced levels of oxidative stress and DNA damage. Importantly, metformin was no longer protective with deletion of AMPKα1 and α2 subunits in the retina, suggesting that AMPK is necessary for metformin-induced protection, and that metformin protection is due to local activation of AMPK in the retina [129]. These results suggest that promotion of MT function and retinal metabolism with metformin treatment is neuroprotective to the retina. Therefore, caloric restriction, caloric restriction mimetics, or activators of AMPK are potential neuroprotective therapies that could stimulate retinal energy metabolism homeostasis to prevent retinal degeneration in AMD.

10.4.1.2 Insulin/mTOR

Another metabolic pathway that is a potential neuroprotective target is the mTOR (mammalian target of rapamycin) pathway. mTOR is a key regulator of metabolism and cell growth and is found in two complexes, mTORC1 or mTORC2. mTORC1 regulates cell metabolism and protein synthesis while mTORC2 regulates pro-cell survival mechanisms. Deletion of mTORC1 or mTORC2 individually in the retina does not affect function or survival of cones up to 1 year of age but does result in alterations of outer and inner segment morphology [130]. Deletion of both mTORC1 and mTORC2 in the retina leads to a loss of cone function, but not cone death [131]. These findings suggest that due to the high metabolic activity of healthy cones, the role of mTOR in regulation of metabolism in healthy cones is minimal.

Although the insulin/mTOR signaling pathway may play a minor role in healthy cones, insulin/mTOR signaling may play a larger role in cones under conditions of stress. Using four models of retinitis pigmentosa, Punzo et al. examined changes in gene expression at various points of cone degeneration [116]. Interestingly, 36% of the genes that were upregulated at least twofold upon secondary death of cones were related to cellular metabolism. The insulin/mTOR signaling pathway had the highest number of hits, suggesting that metabolism and mTOR signaling play an important role in the cone death process. Increases in levels of heterodimeric transcription factor hypoxia inducible factor 1 (HIF-1a/b), which increases glycolysis under stressful conditions such as those of low oxygen, and GLUT1, a glucose transporter expressed in the photoreceptors, were observed. This suggests that cone death may be due to compromised glucose uptake in the cones, resulting in starvation. Stimulation of the mTOR pathway with insulin treatment resulted in enhanced survival of cones, while inhibition of insulin with injection of streptozotocin, a drug that kills the insulin-producing beta cells of the pancreas, resulted in decreased survival of cones [116].

Other studies have shown that constitutive activation of mTORC1 is able to preserve function and survival of cones in mouse models of retinitis pigmentosa, and that mTORC1 is required for the protective effect of activating insulin/mTOR pathway [130]. Activation of mTORC1 resulted in increased uptake and utilization of glucose and elevated levels of NADPH. Loss of the mTORC1 accessory protein, RAPTOR, resulted in accelerated retinal degeneration in disease models, but had no effect on retinal function or cell survival when it was deleted in a wildtype retina. Activation of mTORC1 was associated with increased expression of genes related to uptake, retention, and utilization of

glucose [130]. These findings suggest that mTORC1 specifically plays an important role in maintaining cone function in disease, and this protection may be mediated through enhanced uptake, retention, and utilization of glucose.

Although these studies focus on models of retinitis pigmentosa rather than AMD, the dysregulation of metabolism observed in these models may reflect pathological features of AMD under conditions of stress, particularly, when there is a reduction in glucose levels reaching the photoreceptors due to increased glycolysis in the RPE. These results suggest that metabolic imbalance is a major contributing factor to loss of cones, and stimulation of pathways such as the insulin/mTOR signaling pathway may be protective to cones under conditions of reduced glucose levels. Further studies examining the role of insulin/mTOR signaling in energy metabolism in AMD are necessary to further investigate this possibility.

10.4.1.3 CoQ10

Coenzyme Q10 (CoQ10), also known as ubiquinone, is ubiquitously expressed, and is localized primarily in the MT. CoQ10 is a component of the electron transport chain acting as an electron carrier. It also has antioxidant capabilities and can affect metabolism-related gene expression. Studies have shown that CoQ10 levels decline in a variety of tissues in the human body with age, including the retina [132]. A randomized, double-blind, placebo controlled clinical trial investigated the efficacy of using a combination of acetyl-L-carnitine, n-3 fatty acids, and CoQ10 in subjects with early AMD [133]. The aim of this therapy was to target MT lipid metabolism for a metabolic therapy to prevent AMD progression. Treatment resulted in preservation of visual field function, as compared to placebo-treated controls, and a reduction in drusen area [133]. These results suggest that using CoQ10 to improve retinal metabolism may reduce progression of early AMD and may be a potential therapeutic target for the disease. Targeting pathways that enhance energy metabolism or promote energy homeostasis in the retina may represent a suitable target for therapies for AMD.

10.4.2 Oxidative Stress and Mitochondrial Biogenesis

10.4.2.1 Nrf2

Other neuroprotective avenues for AMD include reducing levels of oxidative stress through targeting antioxidant pathways. A reduction in oxidative stress in the RPE and retina would likely help prevent oxidative DNA damage to mtDNA, nuclear DNA, and prevent oxidation of lipids and proteins. Nrf2 is a master transcription factor that regulates the antioxidant response in virtually all cell types. Several studies have focused on targeting Nrf2 to reduce levels of oxidative stress in AMD. In unstressed conditions, Nrf2 is held in the cytoplasm by Keap1, which facilitates its ubiquitination and subsequent degradation. However, in conditions of oxidative stress, Keap1 structure is disrupted, preventing it from interacting with Nrf2, which allows Nrf2 to translocate to the nucleus and activate the antioxidant response element. Studies have shown that AAV-mediated gene therapy delivering an Nrf2-derived peptide that interacts with Keap1 to prevent endogenous Nrf2 degradation is able to increase antioxidant expression and protect retinal function and morphology in response to sodium iodate-induced RPE damage [134]. Other groups have shown that Nrf2 upregulation in the retinas of mice, via AAV-mediated gene delivery, resulted in protection from damaging light levels [135]. Targeting Nrf2 to reduce levels of oxidative stress may protect MT from oxidative damage and help preserve RPE function in AMD.

10.4.2.2 PGC-1a

Another potentially neuroprotective target to enhance mitochondrial bioenergetics is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1α is a transcriptional coactivator that is involved in regulation of many genes involved in energy metabolism, including those involved in MT biogenesis. PGC-1α is activated by increased levels of oxidative stress and by direct phosphorylation by AMPK [136].

Studies have shown that overexpression of PGC-1α in the RPE leads to increased expression of genes associated with fatty acid oxidation, MT respiration, oxidative metabolism, and antioxidants [137]. This increase in antioxidant expression correlated with an increased ability for the RPE cells to cope with oxidative insults, including H_2O_2 and hydroquinone (an oxidant found in cigarette smoke) [137]. As MT dysfunction and elevated levels of oxidative stress in the RPE are associated with AMD, overexpression of PGC-1α may represent an important target for potential therapies for the disease.

Other studies suggest that PGC-1α plays an important role in vivo. Mice heterozygous for PGC-1α that were fed a high fat diet for 4 months exhibited RPE and photoreceptor degeneration. This was associated with an accumulation of lipofuscin, basal laminar deposits, Bruch's membrane thickening, and deposits containing proteins with oxidative damage [138]. This phenotype may be due to reduced MT function, as these mice had decreased levels of mtDNA copy number, reduced activity of MT oxidative phosphorylation Complex I, and increased levels of reactive oxygen species [138]. These findings suggest that decreased PGC-1α expression in AMD may contribute to disease phenotypes. Targeting PGC-1α, through indirect activation using an AMPK activator, such as metformin, other pharmacological activators, or gene therapies may represent a possible neuroprotective therapy for AMD that could enhance MT function and possibly prevent RPE dysfunction.

10.4.3 Neuroprotection in AMD

Dysregulation of MT and energy metabolism in the RPE is likely a contributing factor to AMD pathogenesis. As predicted by the Metabolic Ecosystems Model, metabolic dysfunction in the RPE can lead to dysregulation of metabolism in photoreceptors and a bioenergetic crisis in the entire retina. Therapies that target pathways involved in regulation of energy metabolism or MT activity in the photoreceptors and especially RPE, such as AMPK, insulin/mTOR, CoQ10 and PGC-1α, have the potential to restore or preserve energy homeostasis, and thus retinal health and function (Fig. 10.7). Targeting other pathways such as those involved in regulation of oxidative stress response, like Nrf2, may protect the RPE from oxidative insults that could disrupt energy homeostasis. Although these pathways are strong candidates for targets for neuroprotective therapies for AMD, a better understanding of the underlying mechanisms of the pathology of AMD is essential to develop targeted therapies to prevent or treat AMD.

10.5 Summary

MT regulate cellular energy production, oxidative stress, inflammation, signal transduction, and apoptotic pathways.

MT alterations, including mtDNA damage/mutations and dysfunction, likely play an important role in AMD pathogenesis.

Alterations in MT in AMD may disrupt the highly coordinated metabolic ecosystem of the retina, which can lead to further retinal metabolic stress and retinal degeneration.

Targeting MT function and retinal metabolism may be neuroprotective to the retina and as these pathways may represent the optimal neuroprotective therapies for AMD.

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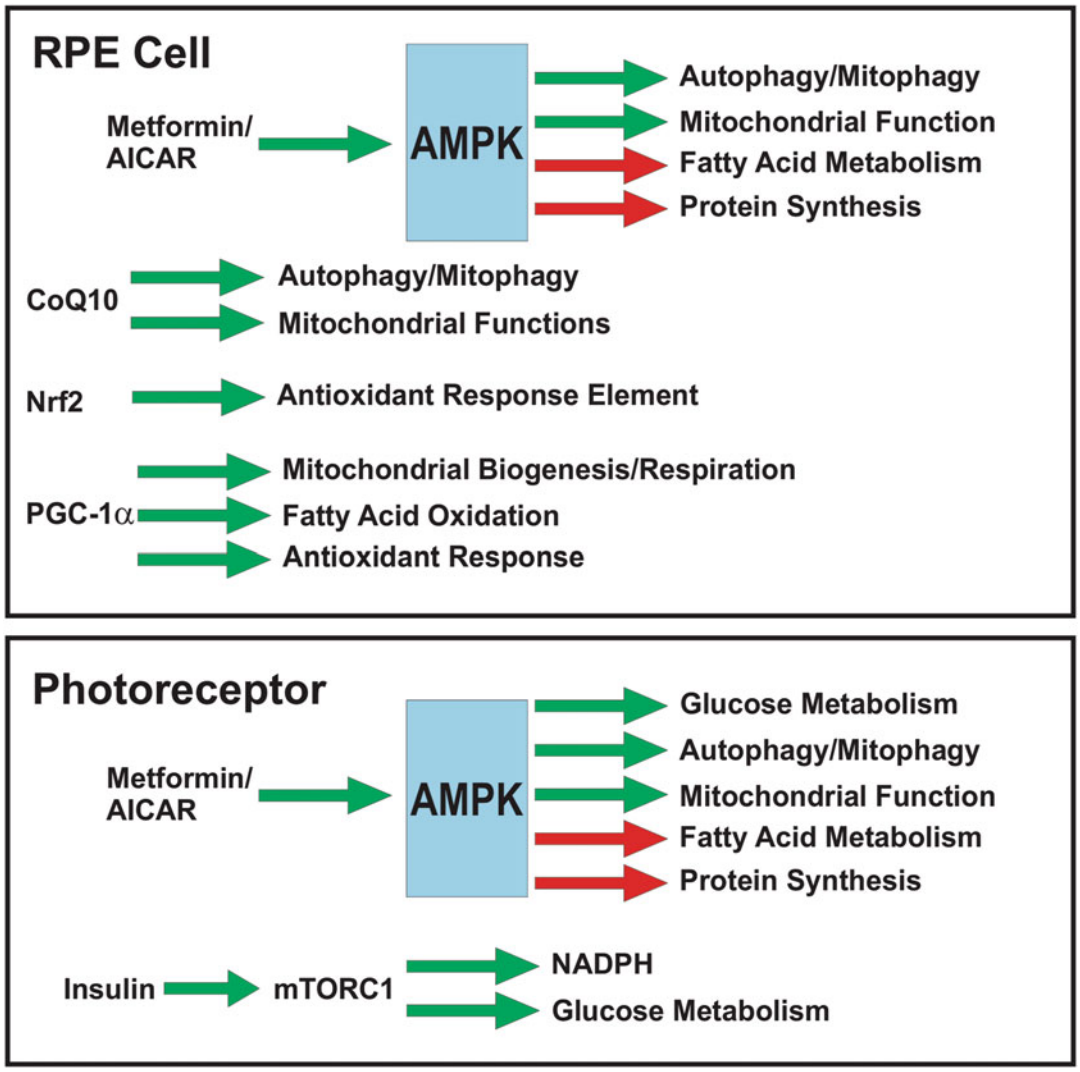

Fig. 10.7 Potential neuroprotective targets for AMD to enhance mitochondrial function and cellular metabolism in the RPE and retina. In the RPE, targeting AMPK, CoQ10, Nrf2, or PGC-1α may promote mitochondrial function and antioxidant responses. The retina, targeting AMPK or mTOR pathways may promote glucose metabolism, enhance mitochondrial function, and NADPH metabolism. Red arrows = inhibitory effect, green arrow = promoting effect

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Cell-Based Therapies for Age-Related Macular Degeneration

11

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Abstract

Age-related macular degeneration (AMD) is a leading cause of blindness worldwide. The pathogenesis of AMD involves dysfunction and loss of the retinal pigment epithelium (RPE), a monolayer of cells that provide nourishment and functional support for the overlying photoreceptors. RPE cells in mammals are not known to divide, renew or regenerate in vivo, and in advanced AMD, RPE loss leads to degeneration of the photoreceptors and impairment of vision. One possible therapeutic approach would be to support and replace the failing RPE cells of affected patients, and indeed moderate success of surgical procedures in which relatively healthy autologous RPE from the peripheral retina of the same eye was transplanted under the retina in the macular area suggested that RPE replacement could be a means to attenuate photoreceptor cell loss. This prompted exploration of the possibility to use pluripotent stem cells (PSCs) as a potential source for "healthy and young" RPE cells for such cell-based therapy of AMD. Various approaches ranging from the use of allogeneic embryonic stem cells to autologous induced pluripotent stem cells are now being tested within early clinical trials. Such PSC-derived RPE cells are either injected into the subretinal space as a suspension, or transplanted as a monolayer patch upon scaffold support. Although most of these approaches are at early clinical stages, safety of the RPE product has been demonstrated by several of these studies. Here, we review the concept of cell-based therapy of AMD and provide an update on current progress in the field of RPE transplantation.

Keywords

AMD · Cell-based therapy · RPE · Embryonic stem cells · Induced pluripotent stem cells · Transplantation

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11.1 Introduction

As per World Health Organization estimates, AMD is one of the leading causes of visual impairment in developed countries. In its advanced stages, the disease severely worsens

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the patient's quality of life. Globally, it is the third leading cause of blindness [1]. AMD is a complex multifactorial disease. Besides the main factor of age, its onset and progression are linked to both genetic and environmental factors including diet, smoking, chronic inflammation and hypertension [2]. The disease has two advanced stages, geographic atrophy (GA) as a severe form of "dry" (non-neovascular) AMD and choroidal neovascularization (CNV) or "wet" (neovascular) AMD (NVAMD). Vision loss in both stages is due to the death of photoreceptors, but the disease etiology is different in these two stages. NVAMD is caused by hyperproliferation of choroidal vessels, some of which penetrate the subretinal space where they leak fluid and blood separating photoreceptors from their supporting cell type, the retinal pigment epithelium (RPE). In the case of dry AMD progressing to GA, photoreceptor cell death is thought to be triggered and secondary to dysfunction and atrophy of RPE cells [3]. In both conditions, the pathology mainly affects the macula resulting in central vision loss and slowly progressing to perimacular regions [4]. While the pathogenesis of AMD is multifactorial and complex, involving interactions between RPE, choroid and the photoreceptors and involving multiple mechanisms including oxidative injury, immune system activation and inflammation (among others), the RPE has become one of the main therapeutic targets because dysfunction and loss of RPE cells seems to precede and play a key role in the changes that then occur in the photoreceptors, the retina, and the choroid.

The RPE is a monolayer of epithelial cells located adjacent to the retinal photoreceptor (PR) outer segments. This monolayer is supported on a proteinaceous Bruch's membrane (BM) that separates it from the choroidal vasculature. The BM actually serves as a bilateral basement membrane, both to the overlying RPE cells and the underlying choriocapillaris. RPE cells within the monolayer are connected via tight junctions [5], and in a fully mature RPE monolayer, a mechanical barrier is thus formed between the retina and the choroidal blood supply, which is part of the blood-retinal barrier. Because of this feature, RPE cells control nutrient and oxygen flow from the choriocapillaris to the PRs, and metabolite flow back from PRs to the choriocapillaris. In addition, RPE cells are critically important for maintaining the health and integrity of PRs: RPE pigment granules absorb scattered light; replenish light-isomerized visual pigment; phagocytose shed outer segments of PRs; and secrete cytokines in a polarized fashion. The polarized nature of the RPE, with characteristic receptors, channels and cellular structures on its apical and basal sides, is a key feature that is required for most of its functions [5]. This apical-basal polarization is induced in cells as they form a confluent monolayer with tight junctions between neighboring cells. Once the tight junctions fully mature, they suppress free flow of receptors and channels between the apical and basal sides of RPE cells, allowing the two membrane sides to behave differently. For instance, predominant location of potassium channels on the apical side and chloride channels on the basal side allow vectorial fluid flow from the apical to the basal sides. Several different retinal degenerative diseases are known to be caused by specific gene mutations that affect some of these RPE functions, underscoring the importance of the RPE in PR and retinal health and survival [3, 6, 7].

With age, RPE cells (that like PRs and other retinal and CNS cells do not naturally divide, regenerate or renew), undergo metabolic changes that may reduce their ability to perform some of their functions. These changes can be exacerbated by environmental and genetic factors. Some of these changes include accumulation of lipofuscin deposits called drusen inside and below the RPE, and these accumulations of "debris" are the hallmark of AMD. Along with reduction of melanin content in the RPE, the changes likely lead to reduced anti-oxidative capacity. These pathological changes in the RPE cells and chronic aberrant inflammatory responses progress over time, and have been linked to development of the AMD [3, 7]. Dry AMD is marked by multiple and often confluent areas of drusen, which can be both internal and external to the RPE. Drusen accumulation has been associated with RPE cell

death and eventually retinal degeneration in dry AMD. Furthermore, degeneration and/or death of RPE cells and subsequent weakening and formation of gaps and breaches in the RPE monolayer can result in choroidal neovascularization, leading to NVAMD.

Attempts to limit vision loss in neovascular AMD included surgical removal of the subretinal pathological choroidal neovascular membrane between the RPE and PRs, laser treatment of extra-foveal CNV and also photodynamic therapy [8–10]. Currently, the mainstay of treatment for curbing and attenuating this form of disease is through the use of anti-VEGF agents that temporarily block choroidal vessel proliferation and reduce permeability of such vessels. Anti-VEGF treatments, that revolutionized and altered the rapid and dramatic course of neovascular AMD, do not, however, fully prevent chronic fibrosis caused by slow leakage of ectopic choroidal vessels and do not arrest the underlying process of progressive RPE dysfunction and loss. Similarly, in the advanced form of dry AMD, formation and expansion of GA is currently largely untreatable. Besides limited benefit obtained by certain dietary modifications (as defined by the AREDS trials [11]) and protection from sunlight and short wavelength light, there is currently no approved drug available that can suppress RPE atrophy or significantly prevent/attenuate GA lesion expansion.

The observations that RPE dysfunction and loss play a critical role in the pathogenesis of AMD coupled with the fact that in humans these cells have an extremely limited potential for regeneration (if at all), suggest the possibility that RPE cell replacement/support may serve as a possible beneficial therapeutic approach. To be effective and maintain visual function, such transplantation needs to occur prior to the secondary loss of the overlying PRs. Once PRs are lost, replacement of both RPE and PR cells, as well as possibly a proper substrate equivalent to Bruch's membrane, may be required. In the following sections, data from basic research studies and early clinical trials that attempt to address this challenge will be presented, with emphasis on generation and use of RPE cells, which in many ways are an easier therapeutic target, as their effects do not require the formation of functional neuronal/synaptic connections. Rather, if a monolayer of healthy RPE cells can be formed in the subretinal space by transplantation, evidence from animal experiments and clinical observations suggest this can support function and viability of the overlying PRs. In advanced stages of disease, once PRs are lost as well, combined RPE + PR grafts may provide regenerative capabilities, but at present this is a more challenging goal and while animal studies show some ability of PR progenitors to survive and partially integrate into the retina, this is as yet not being explored in clinical trials in AMD.

11.1.1 Proof of Concept Studies Suggesting Cell Replacement May Be Beneficial

The etiology of the two advanced stages of AMD and the post-mitotic, non-regenerative nature of RPE cells and PRs suggest that replacement cell-based therapy may be a possible therapeutic approach. This is supported by various proof-of-concept procedures and studies that aimed to replace/support the degenerating RPE in order to halt or slow down PR cell death. These included attempts to perform autologous transplantation of RPE from the retinal periphery to the macula, retinal rotation procedures to overlay the macular and particularly foveal cone PRs over healthier RPE, and early attempts to transplant RPE. As detailed here in different sections of this chapter, these studies indeed lend support to the notion that delivery of healthy RPE has the potential to attenuate PR loss.

Gouras et al. reported in 1984 the first ever RPE transplant, which was performed in a monkey eye. Adult post-mortem human RPE cells were cultured in vitro and transplanted as a cell suspension into the subretinal space where the native RPE had been surgically removed [6]. Similar RPE transplants were subsequently performed in rabbits [12] and in rats [13]. All these animal studies confirmed survival of human xenografts. Functional efficacy of RPE

transplants was first tested in 1988 in Royal College of Surgeons (RCS) rats in which PR degeneration is secondary to RPE dysfunction, with a mutation in the MERTK gene causing defective phagocytosis of photoreceptor outer segments (POS) by the RPE cells [14]. As the majority of animal species apart from primates and certain birds do not develop macular structures, there is a lack of appropriate animal models for AMD. Thus, the RCS rat has been extensively used to examine therapeutic approaches for RPE-induced retinal degeneration. Transplanted human RPE cells were indeed demonstrated to phagocytose rat POS in this model [14, 15]. Later on, donor RPE from wild type rats, a human RPE cell line, human embryonic stem cell as well as iPSC-derived RPE cells were also tested and shown to affect disease progression in RCS rats [16–20]. These animal studies, that demonstrated survival and function of transplanted human RPE, provided the first proof-of-concept that RPE transplantation can be developed as a therapy for AMD.

In humans, lack of effective treatments for NVAMD prior to the development of PDT and later anti-VEGF therapies prompted retinal surgeons to attempt macular translocation surgeries, in which foveal PRs were re-positioned above healthier RPE outside the area affected by CNV and injury [21, 22]. These procedures showed that indeed improved survival of PRs and even significant visual acuity gains could be obtained in some cases, but the surgeries are highly complex and the rate of complications is high, including retinal detachment, diplopia, and recurrence of CNV [23]. As a routine treatment for NVAMD this was not practical and now is largely unnecessary, thanks to anti-VEGF treatments, but it did support the notion that approximating macular and foveal PRs to healthier RPE could be a valid therapeutic approach. This approach gained further support from studies of autologous RPE transplantation within the eyes of patients, as outlined here in Section 2a-III.

11.1.2 Delivery as a Cell Suspension or as Sheets of Cells on Scaffolds (Fig. 11.1)

Native RPE cells exhibit several critical characteristics that must also ultimately manifest in transplanted RPE cells to ensure their efficacy and proper function. These include maintaining cell polarity with the correct basal and apical orientation of different proteins and structures such as the Na^+/K^+ ATPase, proteins associated with tight junctions, retinol cycling, blood-retinal barrier and phagocytosis [31, 32]. Thus, structure of the cells and transplantation approach may markedly affect outcome. In general, two main forms of delivery are being tested, the first being injecting the cells in suspension, and the second as cell sheets, with or without a scaffold (Fig. 11.1) [30, 33, 34]. Both approaches have their advantages and disadvantages: subretinal or transchoroidal injection of cells in suspension is simpler and generally less surgically challenging and traumatic (Fig. 11.1a). In contrast, more complex biological formulations such as 3-dimensional RPE sheets on different types of scaffolds, while requiring a more complicated surgical approach, provide the possibility to deliver the RPE cells as an intact, functioning unit with the cells already in the correct polarity, enabling better formation of tight junctions (Fig. 11.1b–f) [35, 36]. In addition, this creates a natural monolayer anatomical structure rather than single cells, RPE clumps or multilayered structures with random orientation and phenotypic variability that can form following simple subretinal cell suspension implantation [25, 37, 38]. This being said, also after delivery in suspension, over time the transplanted cells are often able to layer out, and adjust their polarity by intracellular trafficking of the relevant proteins.

To date, various natural and synthetic scaffolds were used to seed RPE cells and to mimic BM. The scaffold material, design of the surface and dimensions highly affect cell adherence, growth, differentiation, and function

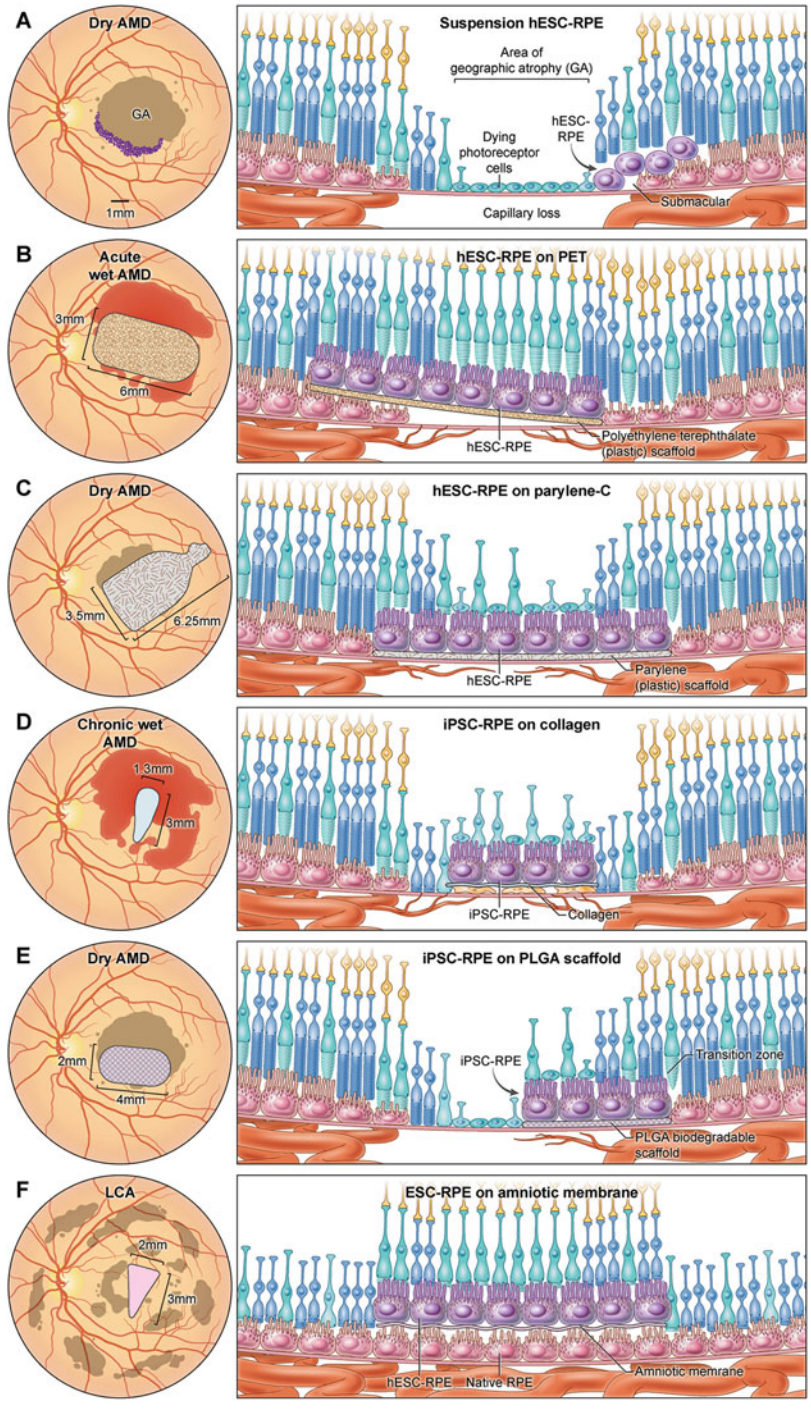

Fig. 11.1 Schematic adapted from Sharma et al. [24] of various ongoing and planned retinal pigment epithelium (RPE) transplant approaches, showing fundus views of the transplants (left) and how the transplants (purple) would be integrated into the subretinal space, their possible impact on retina and choroid, and the various scaffold materials involved (right). (**a**) In the study by Schwartz et al. [25] and in additional trials such as the Cell Cure trial (Table 11.1), human embryonic stem cell (hESC)-derived RPE cells in suspension are injected into the subretinal space in the macular region. hESC-RPE cells in suspension do not initially form a polarized monolayer, but may over time layer out and adopt the correct polarity by intracellular trafficking of the appropriate proteins. (**b**)

[39]. Natural scaffolds include human lens capsule [40–43], extracellular matrix-coated basement membrane [44–46], human amniotic membrane [30, 47, 48] and Descemet's membranes [49]. Human lens capsules are highly available endogenous basement membranes composed mainly of collagen type IV, laminin, and fibronectin and were shown to be biologically tolerated as a RPE scaffold [43]. Preliminary tests showed that RPE cells well adhere and survive on human lens capsule in culture, enabling the cells to create tight junctions more efficiently than on hydrogel scaffolds [40, 41, 43]. In addition, this tissue was found to have the potential to act as a substitute to BM, which is also damaged in AMD [42]. Human amniotic membranes have been in clinical use for many indications [50, 51], and in particular in ophthalmology [52, 53]. They were found to be well tolerated subretinally and reduce choroidal neovascularization [47]. All naturally occurring membranes that were investigated demonstrated high ability to support RPE growth and function, and to preserve the polarized monolayer structure of RPE cells grown upon them [40, 41, 47–49, 54] (Fig. 11.1f demonstrates schematic of the proposed use of a gelatin-embedded ESC-RPE patch on an amniotic membrane in patients with Leber congenital amaurosis [30]).

Natural polymer scaffolds were also investigated for their potential to serve as a scaffold for RPE growth and delivery. These include collagen [55], gelatin films [56], hydrogel [41], basement membrane explant layers [44, 57], cryoprecipitate [58], bacterial cellulose [59], microspheres of cross-linked fibrinogen [60], and silk fibroin [61–64]. Overall, they showed diverse potential in terms of cell survival and biocompatibility [58, 65, 66], but in general were less effective than natural scaffolds [41] and were associated with a serious risk of inducing significant inflammatory responses leading to transplant rejection and cell death.

A third group of scaffolds are the synthetic polymers such as poly lactic-co-glycolic acid (PLGA) [18, 67], polydimethylsiloxane (PDMS) sheets with laminin coating (PDMS-PmL) [68], poly-l-lactic and poly lactic-co-glycolic acid (PLLA/PLGA) [29, 69, 70], methacrylate/methacrylamide copolymer (PEGDMA) hydrogel [41], mesh-supported submicron parylene-C membrane (MSPM) [71], polyester [26], poly(-ε-caprolactone) (PCL) [72] and parylene C synthetic material [27] (Fig. 11.1). Studies have shown the effectiveness of these artificial scaffolds in maintaining normal RPE function and morphology, and some of them have advantages that include biodegradability, good microstructure control enabling selective transport, cell adhesion, and tissue support. On the other hand, the biodegradable materials may carry a higher risk of provoking subretinal

Fig. 11.1 (continued) Da Cruz et al. [26] transplanted an hESC-RPE patch over the area of acute choroidal neovascularization (CNV) in two patients. This 3 × 6-mm transplant was intended to help in stopping CNV growth and activity while rescuing photoreceptors that are still viable in this area. (**c**) Kashani et al. [27] used an hESC-RPE patch on a parylene scaffold, transplanted subretinally in the area of geographic atrophy (GA). Shifting of the preferred retinal location for fixation to the area of the hESC-RPE patch was observed in three patients, suggesting that the patch was able to recover and support activity of some photoreceptors in the transplanted region. (**d**) Mandai et al. [28] tested the first autologous iPSC-RPE patch in an acute wet (neovascular) AMD patient. This patch was transplanted in a macular region that was fibrotic due to chronic vessel leakage. One-year follow-up with this patient revealed the absence of new leaks. (**e**) Sharma et al. [29] propose to transplant an autologous iPSC-RPE patch using a poly-(lactic-coglycolic) acid (PLGA) scaffold at the border of the GA lesion. This patch is intended to cover parts of the transition zone where the PRs are still alive to slow down or halt the expansion of the GA lesion. (**f**) Ben M'Barek et al. [30] propose to test a gelatin-embedded hESC-RPE patch on an amniotic membrane in patients with Leber congenital amaurosis (LCA). This patch will be transplanted on top of dysfunctional native RPE cells such that over the long term, the new RPE patch will integrate into the host RPE monolayer in place of diseased cells [Adapted and printed by permission from the review by Sharma R, Bose D, Maminishkis A, and Bharti K.: Retinal Pigment Epithelium Replacement Therapy for Age-Related Macular Degeneration: Are We There Yet? Annu Rev. Pharmacol Toxicol. 2020 Jan 6;60:553–572. doi: https://doi.org/10.1146/annurev-pharmtox-010919-023245. PMID: 31914900]

inflammatory reactions as compared with nondegradable materials [73]. In order to gain the synthetic scaffold properties on the one hand and the biocompatibility of the natural scaffolds on the other hand, combination scaffolds were created including chitosan-graft-poly(varepsilon-caprolactone)/polycaprolactone (CS-PCL/PCL) [74], Antheraea pernyi silk fibroin (RWSF), polycaprolactone (PCL), and gelatin (Gt) [75]. RPE cells grew and differentiated on these scaffolds without inflammatory response or rejection [75, 76].

The second strategy of RPE replacement therapy is delivery as a cell suspension, which was shown to be successful in terms of PR rescue [77] and phagocytosis activity [78]. The cells can be injected into the subretinal space following vitrectomy using a narrow gauge cannula with minimal surgical trauma, and transchoroidal injections (penetrating into the subretinal space from the choroidal side after advancing a cannula via the suprachoroidal approach) have also been used [79]. Healing following delivery of cells in suspension is rapid, there is no need to leave silicone oil or gas in the eye, which is required when scaffolds are inserted via relatively large retinotomies and collateral retinal injury is minimal. However, the cells are initially not in their correct polarity and may form cell aggregates and clumps in animal models and humans [25, 38]. Over time, the cells do seem to layer out and the fact that they can maintain viable PRs above the grafts in both animal models and in human patients supports the view that they are functional. In general, if only RPE transplantation is performed, both forms of RPE cell transplantation, whether as a cell suspension or as a monolayer on different types of scaffolds can be considered, and both show good safety profiles in terms of tumorigenicity and teratoma formation [38, 80]. In the future, if composite RPE and PR grafts will be developed, delivery on scaffolds will probably be required.

11.1.3 Surgical Approach

The surgical technique used to deliver the cells may affect their therapeutic potential and function. Subretinal transplantation of RPE sheets is a challenging surgical procedure, which requires formation of relatively large retinotomies, the use of special instruments and induction of large areas of retinal detachment to deliver the scaffold. In addition, the subretinal manipulation of the sheets and scaffolds may injure the remaining PRs. In these surgeries, the retinotomies usually are sealed using laser photocoagulation retinopexy and silicone oil (that later needs to be removed) is often used at the end of surgery. In contrast, retinotomies caused by injection of RPE cell suspension using 38–41 gauge needles are self-sealing [81]. This technical difference increases the risk of postoperative retinal detachment and epiretinal membrane for RPE sheet implantation and perhaps demands better surgical skills than subretinal injection of cells in suspension, especially in cases in which multiple areas of geographic atrophy need to be treated [81]. No standardized concentration or volume of transplanted cells for either cell formulation was established, and most trials in animal models and humans use a scale-up strategy in order to identify the optimal number and volume to be used. It was found that approximately 60,000 cells are needed to cover the macular area [82], but a larger number may need to be delivered as viability following delivery is partial.

The subretinal transplantation of RPE cells, both as cells in suspension and as sheet formulations, can be accomplished using two main routes [83]:

1. Internal/trans-vitreal [84]—based on pars plana vitrectomy (PPV).
2. External/trans-scleral [85, 86]—through the choroid and Bruch's membrane without penetrating the retina itself.

Many factors influence the choice of surgical approach including eye size, rigidity of the

implant, cell properties, and surgical abilities. To date, RPE cells in suspension were implanted mainly using the trans-vitreal approach. This procedure is considered minimally invasive and traumatic and is based on creation of a subretinal bleb of localized retinal detachment following PPV. The retinotomies can be made using 38–41 gauge needles, which form self-sealing holes [25, 81, 87, 88]. Some surgeons prefer forming a pre-bleb using a solution (such as buffered saline solution, BSS) or air, followed by injection of the cell suspension. Of note, a small air bubble may assist in preventing cell reflux [89]. The complications of this strategy are similar to the well-known ones of PPV surgery in addition to PR atrophy, subretinal hemorrhage and secondary choroidal neovascularization due to bruch's membrane rupture. Also, increased risk of epiretinal membrane formation and proliferative vitreoretinopathy (PVR) were described, which may be related to RPE cell reflux into the vitreal cavity during and after the injection.

Several devices have been developed in order to transplant RPE sheets using a trans-vitreal approach [90–92]. In general, most trans-vitreal transplantation procedures are based on performing a standard 3 port vitrectomy followed by formation of a localized retinal detachment bleb by injection of BSS into the subretinal space. Afterward, a retinotomy is created (usually at the border of the bleb), and the sheet of cells is delivered into the bleb using various devices, needles or manipulators, including devices that allow to partially roll the sheets in order to minimize the size of the retinotomies [90–92]. Finally, laser is often applied along the retinotomy, an air-fluid exchange is performed and intravitreal tamponade using either gas or silicone oil is left in the eye [91, 93–95].

In many animal studies and in one human clinical trial [91–93, 96], subretinal delivery of cell preparations in suspension was performed via a trans-scleral, trans-choroidal approach. In rodents and particularly rats (such as the RCS rat model), in view of the large size of the lens and small vitreal cavity, this is often the preferred mode of delivery. In a study delivering umbilical cord-derived cells to the subretinal space in patients with AMD, a microcatheter inserted into the suprachoridal space through a peripheral scleral cutdown was used to reach the macular area and then a small needle was advanced to penetrate into the subretinal space from the choroidal side. The cells were then injected following formation of a small pre-bleb. The rate of surgical complications including inadvertent retinal perforations into the vitreal space and retinal detachments was high, leading the authors to conclude that this surgical approach requires improvement [79]. This study is further described in section 11.2(b), and indeed a modified device for subretinal delivery via the trans-choroidal approach is being developed by Orbit Biomedical and currently being tested in a hESC-derived RPE clinical trial conducted by Cell Cure Neurosciences Ltd. (NCT01226628, unpublished data).

The different routes of delivery share complications associated with the surgical technique including retinal detachment, PVR, subretinal hemorrhage, PR injury secondary to mechanical disruption, flow, and toxicity. Specific potential complications of the trans-scleral trans-choroidal approach include severe rupture of Bruch's membrane, retinal breaching, choroidal trauma, suprachoroidal hemorrhage, and an increased risk of inflammatory and immune responses [97].

11.2 Overview of Clinical Trials

Currently, transplantation of RPE cells from various sources, via different modes of delivery and at different stages/forms of disease, form the main efforts for treatment of AMD that are already being tested in early clinical trials (Table 11.1). These efforts and studies will be the focus of this section. Other cell types including bone marrow and umbilical cord-derived cells and neural stem cells were tested in a limited fashion in a small number of trials and will also be described. Attempts to transplant PRs or PR-progenitors have not yet matured to clinical trials in AMD, but retinal progenitor cells (RPCs) derived from fetal tissue are being transplanted into the vitreous

Table 11.1 RPE transplantation in AMD: ongoing clinical trials

NCT Number	Title	Interventions	Sponsor/ collaborators	Age	Phases	Enrollment	Study Type	Start Date	Primary completion date	Locations
Active recruiting										
NCT02286089	Safety and efficacy study of hESC-derived RPE in suspension (OpRegen) for treatment of advanced dry-form age-related macular degeneration	Dry AMD with GA	Lineage cell therapeutics| CellCure neurosciences Ltd.	50 years and older	Phase I/IIa	24	Interventional	April 2015	December 2020	Retina Vitreous Associates Medical Group, Los Angeles, California, United States| Byers Eye Institute, Stanford School of Medicine, Palo Alto, California, United States| Retinal Consultants Medical Group, Sacramento, California, United States| West Coast Retina Medical Group, Inc, San Francisco, California, United States| Hadassah Ein Kerem University Hospital, Jerusalem, Israel|Rabin Medical Center, Petah Tikva, Israel|Tel Aviv

(continued)

Table 11.1 (continued)

NCT Number	Title	Interventions	Sponsor/ collaborators	Age	Phases	Enrollment	Study Type	Start Date	Primary completion date	Locations
										Souraski Medical Center, Tel Aviv, Israel
NCT03046407	Treatment of dry age-related macular degeneration disease with retinal pigment epithelium derived from human embryonic stem cells	Dry AMD	Chinese Academy of Sciences|The First Affiliated Hospital of Zhengzhou University	55 years to 80 years	Phase I/IIa	10	Interventional	September 2017	January 2019	The first affiliated hospital of Zhengzhou university, Zhengzhou, Henan, China
NCT02755428	Subretinal transplantation of retinal pigment epithelium in treatment of age-related macular degeneration diseases	Dry AMD	Chinese Academy of Sciences|Beijing Tongren Hospital	55 years to 80 years	Phase I/IIa	10	Interventional	January 2018	January 2019	Beijing Tongren Hospitol, Capital Medical University, Beijing, Beijing, China
Active not recruiting										
NCT02868424	Treatment of age-related macular degeneration by fetal retinal pigment epithelial cells transplantation	Dry AMD	The First Affiliated Hospital with Nanjing Medical University	55 years to 90 years	Phase I	6	Interventional	February 2016	August 2018	The First Affiliated Hospital with Nanjing Medical University Nanjing, Jiangsu, China

NCT02590692	Study of subretinal implantation of human embryonic stem cell-derived RPE cells in advanced dry AMD (monolayer on parylene membrane)	Dry AMD	Regenerative patch technologies, LLC	55 years to 85 years	Phase I/IIa	16	Interventional	October 2015	July 2019	Regenerative Patch Technologies, LLC, California, United States
NCT01691261	A study of implantation of hESC-derived RPE (monolayer on polymer scaffold) in subjects with acute wet age-related macular degeneration	Wet AMD	Pfizer| University College, London	60 years and older	Phase I	2	Interventional	June 2015	December 2019	Moorfields Eye Hospital NHS Foundation Trust, London, United Kingdom
NCT02749734	Clinical study of subretinal transplantation of hESC-derived RPE (suspension) in treatment of macular degeneration diseases	Macular degeneration| Stargardt's macular dystrophy	Southwest Hospital, China	18 years to 75 years	Phase I/IIa	15	Interventional	May 2015	December 2019	Southwest Hospital, Chongqing, Chongqing, China
NCT02463344	Long term follow up of subretinal transplantation of hESC	AMD	Astellas Institute for Regenerative Medicine|	55 years and older	Long term follow up of phase I/IIa trial	11	Observational	July 2012	December 2019	Jules Stein Eye Institute, UCLA School of Medicine, Los Angeles,

(continued)

Table 11.1 (continued)

NCT Number	Title	Interventions	Sponsor/ collaborators	Age	Phases	Enrollment	Study Type	Start Date	Primary completion date	Locations
	derived RPE (suspension) in patients with AMD		Astellas Pharma Inc.							California, United States| Bascom Palmer Eye Institute, Miami, Florida, United States| Mass Eye and Ear, Boston, Massachusetts, United States| Wills Eye Institute-Mid Atlantic Retina, Philadelphia, Pennsylvania, United States
Completed studies										
Not available	Adult retinal pigment epithelial transplantation (allogeneic) in exudative age-related macular degeneration	Exudative AMD	Washington University School of Medicine in St Louis, Missouri, USA	60 years and older	Phase I	12	Interventional	1996	1998	Washington University School of Medicine in St Louis, Missouri, USA
Not available	Transplantation of RPE (fetal RPE) in age-related macular degeneration: Observations in disciform	AMD	Karolinska Institute, Stockholm, Sweden	Not available	Phase I	13	Interventional	1994	1996	Karolinska Institute, Stockholm, Sweden

	lesions and dry RPE atrophy									
Not available	Allogenic fetal retinal pigment epithelial cell transplant in a patient with geographic atrophy	Dry AMD	Johns Hopkins University School of Medicine	65 years	Phase I	1	Interventional	1997	1999	Johns Hopkins University School of Medicine
Not available	Autologous peripheral retinal pigment epithelium translocation in patients with subfoveal neovascular membranes	Subfoveal neovascular AMD	The Rotterdam Eye Hospital, Schiedamsevest 180, 3011 BH, Rotterdam, Netherlands	37 years to 92 years	Phase I	8	Interventional	February 2001	July 2002	The Rotterdam Eye Hospital, Schiedamsevest 180, 3011 BH, Rotterdam, Netherlands
Not available	A comparison of autologous transplantation of retinal pigment epithelium (RPE) monolayer sheet graft with RPE-Bruch's membrane complex graft in neovascular age-related macular degeneration	Subfoveal neovascular AMD	Peking University Third Hospital, Beijing, China	Not applicable	Not available	80	Retrospective	March 2004	August 2013	Peking University Third Hospital, Beijing, China
NCT00401713	Transplantation of autologous RPE versus translocation of	AMD and submacular CNV	The Ludwig Boltzmann Institute of Retinology and	50 years and older	Not applicable	40	Interventional	February 2004	September 2008	Department of Opthalmology, Rudolf Foundation

(continued)

Table 11.1 (continued)

NCT Number	Title	Interventions	Sponsor/ collaborators	Age	Phases	Enrollment	Study Type	Start Date	Primary completion date	Locations
	autologous RPE and choroid in AMD		Biomicroscopic Laser Surgery							Clinic, Vienna, Austria
Not available	A study of transplantation of autologous induced pluripotent stem cell (iPSC) derived retinal pigment epithelium (RPE) cell sheet in subjects with exudative age-related macular degeneration	Exudative AMD	RIKEN Laboratory of retinal regeneration, Kobe, Hyogo, Japan	50 years and older	Not available	2	Interventional	October 2013	2014	Kobe City Medical Center General Hospital, Kobe, Hyogo, Japan

Based on information in clinical trials website (https://www.clinicaltrials.gov/; accessed October 1st, 2019)

(jCyte, NCT03073733) and in the subretinal space (ReNeuron, NCT02464436) in patients with retinitis pigmentosa.

(a) **RPE cell replacement**

For RPE replacement therapy in humans, attempts have been made using RPE cells from the following main sources:

I. Allogeneic adult RPE: Harvested from adult cadaver eyes
II. Allogeneic fetal RPE: Harvested from aborted fetus eyes
III. Autologous RPE: Obtained from the same eye of the same patient
IV. Allogeneic ESC-derived RPE: Generated in vitro from embryonic stem cells
V. Autologous iPSC-derived RPE: Generated in vitro from induced pluripotent stem cells derived from the same patient
VI. Allogeneic iPSC-derived RPE: Generated in vitro using HLA-matched, non-matched, or a universal donor induced pluripotent stem cells

In the following sections, these approaches and the relevant clinical trials will be detailed.

I. Allogeneic Adult RPE

The idea of "patching" or "bandaging" the area of choroidal neovascularization in NVAMD was raised and explored two decades ago. In the 1990s, much before researchers differentiated RPE cells from ESCs or iPSCs, retinal surgeons were testing allogeneic adult RPE transplants in the eyes of AMD patients [12–14]. Patients with CNV were considered an ideal choice for two reasons: (1) for lack of better treatment, subretinal CNV removal surgeries were a common practice, performed to try and arrest the neovascular process, (2) because of the rapid onset of symptoms in NVAMD, at least some photoreceptors were usually still viable when patients would come to the clinic with signs of vision loss. The rationale was that in addition to removal of the CNV, if the area of CNV could be "patched" with a "new" RPE sheet, this would stop further bleeding and protect overlying photoreceptors from degenerating. The easiest source of RPE tissue available at that time was from cadaver eyes. Since this was an allogeneic source, it required heavy immunosuppression limiting the success of this procedure. Tezel et al. reported a clinical trial of 12 NVAMD patients in which along with surgical removal of subfoveal CNV, a sheet of adult allogeneic RPE was transplanted [98]. No significant change in best-corrected visual acuity, contrast sensitivity, or reading speed was observed in the follow-up visit after 1 year. All patients were administered a triple immune-suppression drug regimen employed for renal transplantation both preoperatively and postoperatively. Five out of the twelve patients were able to continue the immune-suppression regimen for 6 months post-surgery and no sign of transplant rejection was observed in these patients during this period. The immunosuppression regimen had to be stopped midway in the remaining seven patients because of development of serious adverse effects. Soon after immunosuppression was stopped, at 6 months or before 6 months, intragraft fibrosis was observed in all patients, indicating that systemic immunosuppression is required for allogeneic RPE transplant. Primarily because of immunosuppression-related complications and a rather invasive procedure that resulted in moderate success, this form of treatment for NVAMD was not frequently used. Additional pertinent complications observed were PVR and transplanted cell migration to extrafoveal locations. Nevertheless, this study helped to establish that transplantation of RPE sheets was a feasible approach as a potential treatment for AMD patients.

II. Allogeneic Fetal RPE

Alongside allogeneic adult RPE transplants, attempts were also made to transplant RPE cells from fetal eye tissue (fRPE) [99, 100]. Using microarray-based analysis, the mRNA expression profile of cultured fRPE was found to closely resemble both native fetal and native adult RPE cells [101]. Initial results with encouraging outcomes led to the suggestion that PR rescue was likely induced by cytokines secreted by fRPE cells [101–103]. Algvere et al. (in a study

that included 13 patients), reported that fRPE transplants were relatively better accepted by non-exudative AMD patients even without immunosuppression. However, multiple cases with leakage on fluorescein angiography and fibrosis were still observed. Similar observations were made by Weisz et al. (1 patient) [100]. In both of these studies, no change in vision of the patients was observed in the follow-up visits. Further use of fRPE was limited because of two main concerns and limitations: ethical and lawful procurement of fRPE is one major concern, and limited amount of material obtained from a given donor eye, combined with limited ability to amplify these cells, prevent scaling up of this potential therapy. Recently, attempts have been made to optimize fRPE culture conditions for increased production of cells [104]. This then prompted researchers in China to conduct a prospective study, which included six AMD patients treated by three different doses of fRPE cells ranging from 100,000 to 500,000 cells (NCT02868424). Results of this study are not yet publicly available. The use of fetal RPE cells, which seemed to fare better than allogeneic adult RPE cells, suggested that success of transplant may depend on the source of RPE cells and that "young" RPE cells may provide an advantage.

III. Autologous RPE

The limited success of allogeneic RPE transplants when not accompanied by aggressive immunosuppression confirmed the notion that the diseased eye, and particularly in the case of NVAMD, may not be immune-privileged [56, 104, 105]. Although improved transplant survival was observed with long-term systemic immunosuppression [7, 98, 106], this regimen led to severe systemic side-effects in the elderly AMD patients. Autologous transplantation, utilizing cells of the host, can prevent rejection and circumvent the need for such immunosuppressive treatment and its complications.

Autologous RPE transplantation was pioneered in 2002 by Binder and colleagues in NVAMD patients [107, 108]. Following vitrectomy and CNV excision, a subretinal transplant of at least 1500 autologous RPE cells was delivered. The autologous RPE cells for this purpose were harvested from the nasal side of the optic disc. Improved multifocal electroretinographic (mfERG) response density was observed in the treatment group at 3 and 6 months post-transplantation, as compared with patients treated by CNV excision alone, without autologous RPE transplant. Reading acuity also improved in these patients. However, no significant improvement was detected in distance acuity and the improvement of mfERG responses was not maintained at longer follow-up visits [107]. It is possible that the transient improvement of mfERG responses observed was due to excision of the CNV and control of the subretinal neovascular process, and/or transient cytokine secretion from the transplanted RPE cells. Although RPE cells transplanted in suspension were able to orient themselves to form a monolayer in vivo, it is possible that because of AMD-related changes in Bruch's membrane, not all transplanted RPE cells formed a polarized monolayer [45]. If RPE cells do not form a monolayer, these non-adherent cells, in the long term, can either form aggregates or undergo apoptosis [38, 57].

Considering the importance of forming a polarized monolayer by attaching to Bruch's membrane, in some studies adhesion promoting supplements were co-transplanted with the autologous RPE cells. In a study by van Meurs et al., poly-L-lysine, which when absorbed to a substrate (in this case Bruch's membrane) increases the number of positively charged sites available for cell binding, was injected in the subretinal space prior to injection of autologous RPE cells in suspension [109]. However, while vision stabilized in five of the eight patients treated in this fashion, only one showed pigmentation at the site of transplant and the other three patients developed retinal detachment secondary to PVR. The authors concluded that translocation of autologous peripheral RPE cells after membrane extraction was technically possible, but was associated with a high rate of PVR and had no measurable positive effect on functional outcome.

Because of RPE cell suspension-associated complications and the lack of long-term visual benefits, an alternative approach using autologous RPE monolayer sheets was then tested. Post CNV excision, transplantation of autologous RPE sheet transplants was attempted in NVAMD patients in a study by Lu et al. [110]. The donor RPE monolayer (relatively healthy RPE) was harvested from a peripheral site of the retina and translocated to the macula. Vision improvement was observed in this study, suggesting that autologous transplantation of intact RPE sheets could improve visual outcomes of AMD patients. However, the surgical procedure was traumatic because of the large retinotomy required, retinal detachment of the donor site and the treatment site, and the very complex and risky maneuver required to translocate the transplant.

Although, none of these early clinical interventions was safe and efficient enough to mature into a commercially approved therapy, they helped develop surgical procedures for delivery of RPE cells and RPE sheets in the subretinal space. These studies also provided sufficient proof-of-concept that RPE transplantation has the potential of slowing down AMD disease progression.

IV. Allogeneic hESC-Derived RPE

As RPE transplantation strategies were evolving, regenerative medicine based on pluripotent cells was also progressing rapidly. Stable cell lines of human embryonic stem cells (hESCs) extracted at the blastocyst phase were first established in 1998 [111] and soon thereafter protocols were developed for differentiation of hESCs to RPE cells, starting in 2004 [112–115]. Initially, protocols relied on spontaneous differentiation: investigators, while growing ESCs in culture to explore their ability to form different cell types, identified and enriched by selection pigmented cells that spontaneously appeared in culture. Characterization of these cells showed that they closely resembled RPE cells [113, 114]. In 2009, Idelson and colleagues developed a directed differentiation protocol based upon the addition of Nicotinamide and Activin A to culture media at specific time-points. This allowed enhancement of the differentiation of hESCs into RPE [19]. Additional protocols were consequently identified by multiple groups to developmentally guide ESCs into retinal and RPE lineages by the use of other specific growth factors. This change not only improved the differentiation efficiency, it also improved reproducibility of differentiation, homogeneity and hence safety of the product, and also likely improved functionality of derived RPE cells [112, 115, 116].

The ability to derive RPE-like cells from hESCs led to the first attempts to transplant these cells in AMD (as well as Stargardt disease) eyes. The first preliminary report by Schwartz, Lanza, and colleagues on the use of hESC-derived RPE cells in suspension to treat AMD patients with GA appeared in 2012 [25] (Fig. 11.1a), followed by a more comprehensive report of this phase I safety study in nine AMD patients (as well as nine Stargardt patients) in 2015 [117]. Following vitrectomy, spontaneously-differentiated hECS-RPE cells in suspension were administered via subretinal injection at the border area of GA lesions. Patients were given systemic immunosuppression to avoid rejection of these allografts. Although no adverse events related to transplanted cells were observed, immunosuppression related side-effects were noted. Post-surgery, transplanted cells identified by their pigmentation and by OCT changes were observed in the subretinal space in the area of transplant in some of the eyes. Visual improvement was reported in some of the eyes that received transplanted cells, but no correlation could be drawn between the density and location of transplanted RPE cells and vision improvement [81].

An additional study delivering hESC-derived RPE cells in suspension was launched in 2015 by Banin, Reubinoff and colleagues (NCT02286089). In this case RPE cells were derived from hESCs according to the directed-differentiation protocol developed by Idelson et al. [19]. To date, 16 patients with advanced dry AMD and GA were transplanted, and systemic immune suppression was used for the first

few months. The overall safety profile is good with epiretinal membranes, the majority of them mild and not requiring intervention, being the main adverse event. Various imaging observations suggest survival and possible efficacy of the cells (unpublished data).

In 2018, Da Cruz, Coffee and colleagues reported on the transplantation of an hESC-derived RPE patch in two AMD patients with severe exudative AMD [26] (Fig. 11.1b). hESCs were differentiated into RPE cells using a spontaneous differentiation protocol similar to the Schwartz et al. approach [25] (Fig. 11.1a). However, differentiated cells were seeded on a vitronectin-coated polyester sheet to form a confluent monolayer. A 6 mm × 3 mm patch of hESC-RPE on the polyester sheet was then transplanted into the subretinal space under the macula. Since this was again an allograft, patients were immunosuppressed, but the authors adopted a local immunosuppression protocol to avoid systemic side-effects. Both patients showed improved visual acuity and preferred fixation in retinal areas above the grafts. It is also worth noting that neither of the two patients showed new signs of subretinal bleeding, suggesting that the RPE patch can at least stop further incidence of CNV. One of the two patients developed an inferior retinal detachment due to PVR and there were some adverse events that were not deemed to be associated with the cells or patch, but in general safety signals were good. Overall, this two patient study provided hope that transplanting hESC-RPE on a scaffold is possible and has the potential to change disease course.

An additional study transplanting hESC-RPE grown on a synthetic scaffold in the eyes of dry AMD patients with GA was reported by the group of Kashani, Humayun and colleagues [27] (Fig. 11.1c). In this case hESC-derived RPE cells were grown on a nano-engineered parylene C scaffold and a 3.5 mm × 6.25 mm patch was transplanted into the subretinal space following vitrectomy. The parylene C scaffold used was 6 μm thick and contained multiple circular ultrathin areas of 0.3–0.4 μm to mimic the diffusion properties of Bruch's membrane. Vision improvement was observed at 4 months post-surgery in one out of the four patients who received a successful transplant and the other three did not lose vision. Microperimetry testing suggested fixation over the area of the patch in some of the patients. Furthermore, no adverse events related to the patch or the transplantation procedure were observed. The patch was found to integrate under the retina, suggesting that RPE-patch transplantation may be a viable approach for dry AMD-GA patients as well.

V. Autologous iPSC-Derived RPE

Development and advances in deriving autologous induced pluripotent stem cells (iPSC) has provided another source for RPE cells. Using iPSC technology, the patient's own cells can be reprogrammed to a pluripotent state and differentiated to an RPE fate, thereby providing an avenue for autologous therapy, which likely eliminates the requirement for immunosuppression. An autologous iPSC-derived cell therapy approach, however, does require an extremely robust manufacturing process so that RPE cells can be derived from multiple different patients in a safe and timely manner. Several reports have suggested that iPSCs may acquire potentially oncogenic mutation or chromosomal alterations during the reprograming process [118–120]. Therefore, much effort is focused on manufacturing of iPSCs that are free of such changes [118, 120]. Furthermore, the autologous iPSC manufacturing process is logistically challenging. Despite these difficulties, two autologous iPSC-RPE studies have reached clinical application.

In Japan, the group of Mandai, Takahashi and colleagues from the Riken Center for Developmental Biology initiated the first iPSC-derived RPE clinical trial [28] (Fig. 11.1d). In this case a NVAMD patient with active CNV refractory to anti-VEGF treatment was treated using an autologous iPSC-RPE patch without scaffold support [28]. A 1.3 mm × 3 mm sheet of RPE monolayer was transplanted in the subretinal space after surgical excision of the CNV. The authors noted that part of the patch folded over itself, but they were

able to deliver it into the subretinal space. No immunosuppression was given and no associated complications were observed. Following surgery additional anti-VEGF injections were not required and no new signs of subretinal hemorrhage were seen. Visual acuity did not significantly change in this one patient over 4 years of follow up [34]. Unfortunately, this trial was suspended when chromosomal alterations were detected in iPSCs generated during the manufacturing of the RPE patch for the second patient [121]. This landmark study provided the first ever autologous iPSC-RPE-patch transplant in AMD patients, but it also highlighted manufacturing challenges associated with an autologous iPSC-based therapeutic approach.

Recently, the team of Bharti and colleagues from the National Eye Institute at the NIH developed an autologous iPSC-RPE patch on a biodegradable PLGA scaffold for administration in AMD patients with GA [29] (Fig. 11.1e). This group demonstrated successful clinical-grade manufacturing of iPSCs and iPSC-derived RPE from three AMD patients, and the iPSCs were shown to be free of potentially oncogenic mutations. While a thorough analysis of differences between the Mandai et al. and Sharma et al. manufacturing processes was not presented, the authors suggested that the use of CD34+ blood progenitor cells as the source for iPSCs may have assisted in preventing occurrence of oncogenic alterations [28, 29]. In comparison, Mandai et al. used patient fibroblasts for deriving the iPSCs. Because of their progenitor cell nature, CD34+ cells retain their proliferative potential and when forced to divide under reprograming conditions may not undergo genomic stress. However, more work is required to test this hypothesis. The group also published safe integration of a 4 mm × 2 mm iPSC-derived RPE patch in a laser-induced RPE injury pig model. In these injured pig eyes, the AMD iPSC-RPE patch outperformed the control group (PLGA scaffold transplant without any cells) in protecting the overlying PRs. Furthermore, functional integration of the RPE-patch was observed, including the ability to phagocytose pig photoreceptor outer segments. This project provides additional evidence that autologous iPSC-based therapy is feasible and a Phase I/IIa clinical trial is expected to begin in the very near future.

VI. Allogeneic iPSC-RPE

Using similar iPSC technologies and RPE differentiation processes, allogeneic iPSC-RPE transplants have also being proposed, and this approach may be especially useful in relatively genetically homogenous populations in which a limited number of iPSC lines may allow immune compatibility in a high percentage of patients. In more genetically diverse populations, as with the previously listed allogeneic sources of RPE cells, the immune response to the transplanted cells will need to be addressed. One of the key underlying causes of immune rejection is the expression of HLA class I antigens by the RPE cells [122] and also their capability to turn on the expression of MHC class II antigens [123]. Some innovative ideas are being tested including generation of HLA-matched iPSC lines and universal donor stem cell lines. Studies have shown that transplantation of MHC homozygous cells in matched recipients reduces infiltration of inflammatory cells and allows reduced use of immunosuppressive drugs [124, 125]. HLA complex genes located on chromosome 6 represent one of the most polymorphic genes in the human genome. The HLA is divided into three groups of antigens: class I, class II, and class III, and each class has multiple genes allowing many possible variations. Because of this diversity, HLA homozygous iPSC banks that include multiple HLA haplotypes will have to be generated for each geographic and ethnic location. To address this concern, studies are presently ongoing to generate hypoimmunogenic iPSCs [126]. Deuse et al. [126] have shown that inactivating MHC class I and II genes and overexpressing CD47 allows iPSCs to retain their pluripotent potential while allowing to prevent an immune-response even in MHC-mismatched transplants. These findings are promising and suggest a technology to generate universal banks of iPSCs for an off-the-shelf RPE product as opposed to the lengthy, costly and

complicated process of deriving a separate product for each individual patient. However, such cells need to be further tested for complications that may be associated with their immune-cloaking such as increased risk for viral infections and formation of evasive tumors.

(b) **Bone marrow and umbilical cord derived cells**

Cell therapy in AMD has and is being attempted using additional cell types besides RPE. Palucorcel [CNTO-2476] is a preparation of human umbilical tissue-derived cells that were shown to preserve outer nuclear layer structure and attenuate visual function loss following transplantation into the subretinal space in the RCS model [127]. This prompted a clinical safety and dose escalation Phase I/IIa trial of transplantation of these cells in patients with bilateral AMD and GA (NCT01226628). Delivery of the cells to the subretinal space in this trial was performed via a trans-choroidal and not a trans-vitreal approach: a microcatheter was advanced to the posterior pole of the eye in the suprachoridal space through a peripheral scleral cutdown, and then the choroid, Bruch's membrane and RPE were penetrated and cells delivered after forming a small subretinal pre-bleb with viscoelastic. Transplantation was achieved in 33/35 patients in which surgery was attempted, and gains of $\geq$ 10 ETDRS letters and $\geq$15 letters were seen in 10 and 7 eyes, respectively. However, the rate of surgical complications was high with retinal perforation (into the vitreous) occurring in 13/35 cases and retinal detachment developing in 6/35 eyes. The authors concluded that palucorcel was well tolerated and may be associated with improvement in visual acuity, but that the surgical approach requires modification [79].

Bone marrow-derived stem cells (BSMCs) have also been transplanted in patients with AMD. In a trial in Brazil (NCT01518127) autologous CD34+ cells separated from the bone marrow were injected into the vitreous of 10 patients with dry AMD and GA. The study concluded that the procedure is safe, and is associated with significant improvements in BCVA and macular sensitivity threshold, with patients who have small areas of atrophy showing a better response. The assumption is that a paracrine effect of CD34 + cells underlies the functional improvement. It should be noted that while all patients completed the 6 month follow up, only 6 patients were evaluated at the 12 month time point [128]. Intravitreal delivery of autologous CD34+ BMSCs was also reported to be safe by Park et al., but this study included only six eyes, two of which were in patients with AMD (NCT01736059) [129].

(c) **Neural stem cells**

The human central nervous system stem cell line (HuCNS-SC, StemCells, Inc., USA) was authorized by the US Food and Drug Administration (FDA) for testing in the lysosomal storage disorder neuronal ceroid lipofuscinosis (NCL) (NCT00337636). The first-in-human clinical trial involving transplantation of a purified population of human neural stem cells for a neurodegenerative disorder was completed in 2009 when six patients with NCL underwent direct neurosurgical transplantation of allogeneic HuCNS-SCs into the cerebral hemispheres and lateral ventricles. The study showed surgical feasibility without adverse effects directly attributed to the donor cells [130]. A similar 1-year, open-label phase I study was undertaken to evaluate safety in four patients with Pelizaeus-Merzbacher disease (PMD) (NCT01005004) [131]. In addition, the same cells were used in a phase I/II trial for the treatment of thoracic spinal cord injury (SCI), conducted in 12 patients in Zurich and two North America sites (NCT01321333) and in a phase II clinical trial (NCT02163876) examining safety and efficacy of HuCNS-SC for cervical SCI. No final results were published for these studies.

Attempts were then made to examine whether these cells may be beneficial in the context of retinal degenerative disease. In vivo preclinical studies in royal college of surgeons (RCS) rats using HuCNS-SC showed photoreceptor and visual function preservation with limited proliferation, phagocytic capacity and no tumor-like

formation [132, 133]. StemCells Inc. then initiated a 1-year Phase I/II clinical trial using HuCNS-SC® human neural stem cells to treat dry AMD (NCT01632527). The study included 15 patients, divided into two sequential cohorts: cohort I included 8 patients with BCVA ≤20/400 in the study eye, who were transplanted with 200,000 (4 patients) and one million (4 patients) cells. Cohort II consisted of 7 patients with BCVA of 20/320 to 20/100 in the study eye, who underwent transplantation of one million cells. The cells were injected in one single subretinal injection. Interim results of cohort I showed a 70% reduction in the rate of geographic atrophy (GA) expansion as compared with the control eye and a 65% reduction in the rate of GA as compared with the expected natural history of the disease. In addition, a positive safety profile was observed. This study was due to end in June 2015, but after the interim results detailed here final results have not yet been posted. A long-term follow-up study over 4 years was recently terminated due to financial reasons and not due to safety concerns (NCT02137915) [134], but it is not clear whether the promising interim results were maintained in the long term.

The multiple studies and trials summarized so far are very positive for the field, as pursuing these varied approaches increases our understanding of the possibilities and challenges associated with cell therapy and particularly RPE-based therapy in AMD. There are some preliminary signs of success: long-term cell survival was seen in some cases, there is evidence of transplant-recipient tissue integration, PR rescue, and even vision improvement in few patients. Multiple obstacles were also observed, including limited tolerance for long-term systemic immunosuppression in elderly patients, cell migration and/or proliferation in the vitreous cavity, and surgical challenges that are particularly associated with delivery in NVAMD patients in which the CNV needs to be addressed/excised and also when subretinal delivery of cells on scaffolds is performed, which require a relatively large retinotomy.

11.3 What's Under Development: Preclinical Studies to Derive PRs

While RPE transplantation using various sources and techniques of delivery is already in multiple clinical trials (as detailed above), this form of treatment will be effective for preservation of vision in AMD only if performed prior to loss of the PRs. Ideally, once proven safe and effective, such transplantation will be carried out in early phases of disease, as RPE changes and small areas of RPE loss and atrophy just begin to appear. Then, the new, healthy cells will be able to provide support and sustain the still viable PRs of the host. However, in advanced stages of disease, once significant numbers of PRs have been lost, transplantation of RPE cells alone will not suffice to regain vision. This situation occurs not only in advanced AMD, but also in other retinal and macular degenerations such as retinitis pigmentosa, Stargardt disease and others. As such, multiple groups are exploring the possibility of supporting/replacing not only RPE cells but also PRs, and perhaps as combined grafts. Preliminary studies showed that multiple intrinsic factors can induce formation of RPCs, followed by differentiation into PR cells (rods or cones) and finally subtypes of PRs by expressing their specific characteristics [135–139]. Pioneering research studies by Sasai and colleagues established protocols that allow the self-organization of eyecup-like structures consisting of self-organized, complex, stratified 3D retinal tissue, which in many ways follows the path of embryonic eye development, yielding PRs and additional retinal elements [140–142]. Reh and colleagues reported the differentiation of embryonic stem cells to retinal cells [143]. This protocol was extensively modified in order to increase the efficacy of the differentiation into PRs [144, 145]. Further enhancement was accomplished with the development of 2D/3D protocols [146–149]. Gamm and colleagues differentiated embryoid bodies (EB) in suspension, cells were

plated on laminin-coated plates and then the neuroepithelial structures were grown in suspension leading to formation of optic vesicle (OV)-like structures [150]. These floating structures continued on a path of ocular and retinal differentiation, producing mature PRs [150–152]. Bi-layered optic cup-like structures developed, leading to differentiation into RPE and PRs organized in a rosette-like shape [141, 153–156]. Formation of PR outer segments in this model is very slow, but this ground-breaking progress in retinal organoid production brings us significantly closer to implementing retinal cell replacement therapy beyond RPE alone. Still, there are significant hurdles on the path of making this technique commercial and technically applicable for PR production and cell replacement therapy including large scale-production, assuring homogeneity of the product, automation of the procedure, and cryopreservation [157].

11.4 Challenges and Conclusion

While progress in the development of cell-based therapies for AMD is accelerating, major challenges still exist. The main impediments to large scale clinical translation of such treatment include the following: (i) identifying the optimal ways of delivering the transplants, especially patches, to the treatment site in the subretinal space, (ii) proving and improving survival and retention of the transplanted RPE cells at the transplant site, (iii) overcoming and managing the innate immune response in the case of allogeneic transplants, (iv) enhancing integration of the transplanted cells with recipient tissue such that in the case of RPE transplantation a polarized monolayer of RPE cells is formed and can physiologically interact with the overlying PRs, and in the case of PRs, the correct synaptic connectivity is achieved, (v) addressing and eliminating the risk of tumor formation and oncogenic transformation in stem cell- and iPSC-derived transplants. The fact that multiple groups are testing varied cell preparations and delivery methods is of much benefit at this early stage of development of cell-based therapies and increases the likelihood that safe and efficient treatments will be forthcoming.

An important cautionary note to be made is that in this current era of much "hype" that is associated with cell-based therapy, there are occasionally attempts to provide treatments that are not properly tested, regulated or approved. One of the worst outcomes in this regard occurred when three women with AMD received bilateral intravitreal injections of autologous adipose tissue-derived "stem cells" in a clinic in Florida. While a "trial" was listed on ClinicalTrials.gov (NCT02024269), it was not regulated in any manner and was not FDA approved. Furthermore, the patients paid for the treatment. Within a week, the patients experienced severe complications including ocular hypertension, hemorrhagic retinopathy, vitreous hemorrhage and combined traction and rhegmatogenous retinal detachment. Despite attempts to treat the complications at other centers, all patients ultimately suffered severe and permanent visual loss, to the level of no light perception in 2/6 eyes, light perception in one eye, two eyes at hand motion acuity and one eye at 20/200 [158]. Additional "stem-cell clinics" are apparently treating patients using unproven and unregulated therapies, and it is important to warn patients not to fall for such bogus "trials" [159].

In summary, there is good reason to believe that cell-based therapy and especially stem cell-based treatments are poised to become the next big revolution in medicine in general and in the eye and retina in particular. Indeed, retinal and macular degenerations, with emphasis on AMD, are currently the "testing ground" for these novel therapies that carry the potential to support and replace dysfunctional and degenerating retinal cells, with RPE cells being a main target. Preliminary results of the efforts and trials described here provide hope that better treatments for these blinding diseases are forthcoming.

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Current Management of Age-Related Macular Degeneration

12

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Abstract

Age-related macular degeneration (AMD) remains a leading cause of blindness worldwide. The assessment and management of patients with this condition has evolved in the last decades. In this chapter, current standards for diagnosis, follow-up, and treatment of patients with AMD are reviewed and summarized. Namely, we highlight how current assessment has moved from conventional ophthalmoscopy and fluorescein angiography testing to a multimodal approach, and its important advantages. Alternatives to visual acuity for functional assessment of patients with AMD are also presented. Regarding strategies for follow-up and treatment, we provide specific information for the different stages (i.e., early, intermediate, and late) and forms (for example, choroidal neovascularization and geographic atrophy) of AMD. Specifically, we discuss the relevance and options for self-monitoring and non-pharmacological interventions. Additionally, a summary of the important trials (both on exudative and non-exudative AMD) that have helped inform clinical practice is provided, including data on antiangiogenic agents currently available, and outcomes of the different regimens that have been studied. The influence of advances in imaging on treatment strategies is also discussed.

In summary, this chapter is a resource for all clinicians engaged in providing *state of the art* care for patients with AMD, and can help improve diagnosis, management, and outcomes of individuals with this blinding condition.

Keywords

Age-related macular degeneration · Diagnosis · Disease management · Choroidal neovascularization · Geographic atrophy · Intravitreal injections · Office visits · Optical coherence tomography · Photodynamic therapy · Visual acuity

12.1 Current Standards for Diagnosis and Assessment of Non-Exudative AMD

Age-related macular degeneration (AMD) has historically been diagnosed based on a dilated fundus exam, and this remains the gold standard. All current, validated AMD classification schemes are based on color fundus photographs (CFP). Multiple grading systems have been proposed, but there is no universal consensus. The

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most widely accepted grading systems include the Age-Related Eye Disease Study (AREDS) classification scheme [1] and severity scale [2], the International Classification [3], and, more recently, the clinical classification developed by the Beckman Initiative [4]. These classifications differ in the criteria used to define the presence of AMD, and the early and intermediate stages of the disease (i.e., drusen number and sizes). These differences have an impact both on clinical practice and on research. For example, the absence of a clear definition of when AMD is present (versus normal aging) is one of the reasons for the failure to diagnose AMD in an important number of cases. A recent study [5] looked at a group of adults 60 years or older considered to have normal macular health in both eyes according to a dilated eye examination by primary care ophthalmologists and optometrists. The authors found that approximately 25% of these eyes had macular characteristics consistent with AMD, as assessed by fundus photography and trained graders. A clear and unified definition of AMD, and a single standard and accepted classification system, would facilitate the diagnosis of this condition, patients' follow-up, and assessment of outcomes. The implementation of retinal imaging modalities in primary eye care settings, as well as the ongoing development of artificial intelligence applied to images of patients with AMD [6, 7], could also contribute to improve the current underdiagnosis of this condition.

The hallmark findings of non-exudative AMD are macular drusen and focal pigmentary changes, which are present across all stages and forms of AMD [1]. Classic drusen are histologically located between the retinal pigment epithelium (RPE) and Bruch's membrane, and appear as focal, whitish yellow excrescences deep to the retina. In general, drusen are considered by their size. They can be round and discrete, measuring less than 63 μm (small drusen); medium-sized drusen, 63 to less than 125 μm; and soft, which are ill defined, with non-discrete borders, measuring 125 μm or greater [1]. Small or hard drusen are commonly identified in many populations, and do not carry an increased risk for the development of neovascularization [8]. Medium-sized drusen carry a low risk of developing late AMD [9]. In contrast, large, soft, confluent drusen are age-related and associated with AMD and a higher risk for developing advanced AMD [9]. Focal pigmentary changes also have been associated with an increased risk of developing soft drusen and geographical atrophy [9, 10].

Advances in imaging over the years have enabled a greater understanding of disease pathophysiology and have offered important diagnostic value. Among available imaging modalities, optical coherence tomography (OCT) is one of the most widely used, and has served as an essential adjunct in monitoring non-exudative AMD [11–13]. OCT is a non-invasive imaging method capable of providing cross-sectional images of the retina, RPE, and choroid. The initial devices were time-domain and had limited resolution capacity. However, spectral-domain OCT, now widely used worldwide, provides high-quality and high-resolution imaging, and thus has a crucial role not only in the initial diagnosis and prognostic assessment of patients with AMD, but also in follow-up [14]. For example, OCT enables detection of classic drusen, changes in their overall volume, as well as evaluation of retinal and RPE thickness both qualitatively and quantitatively (automated algorithms for quantification are available with the Cirrus OCT, Carl Zeiss Meditec, CA, USA). Additionally, OCT has enabled clinicians and researchers to identify lesions of prognostic value. Examples include subretinal drusenoid deposits (SDD) and outer retinal tubulations. SDD [15, 16] have been proposed as an independent risk factor for AMD progression [17]. SDD can also be identified with other imaging modalities, such as infrared and fundus autofluorescence [18, 19], but spectral-domain OCT has the highest sensitivity (95%) and specificity (98%) to identify these deposits [20]. Outer retinal tubulations, identified on OCT as a circular or ovoid hyperreflective band around a hyporeflective core located in the outer nuclear layer [21], appear in cases of advanced disruption of the outer retina, but have been associated with a slower rate of enlargement of geographic atrophy (GA) lesions [22]. Importantly, in eyes with neovascularization, the

hyporeflective lumen of these lesions may be misdiagnosed as intraretinal or subretinal fluid. Their recognition is important to avoid unnecessary treatment. Other qualitative and quantitative OCT features, such as ellipsoid zone disruption, drusenoid RPE detachment, or RPE drusen volume, have also been suggested as potential OCT biomarkers for risk of AMD progression to advanced AMD [23–25].

The assessment of geographic atrophy, one of the forms of late AMD, has also changed over time. Classically, GA has been defined based on CFP, where it is seen as one or more well-delineated areas of hypopigmentation or depigmentation due to absence or severe attenuation of the underlying RPE [1]. The large, deep choroidal vessels are usually readily visualized in these areas. Different classification schemes consider different criteria in terms of size and foveal involvement, as recently reviewed by the Classification of Atrophy Consensus (CAM) group [26], a consortium of retina specialists. However, advances in retinal imaging technology, including high-resolution OCT, have markedly improved the detection and study of GA morphology. The CAM group recently provided recommendations on the use of imaging modalities to detect and quantify atrophy [27]. The authors highlighted that the imaging protocols to detect, quantify, and monitor progression of atrophy should include CFP, as well as confocal fundus autofluorescence (FAF), confocal near-infrared reflectance (NIR), and high-resolution OCT volume scans. Despite being originally developed for clinical trials, these recommendations can be easily translated to clinical practice. Currently, FAF imaging together with OCT are the most commonly used modalities [27]. Figure 12.1 presents an example of progression of GA demonstrated using FAF images.

12.2 Current Standards for Diagnosis and Assessment of Exudative AMD

The late forms of AMD include geographic atrophy (GA), and choroidal neovascularization (CNV), also known as "exudative AMD." Both manifestations are not mutually exclusive. GA can develop in eyes with CNV effectively treated with intravitreal anti-vascular endothelial growth factor (VEGF) injections; and CNV can appear in eyes with pre-existing GA [28, 29].

In addition to funduscopy, fluorescein angiography (FA) is the gold standard to diagnose and classify CNV [30]. Classically, three types of CNV have been described: (i) type 1 CNV, also known as occult choroidal neovascularization, which refers to new blood vessels that proliferate underneath the RPE—on FA, it presents as a fibrovascular pigment epithelial detachment (PED: an area of irregular elevation of the RPE, often with stippled hyperfluorescence present in the midphase of the angiogram and leakage or staining by the late phase) or late leakage from an undetermined source (speckled hyperfluorescence with dye pooled in the subretinal space in the late phase); (ii) type 2 CNV, or classic CNV, which is characterized by the development of new blood vessels between the neurosensory retina and the RPE—on FA, it is characterized by a bright, often "lacey," early hyperfluorescence exhibiting prominent leakage in the late phase; and (iii) type 3 CNV, also known as retinal angiomatous proliferation (RAP), which is characterized by the formation of a retinal–retinal anastomoses, which then extends beneath the neurosensory retina to become subretinal neovascularization. Indocyanine-green angiography (ICGA) provides additional information on choroidal vasculature [31], and it is recommended when polypoidal choroidal vasculopathy (PCV) is suspected [32]. Despite the ongoing debate [32], PCV is considered a variant of exudative AMD, which is characterized by the presence of orange-red nodules and serosanguinous pigment epithelial detachments on ophthalmoscopy. ICGA enables the visualization of polyps and branching vascular networks in this condition, which are often difficult to detect on FA.

Regardless of the form of neovascularization, OCT is currently considered an important adjunct to FA and ICGA, especially to monitor the presence of intraretinal and subretinal fluid over time.

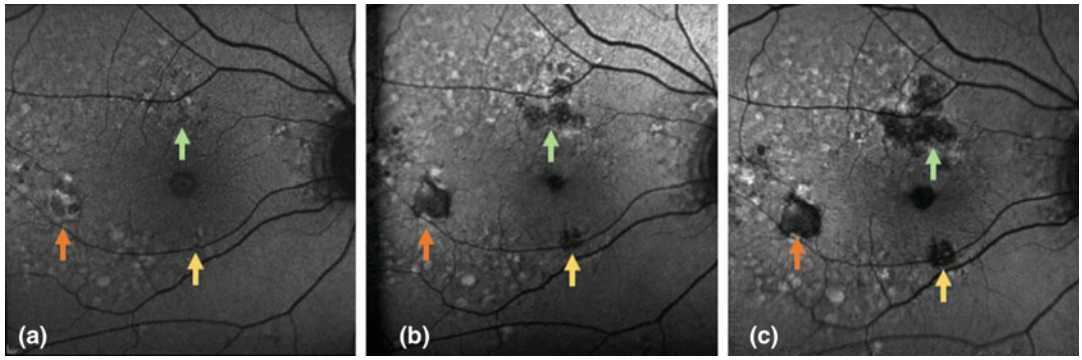

Fig. 12.1 Fundus autofluorescence images of a right eye with progression of geographic atrophy over 3 years. As shown, initial areas of hypo-autofluorescence increased in size during this time period (marked as colored arrows). (**a**) Baseline, (**b**) 2 years later, (**c**) 3 years after baseline

Indeed, data suggest that at least in the USA several clinicians currently rely solely on clinical examination and OCT to determine whether a treatment regimen is adequate in controlling disease activity [33]. Since the advent of drugs that inhibit vascular endothelial growth factor (VEGF), one of the strategies for following eyes with wet AMD has been to use OCT to guide treatment frequency based on the status of exudation in the macula.

More recently, OCT angiography (OCTA) has also been used for detailed qualitative and quantitative characterization of CNV in AMD [34, 35]. OCTA is commercially available both for spectral-domain and swept-source devices, and relies on blood flow detection based on motion contrast. Compared to FA, its greatest advantage is being non-invasive; however, it does not allow for the identification of leakage and projection artifacts can occur [36]. The full clinical value and optimal application of OCTA are still being defined [27], but it has been suggested that it enables more distinct characterization of neovascular patterns than FA, since there is less light scattering and less obscuration by overlying subretinal hemorrhages or exudation. Another interesting application includes the study of quiescent neovascular membranes, which are defined as CNV in the absence of exudation. The clinical and prognostic value of quiescent CNV remains to be established. Namely, there is still no consensus on the best approach to manage these lesions, especially if their size is increasing despite the absence of exudation. Recently, de Oliveira Dias et al. suggested that risk of exudation is greater for eyes with documented subclinical CNV on OCTA, compared with eyes without detectable CNV [37].

It is important to note that individuals with neovascularization in one eye have increased risk of developing it in the fellow-eye, so close follow-up may be warranted [38, 39]. This can include more frequent clinic visits for dilated fundus examination and retinal imaging, or by encouraging vigilant home monitoring, as described later in this chapter.

12.3 Functional Assessment of AMD Patients

Visual acuity (VA) is currently the most widely accepted and universally used functional outcome measure for AMD, both in clinical practice and clinical trials or observational studies. However, VA has well-recognized limitations in characterizing visual impairment of AMD, especially early in the course of disease [40]. VA loss typically occurs late in the disease course [41], making it a less useful measure of retinal function in early and intermediate AMD. Therefore, other

functional outcome measurements have been explored [42]. These include contrast sensitivity [43], low-luminance visual acuity, photopic or scotopic light sensitivity [44, 45], and dark adaptation (DA) [41]. DA is promising, and there is currently a commercially available U.S. Food and Drug Administration (FDA)-approved device [46]. Studies have shown that DA can differentiate AMD from healthy eyes, and has correlations to the different stages of AMD based on conventional CFP classification schemes [41, 46, 47]. More recently, an association between AMD features identified on OCT and time to dark-adapt has also been described, including the presence of SDD and ellipsoid changes [48]. Figure 12.2 shows an example of an eye with SDD and prolonged time to dark-adapt.

12.4 Management of Non-Exudative AMD

There are currently no proven therapies for the non-exudative form of AMD or limited options to halt progression from the early/intermediate AMD stages to late disease. Certain behavioral modifications may be beneficial in reducing risk of advanced AMD. Smoking is considered the most important modifiable risk factor for AMD [49–51]. Smoking cessation should be strongly recommended to patients since the risk of developing AMD in individuals who have not smoked for more than 20 years is comparable to the risk in nonsmokers [52]. There is also extensive literature on dietary interventions that may be beneficial [53, 54]. In general, a diet similar to the Mediterranean diet, rich in fruits, vegetables, and fish, is recommended. Other risk factors, such as hypertension or obesity, have also been linked to AMD risk, but available data are inconsistent [53, 55]. Considering the benefit of controlling these risk factors for reduction in cardiovascular risk, patients may be advised to discuss these risk factors and their appropriate management with their primary care physicians.

For patients with intermediate AMD, the dietary supplements studied by the Age-Related Eye Disease Study (AREDS) group are recommended. The initial AREDS trial evaluated the effect of daily oral therapy with high doses of vitamin C (500 mg), vitamin E (400 international units), beta-carotene (15 mg), zinc (80 mg as zinc oxide), and copper (2 mg as cupric oxide), and showed that, in patients with intermediate AMD in at least one eye, the formula was able to reduce the progression to advanced AMD by 25% at 5 years [1]. The AREDS 2 trial followed [56] to investigate the role of omega-3 fatty acids in reducing progression of AMD and whether beta-carotene was necessary for efficacy due to concerns of possible associations with lung cancer in smokers. A new formulation was proposed, where lutein (10 mg) + zeaxanthin (2 mg) were introduced to substitute for beta-carotene. Beta-carotene was associated with a twofold increase in the risk of lung cancer. There was an incremental benefit with lutein and zeaxanthin versus beta-carotene in preventing progression to advanced AMD, especially in persons who had the lowest intake of dietary intake of lutein. When lutein and zeaxanthin were compared with beta-carotene, there was a 25% increased beneficial effect. The currently recommended formulation consists of vitamin C (500 mg), vitamin E (400 international units), lutein (10 mg) + zeaxanthin (2 mg), zinc (80 mg as zinc oxide), and copper (2 mg as cupric oxide). Recently, investigations have been performed to assess whether genotype at certain loci associated with AMD risk may impact benefit from supplementation with the AREDS formula [57–64]. These studies remain controversial and routine genetic testing prior to supplementation has not been recommended or widely adopted. Three independent groups evaluated the data from the AREDS researchers and the data from the non-AREDS researchers and concluded that there was no evidence to support genetic testing prior to initiating supplementation with the AREDS formula [65]. A prospective study would be required to determine whether there in fact is any association between genotype and response to AREDS supplementation.

For all patients with non-exudative AMD, the use of the Amsler grid [66] to assess for new metamorphopsia is recommended. Early detection of neovascular disease remains a priority. It

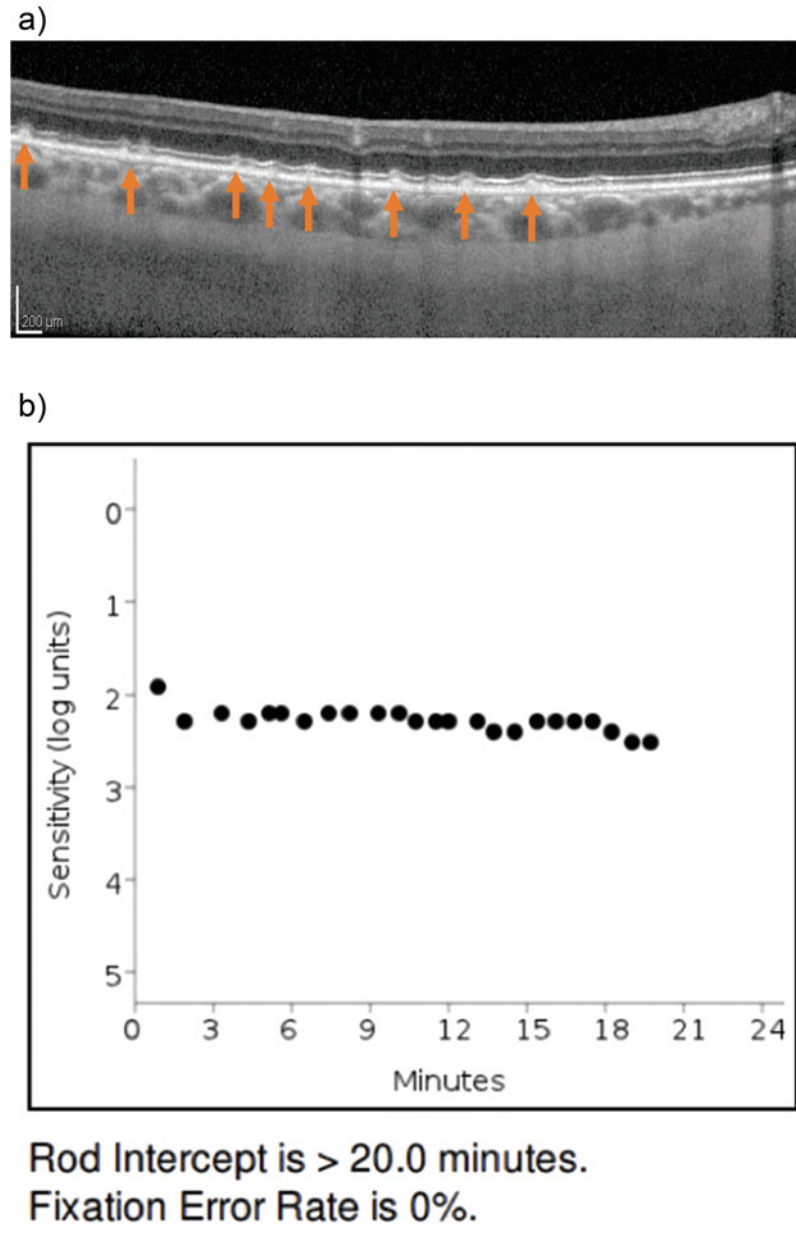

Fig. 12.2 (**a**) Optical coherence tomography showing multiple subretinal drusenoid deposits (orange arrows); (**b**) dark adaptation curve of the same eye, where it is shown that the rod intercept time is not achieved within the test period (20 min, standard available commercial test)

has been shown that treatment of choroidal neovascularization within 1 month of detecting symptoms is more likely to result in better visual outcomes [67]. Several technologies for home monitoring currently exist, including ForeseeHome™. ForeseeHome™ is a self-administered test that uses preferential hyperacuity perimetry to measure visual field defects using 500 retinal data points over 14° of a patient's central visual field. The AREDS Home Monitoring of the Eye (HOME) Study compared visual acuity at the time of choroidal neovascularization diagnosis between 1520 at-risk dry AMD patients who were randomly assigned to use the device plus standard-of-care (self-monitoring with Amsler grid and routine clinic visits) and a control group utilizing standard-of-care alone [68]. Their results showed that patients with

high risk for developing CNV may benefit from frequent and regular home-screenings with highly sensitive technology.

12.4.1 Geographic Atrophy

There is currently no approved treatment to slow or halt the progression of geographic atrophy (GA). One of the most explored potential targets for therapies has been the complement system. Although the pathophysiology of GA is incompletely understood, overactivation of the complement has been implicated in its the pathogenesis [69], and genome-wide association studies [70] have also suggested a central role of the complement system in AMD. Several clinical trials have been performed targeting different complement cascade components. The largest studies conducted to date used lampalizumab, an antigen-binding fragment of a humanized monoclonal antibody that inhibits complement factor D, and failed to show a reduction in GA enlargement as compared to sham during 48 weeks of treatment [71] (Fig. 12.3). Other complement components have also been attempted as potential targets, including with eculizumab, which also failed a phase II trial [72].

Other drugs targeting different pathways involved in GA pathogenesis have been studied and failed to show efficacy. These include attempts to modulate the visual cycle (such as with emixustat; hydrochloride [Acucela]; a small non-retinoid molecule that specifically binds and inhibits RPE65 and its active site, and fenretinide, an oral synthetic derivate of vitamin A), neuroprotection (such as with an implant producing ciliary neurotrophic factor, NT-501), and amyloid beta aggregation (with an anti-amyloid beta monoclonal antibody, GSK933776; GlaxoSmithKline) [72]. Gene and stem cell therapies have also been attempted but remain in their infancy [73, 74].

12.5 Management of Exudative AMD

12.5.1 Photodynamic Therapy

In 2000, photodynamic therapy (PDT) with verteporfin was approved as the first pharmacologic therapy for exudative AMD. It consists of a two-step procedure involving intravenous infusion of verteporfin, a photosensitizing dye that accumulates preferentially in neovascular membranes, followed by dye activation with infrared (689 nm) laser light [75]. This process results in direct cellular injury, including damage to vascular endothelial cells and vessel thrombosis; and it promotes closure of choroidal neovascular complexes, with relative sparing of the overlying retinal structures [76–78].

Two large, prospective, randomized controlled trials led to the approval of PDT for neovascular AMD: the TAP—Treatment of AMD with Photodynamic Therapy Study—Study, and the VIP—The Verteporfin in Photodynamic Therapy Study—Trial [79, 80]. The TAP Study demonstrated lower rates of moderate vision loss through 2 years in patients with predominantly classic subfoveal CNV treated with verteporfin PDT (47%) compared to placebo (62%). For occult with no classic CNV lesions, the VIP Trial showed that verteporfin PDT treatment demonstrated greater efficacy than placebo in preventing moderate vision loss (percentage of eyes losing less than 15 Early Treatment Diabetic Retinopathy Study [ETDRS] letters: 46.2% versus 33.3%) in a 24-month period. Side-effects included hemorrhage, neurosensory detachment, and choroidal infarction.

Currently, antiangiogenic therapy has largely replaced verteporfin PDT therapy as the preferred treatment modality for neovascular AMD as it achieves better visual outcomes. However, verteporfin PDT is still considered in patients with systemic or ocular contraindications for intravitreal administration of antiangiogenic drugs, and it is an important option for the

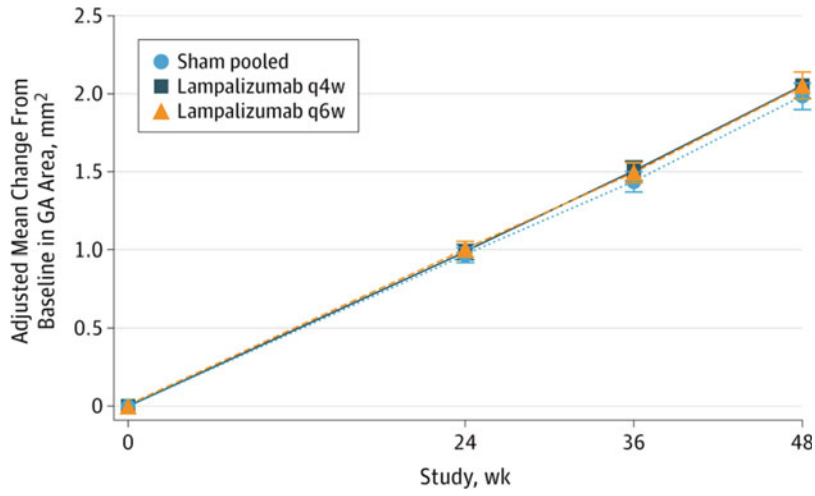

Fig. 12.3 Overall results from phase 3 trials of lampalizumab for geographic atrophy due to macular degeneration. Graph shows adjusted mean change in area of geographic atrophy from baseline to week 48 (measured on fundus autofluorescence imaging). Reprinted with permission from *JAMA Ophthalmol*. 2018 Jun 1;136(6):666–677

treatment of polypoidal choroidal vasculopathy (PCV). Figure 12.4 presents a color fundus photograph and indocyanine-green angiography of an eye with PCV. The EVEREST II trial demonstrated that verteporfin PDT combined with ranibizumab resulted in greater visual acuity improvement (8.3 versus 5.1 letters) than monotherapy with ranibizumab, and complete resolution of lesions with fewer ranibizumab injections [81].

12.5.2 Anti-VEGF Therapies

Vascular endothelial growth factor (VEGF) plays an important role in intraocular neovascularization in a number of conditions. VEGF-A acts via the VEGF receptor 2 (VEGFR2) and is thought to be the main stimulator of angiogenesis and vascular permeability in neovascular AMD [82]. Four different VEGF-A isoforms have been identified in humans as a result of alternative RNA splicing: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ [83]. Among them, $VEGF_{165}$ is the most prevalent in ocular neovascularization processes [84, 85]. In the last decade, anti-VEGF

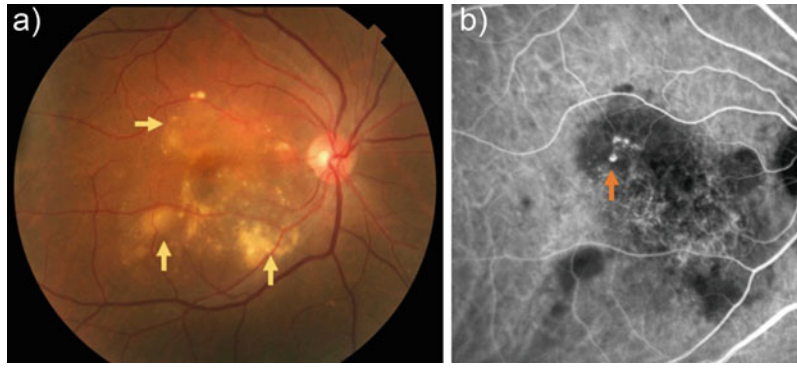

Fig. 12.4 (**a**) Color fundus photograph of a right eye with polypoidal choroidal vasculopathy, where extensive lipid exudation (yellow arrows) is observed in the macular area; (**b**) indocyanine-green angiography of the same eye, where multiple focal areas of hyperfluorescence (i.e., polyps; orange arrows) are seen arising from the choroidal circulation

therapy has become first-line treatment for neovascular AMD. Four major agents have been evaluated and widely used.

(a) *Pegaptanib*

Pegaptanib sodium (Macugen; Eyetech/Valeant Pharmaceuticals) was the first VEGF-A inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2004 for the treatment of neovascular AMD. Pegaptanib sodium is an RNA oligonucleotide ligand (or aptamer) that binds and inhibits $VEGF_{165}$ with high affinity and specificity [86]. Its approval for clinical use was based on two prospective, double-masked, randomized, controlled phase III clinical trials, known as the VEGF Inhibition Study in Ocular Neovascularization (VISION) Study [87]. In these trials, patients with neovascular AMD were randomized to receive intravitreal injections of pegaptanib sodium (0.3, 1.0, or 3.0 mg) or sham injection every 6 weeks for 48 weeks. At 2 years, there was a higher proportion of patients gaining vision for those assigned to 2 years of 0.3-mg pegaptanib than those re-randomized to discontinue pegaptanib after 1 year or receiving usual care [88].

(b) *Ranibizumab*

Ranibizumab (Lucentis, Genentech, Inc., South San Francisco, CA) was approved by the FDA in 2006 for the treatment of neovascular AMD. Ranibizumab is a 48-kilodalton (kDa) recombinant, humanized immunoglobulin G1 (IgG1) monoclonal antibody fragment (kappa isotype) that binds with high affinity to all isoforms of VEGF-A [89]. FDA approval was based on results from two landmark trials: the Minimally Classic/Occult Trial of Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA) [90] and Anti-VEGF Antibody for the Treatment of Predominantly Classic CNV in AMD (ANCHOR) [91]. The MARINA trial was a phase 3, randomized, multicenter, double-blind, sham-controlled, 2-year study. Patients with minimally classic or occult CNV secondary to AMD were randomized to receive monthly intravitreal ranibizumab (0.3 or 0.5 mg) or sham injections [90]. Overall, 95% of patients treated with ranibizumab lost less than 15 letters at 1 year compared with 62% of patients receiving sham injections. In addition, visual acuity improved by 15 or more letters in 34% of the 0.5 mg ranibizumab-treated group versus 5% in the sham-injection group at 2 years.

In phase 3, international, multicenter, randomized, double-blind ANCHOR trial, patients with predominantly classic lesions were randomly assigned to monthly intravitreal injections of ranibizumab (0.3 or 0.5 mg) plus sham verteporfin photodynamic therapy (PDT) or to verteporfin PDT plus monthly sham injections. At 1 year, 95% of those treated with ranibizumab and 64% of patients treated with PDT lost fewer than 15 letters compared with baseline [91]. In terms of visual outcomes, 41% of 0.5 mg ranibizumab-treated patients gained 15 or more letters compared with 6% of the PDT group at 2 years [92].

(c) *Bevacizumab*

Bevacizumab (Avastin, Genentech, Inc., South San Francisco, CA) is a 149-kDa full-length humanized, monoclonal IgG1 antibody that binds all isoforms of VEGF-A, and is almost three times the size of the ranibizumab molecule [93]. Bevacizumab was approved in 2004 as first-line therapy for patients with metastatic colorectal cancer, as it was shown to inhibit angiogenesis and tumor growth [93]. The first open-label prospective clinical study using intravenous bevacizumab for neovascular AMD was the Systemic Avastin for Neovascular AMD (SANA) study [94]. Eighteen participants were treated with two or three intravenous infusions of bevacizumab (5 mg/kg) at 2-week intervals. Systemic bevacizumab was associated with a decrease in central retinal thickness of 112 um and a 14-letter gain in visual acuity at 24 weeks. Ten patients developed mild hypertension that was controlled with systemic medications.The use of intravitreal bevacizumab for the treatment of exudative AMD was first described in 2005 in a 63-year-old woman with subfoveal CNV [95]. She received a single intravitreal injection of 1 mg bevacizumab. At 1 week, there was

resolution of subretinal fluid on OCT. This effect was maintained at 4 weeks [95]. Since then, intravitreal bevacizumab has gained widespread acceptance due to its effectiveness, safety profile, and its inexpensiveness compared with other anti-VEGF intravitreal therapies. Large clinical trials such as the Comparison of AMD Treatment Trials (CATT Study) and the Inhibition of VEGF in Age-related Choroidal Neovascularization (IVAN) study also showed that monthly injections of bevacizumab or ranibizumab resulted in approximately the same visual outcomes at the end of 1 and 2 years [96, 97].

(d) *Aflibercept*

Aflibercept (EYLEA, Regeneron Pharmaceuticals, Inc., Tarrytown, NY) is a 115-kDa recombinant, chimeric, decoy receptor comprised of VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) fused to the Fc portion of human IgG1 [97]. This protein binds VEGF-A, VEGF-B, and placental growth factor (PlGF) and has a 100-fold greater binding affinity for VEGF-A [98]. Aflibercept was approved in 2011 based on the VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW 1 and 2) trials [99]. VIEW 1 and 2 were two phase 3, randomized, double-blind, multicenter, non-inferiority studies that compared ranibizumab with aflibercept in patients with wet AMD. Patients were randomized to four different therapy groups: 2 mg aflibercept every 4 weeks; 0.5 mg aflibercept every 4 weeks; 2 mg aflibercept every 8 weeks; and 0.5 mg ranibizumab every 4 weeks. All treatment regimens were initiated with 3 monthly doses.
These studies demonstrated that 2 mg aflibercept injections administered every 8 weeks following a 3-month loading period had similar improvements in anatomic and visual outcomes to those obtained with monthly ranibizumab injections [99].

(e) *Brolucizumab*

Brolucizumab (Beovu, Novartis) is a 26-kDa, humanized, single-chain antibody fragment that inhibits all VEGF-A isoforms [100]. The HAWK and HARRIER trials were phase 3, randomized, double-masked, multicenter, non-inferiority studies comparing brolucizumab with aflibercept in patients with neovascular AMD. In the HAWK trial, patients were randomized to aflibercept (2 mg) or brolucizumab (3 mg or 6 mg). In the HARRIER trial, patients received either brolucizumab at 6 mg or aflibercept at 2 mg [100]. After 3 monthly loading doses, brolucizumab-treated patients received an injection every 12 weeks with the option to decrease to every 8 weeks at each disease activity assessment. Aflibercept was given at a fixed 8-week interval dose. At 48 weeks, brolucizumab was found to be non-inferior to aflibercept with respect to mean change in visual acuity from baseline (Fig. 12.5). Additionally, central subfield thickness reductions were greater in the brolucizumab arm compared to the aflibercept arm at 16 weeks and 48 weeks [101].

12.5.3 Anti-VEGF Treatment Regimens

In both the MARINA and ANCHOR trials, ranibizumab was administered monthly for 24 months [90, 91]. In routine clinical practice, patient adherence to monthly treatment schedules has proven difficult. There has been great interest in identifying alternative dosing strategies that reduce the number of anti-VEGF injections without compromising visual acuity outcomes. These alternative dosing regimens include a pro re nata (PRN) regimen, where retreatment is given at monthly visits if there is fluid accumulation or hemorrhage, and a "treat-and-extend" regimen where treatment intervals are lengthened until signs of recurrent fluid.

(a) *As-Needed Treatment*

In as-needed (PRN) treatment regimens, injections are given based on the presence of active neovascular AMD. The PRN dosing requires the same number of visits as the fixed-monthly interval, but the regimen reduces the injection burden by three to four injections in a year. Monthly visits are required to determine the need for retreatment.

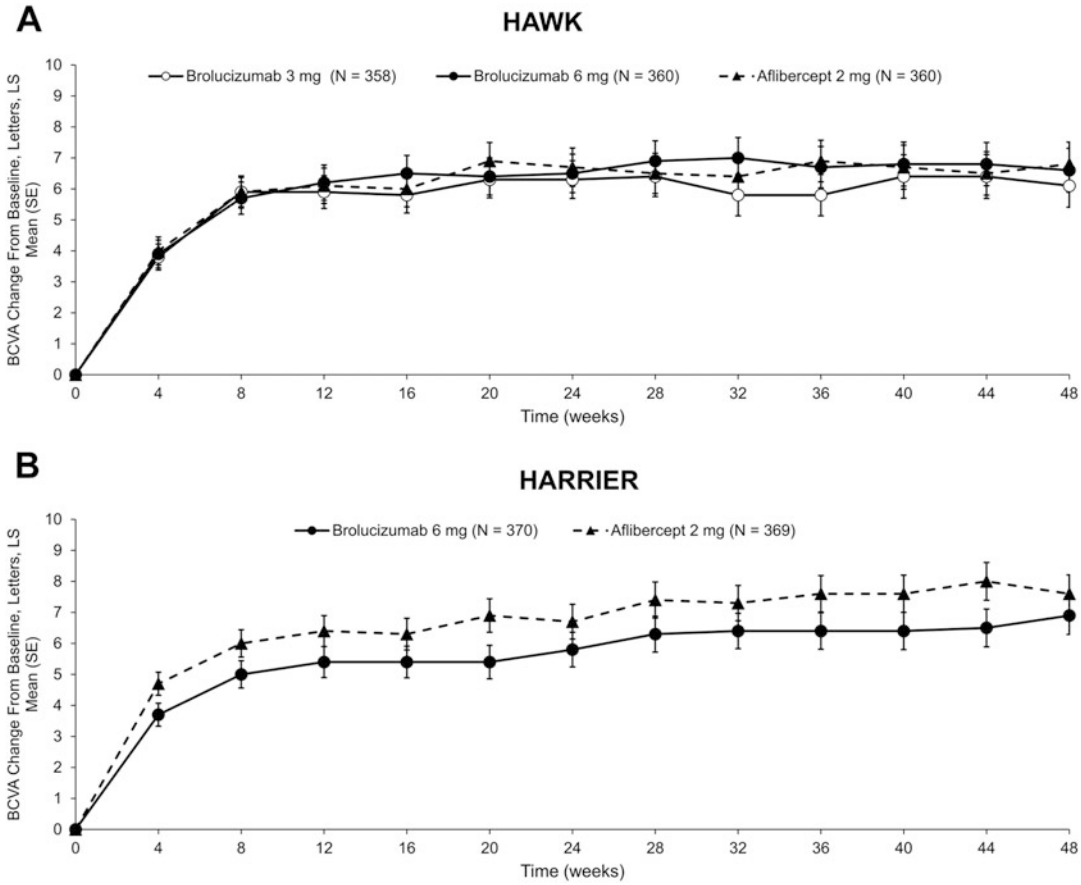

Fig. 12.5 Visual acuity results from a phase 3 trial of brolucizumab versus aflibercept for neovascular age-related macular degeneration (HAWK and HARRIER). The graph shows least-square mean best-corrected visual acuity (BCVA) change from baseline (number of letters) for aflibercept and brolucizumab. Reprinted from *Ophthalmology*. 2019 Apr 12. pii: S0161–6420(18)33018–5. doi: https://doi.org/10.1016/j.ophtha.2019.04.017 with permission

Early prospective studies investigating a PRN approach included the Prospective Optical Coherence Tomography Imaging of Patients with Neovascular AMD Treated with Intra-Ocular Lucentis (PrONTO) study [102] and the Study of Ranibizumab in Patients with Subfoveal Choroidal Neovascularization Secondary to Age-Related Macular Degeneration (SUSTAIN) study [96]. In both studies, patients received three, monthly, intravitreal injections of ranibizumab, followed by monthly office visits. Retreatment was performed if any of the following criteria was met: loss of visual acuity of greater than five letters, increase of at least 100 μm in central macular thickness on OCT, or new hemorrhage.

During the second year in PrONTO, the retreatment criteria were amended to include retreatment if there were any qualitative increase in the amount of fluid detected on OCT. At 24 months, patients required a mean of 9.9 injections and median of 9.0 injections (compared with 24 injections in MARINA and ANCHOR) [102]. In addition, 17.5% of patients did not require further treatments after the initial 3 monthly injections. Mean BCVA outcomes were similar to MARINA and ANCHOR at 24 months.

The SUSTAIN trial was a 1-year, phase 3, multicenter study performed in Europe and Australia evaluating as-needed dosing of ranibizumab in patients with CNV secondary to AMD [96]. While BCVA improved at month 3 (+5.8 letters), visual acuity declined slightly between months 3 and 6, but had a mean improvement of 3.6 letters at month 12. Both studies showed that acceptable patient outcomes can be achieved with an as-needed treatment regimen.

The Comparison of AMD Treatment Trials (CATT Study) was a multicenter, non-inferiority, randomized trial of neovascular AMD patients aged 50 years or older comparing the safety and efficacy of bevacizumab versus ranibizumab on a PRN dosing or monthly fixed dosing regimen [96]. The study's primary outcome was mean change in visual acuity at 2 years. All patients received treatment on initial visit and were followed monthly thereafter for 2 years. After 1 year, patients who were assigned to the monthly treatment groups were re-randomized to monthly or as-needed treatment without change in their drug assignment. At 2 years, a subtle difference emerged with the ranibizumab group gaining more letters than the bevacizumab group. The monthly administration of ranibizumab and bevacizumab led to an average gain of 8.8 letters and 7.8 letters, respectively, while the as-needed regimen led to gains of 6.7 letters and 5.0 letters ($p = 0.046$) [103]. In addition, monthly dosing of either treatment did not protect against vision loss when switched to as-needed dosing in the second year. Patients who switched to as-needed dosing after 1 year of monthly dosing had a mean loss of 2.2 letters ($p = 0.03$) and an increase in subretinal fluid [103].

The Inhibition of VEGF in Age-related Choroidal Neovascularization (IVAN) study was a similar study in the United Kingdom that randomized patients to 0.5 mg ranibizumab or 1.25 mg bevacizumab monthly or as-needed dosing [104]. There were no significant differences found in BCVA between bevacizumab and ranibizumab or between continuous and discontinuous treatments.

(b) *Treat-and-Extend*

The treat-and-extend regimen involves extending intervals between treatments as long as there is no macular fluid present. If fluid is present, the interval between treatments is typically shortened. The goal of treat-and-extend is to find the optimal treatment interval that stabilizes visual acuity and controls disease activity.

The Lucentis (ranibizumab) Compared to Avastin (bevacizumab) Study (LUCAS) was the first prospective, randomized, multicenter trial to use a treat-and-extend protocol [105, 106]. This study ($n = 432$) compared the safety and efficacy of bevacizumab versus ranibizumab for neovascular AMD through 2 years. Both arms were given injections every 4 weeks until there was inactive disease with no induction phase. The minimum treatment interval was 4 weeks and the maximum treatment interval was 12 weeks. After 1 year of treatment, this study found that treat-and-extend with ranibizumab or bevacizumab resulted in mean increases in BCVA of 8.2 and 7.9 letters, respectively. This was comparable to the visual acuity gains in the CATT study of 8.5 and 8.0 letters, respectively, at 1 year [106]. Ranibizumab was found to be equivalent to bevacizumab, with 6.6 and 7.4 letters gained, respectively at 2 years.

More recently, the Treat and Extend (TREND) study, was a 12-month, randomized, multicenter, intervention study to compare the effects of treat-and-extend versus monthly ranibizumab regimens on best-corrected visual acuity in patients [107]. The treatment intervals were extended by 2 weeks at each visit if there was no disease activity with a maximum of a 12-week treatment interview. The study, which included 650 treatment-naive AMD patients aged 50 and older, determined that the 2 treatment regimens resulted in similar visual acuity outcomes and the treat-and-extend regimen resulted in fewer injections (8.7 versus 11.1; Fig. 12.6).

Over the long term, repeated anti-VEGF injections may increase the chance of ocular complications. Infectious endophthalmitis remains one of the most devastating complications of intravitreal injections. In

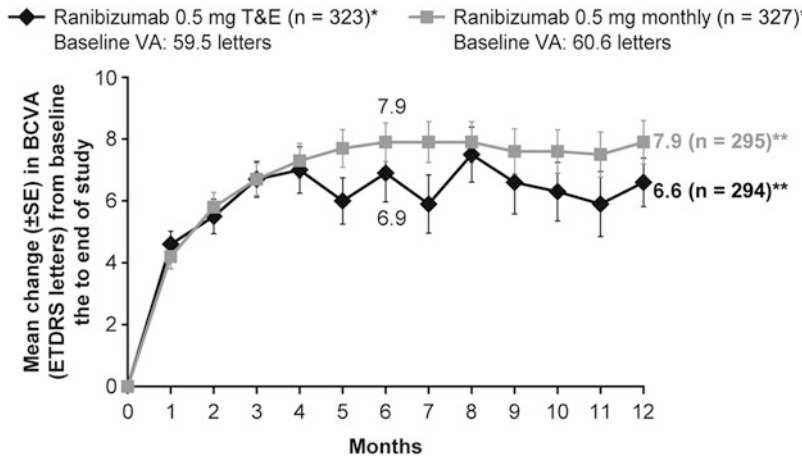

Fig. 12.6 Visual acuity results from the Treat-and-Extend versus Monthly Regimen in Neovascular Age-Related Macular Degeneration (TREND) study demonstrating non-inferiority of ranibizumab administered on a treat-and-extend regimen compared to monthly dosing. Reprinted from *Ophthalmology* 2018;125:57–65 with permission

multicenter clinical trials, the incidence of endophthalmitis has been reported to range from 0.016% to 1.6% [108–110]. Studies have also suggested that chronic anti-VEGF therapy may be associated with the development of macular atrophy, but whether this is part of the natural history of the disease or is treatment-related remains unclear [111]. In addition, long-term or sustained rise in intraocular pressure (IOP) after anti-VEGF injections has been reported, with a greater number of intravitreal injections being associated with a higher risk for sustained IOP elevation [112, 113].

(c) *Tachyphylaxis and Need to Switch Agents*

Most patients with exudative AMD require repeated intravitreal injections. The SEVEN-UP study reported the long-term, 7-year outcomes of 65 AMD patients that had originally enrolled in the ANCHOR, MARINA, and HORIZON studies [114]. Approximately, 68% of patients had active disease on OCT and 50% of the patients required intravitreal treatment at the end of the seventh year [114]. Persistence of active disease may be related to the natural course of the disease, or due to tachyphylaxis to treatment. Tachyphylaxis refers to a diminished response to a certain medication after repeated administrations, and it has been reported in several trials in patients receiving repeated ranibizumab and bevacizumab injections [115, 116]. In such cases, use of other treatment agents is considered.

Patients who fail to respond to anti-VEGF therapy have been designated as nonresponders. There is a range of definitions for nonresponders from morphologic classifications, where nonresponders continue to have persistent subretinal or intraretinal fluid on OCT while under treatment, to functional classifications, where nonresponders have stable BCVA or a worsening of BCVA while under treatment. It has been found that switching nonresponders from ranibizumab or bevacizumab to aflibercept can result in improvements in mean central macular thickness and increase in the time interval between intravitreal injections. However, despite the anatomical improvements reported, functional improvements are rare [117, 118]. The functional and anatomical improvements from switching between ranibizumab and bevacizumab are debatable [119–121]. In addition, it can take as long as a year to notice improvement in vision, so switching early may not be advisable.

(d) *New Strategies*

More recently, the Port Delivery System (PDS) with ranibizumab from Genentech has been developed as a novel device developed to provide extended drug delivery for anti-VEGF agents. The PDS is a permanent, reusable drug reservoir that is surgically implanted through a 3.5-mm scleral incision at the pars plana. There is

a semipermeable membrane that allows continuous passive diffusion of the drug from the reservoir with higher concentration into the vitreous. The device can be refilled in the office with a specialized refill needle. The Long-Acting Delivery of Ranibizumab (LADDER) trial [122] was a phase 2 multicenter trial that enrolled 220 patients randomized in a 3:3:3:2 ratio to PDS with 10, 40, and 100 mg/mL formulations of ranibizumab, or an intravitreal injection of 0.5 mg ranibizumab monthly [123]. The primary endpoint of the study was the time to first required PDS refill. The median time to first refill in the 10 mg/mL arm was 8.7 months; in the 40 mg/mL arm, 13.0 months; and in the 100 mg/mL arm, 15.0 months. At 9 months, the reductions in central retinal thickness measurements and improvements in visual acuity were similar between the PDS 100 mg/mL group and the monthly intravitreal ranibizumab group. Vitreous hemorrhage rate postoperatively was 4.5%.

The potential for longer-term delivery of anti-VEGF and anticomplement therapy through gene therapy platforms is currently being developed [124]. Early phase studies evaluating anti-VEGF agents delivered via adeno-associated viral (AAV) vectors have demonstrated reductions in the need for intravitreal injections.

12.6 Conclusion

In the last two decades, the assessment and management of patients with AMD have dramatically improved. As described in this chapter, this was primarily due to two groundbreaking advances: the development and clinical approval of antiangiogenic injections for the treatment of neovascular AMD; and the continuous and remarkable improvements in the available imaging modalities. Currently, we have treatment strategies that effectively improve vision of patients with CNV; and the ability to visualize retinal and choroidal structures non-invasively and to a near-histological detail, thus recognizing a wide range of AMD phenotypes, which seem to have distinct prognostic implications.

Despite recent advances, limited interventions have shown to slow progression from the early to the advanced forms of AMD, and there are currently no effective treatment options available for patients with geographic atrophy. This is at least partly related to the complex, multifactorial nature of AMD, where multiple mechanisms and pathways are implicated [125]. A better understanding of the pathophysiology of this condition, including the interplay among genetic and environmental risk factors, is required to successfully halt disease progression and effectively treat the atrophic forms of AMD. Therapies reversing neurodegeneration are promising, but it is likely that future strategies will need to address multiple targets to succeed.

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