Age-related Macular Degeneration

Genetic Epidemiologic findings from large European studies

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COLOPHON

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Genetic Epidemiologic findings from large European studies

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genetische en epidemiologische bevindingen van grote Europese studies

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1

INTRODUCTION

GENERAL INTRODUCTION

Age-related macular degeneration

Age-related macular degeneration (AMD) is currently the leading cause of blindness among elderly in the Western world, typically affecting individuals 60 years of age and older. AMD is characterized by drusen, which are small lipid- and protein-rich deposits between the retinal pigment epithelium (RPE) and Bruch's membrane (see Figure 1 for gross anatomy of the eye). In the early stages, AMD presents with drusen and pigmentary changes in the RPE, and both the extent of these pigmentary changes and the size of the drusen are correlated with the risk of developing late-stage disease[1]; choroidal neovascularization (CNV, or "wet" AMD) and geographic atrophy (GA, or "dry" AMD). CNV is characterized by the development of new blood vessels that arise from the choroid and grow into the retina. Due to the poor quality of these vessels, they can leak, causing hemorrhage and initiating the formation of fibrosis. GA is characterized by atrophy of the RPE together with a loss of photoreceptor cells and the choriocapillaris. Both wet and dry AMD develop in the macula and therefore affect visual acuity, particularly when the fovea becomes involved.

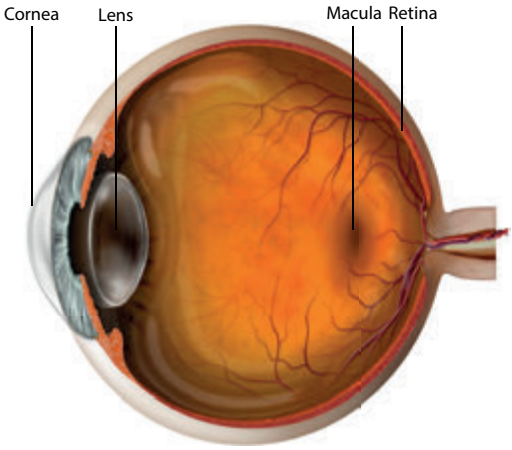

Figure 1. Gross anatomy of the eye. Light enters the eye through the cornea and is focused on the retina by the lens. The retina is the inner lining of the eye and contains the light-sensitive photoreceptor cells, namely the rods and cones. The rods allow vision under low luminance conditions, and the cones provide detailed, color vision. The cones are largely concentrated in the central part of the retina known as the macula, with the highest concentration of cone cells at the midpoint (the fovea). Photoreceptors convert light energy into an electrical signal that is carried by the optic nerve to the brain. The retinal cell layer below the photoreceptors is called the retinal pigment epithelium (RPE), which protects against free radicals and absorbs light[2]. In addition, the RPE takes up lipoproteins from the circulation[3], releases cholesterol into the inter-photoreceptor matrix[4], phagocytes membrane lipids from photoreceptors[5], and releases lipids back into the circulation[6]. Age-related macular degeneration affects the retina, specifically the RPE located in the macula.

Epidemiology of AMD

The prevalence of early and late AMD increases with age, reaching up to 25% among individuals 80-84 years of age[7]. Given our aging population, particularly in the Western world, the number of individuals with AMD is expected to increase rapidly. Indeed, estimates suggest that by the year 2040, the global incidence of AMD could reach 288 million, including 12.5 million cases of late-stage AMD[7]. When corrected for age, the prevalence of AMD is similar between men and women[8,9]. Individuals of European descent are more susceptible to developing AMD compared to Asians and Africans, with a prevalence of 12.3% versus 7.4% and 7.5%, respectively[7]; this difference in prevalence among ethnic groups supports the notion that genetic and/or environmental factors contribute to the development of AMD.

In 2005, the first genetic variant related to AMD was identified in the *CFH* gene (which encodes complement factor H)[10] using a genome-wide association analysis involving 96 patients and 50 controls (Figure 2). The following year, a variant in the *ARMS2-HTRA1* locus was discovered[11]. This locus (see Figure 2) is located between the *ARMS2* (Age-Related Maculopathy Susceptibility 2) and *HTRA1* (HtrA Serine Peptidase 1) genes. Subsequent studies involving larger sample sizes revealed additional variants associated with AMD. For example, a large genome-wide association study (GWAS; see Figure 2) identified a set of 52 independent loci associated with AMD[12], to which an additional 12 loci were added by the largest and most recent GWAS[13]. Studies involving thousands of patients and subjects not only revealed common variants with small effect sizes, but also revealed relatively rare variants with large effect sizes. Together, the genetic variants identified to date are estimated to explain more than half of the genetic heritability of AMD[12]. Since a large percentage of this heritability remains unexplained, the search continues for additional variants associated with AMD, using a variety of techniques such as exome sequencing and whole-genome sequencing.

Risk factors and protective factors

Environmental risk factors for AMD have been the subject of extensive research; after age, smoking is the second-most important risk factor for developing AMD, with a 2-3-fold higher risk of AMD among smokers compared to non-smokers[17,18]. Even former smokers who quit still have a small but significant increased risk of AMD. Many other risk factors have also been associated with AMD, including atherosclerosis and high body mass index[18-20]; however, the effect size of these factors is relatively small compared to smoking, and some studies report conflicting results with respect to these relatively small risk factors.

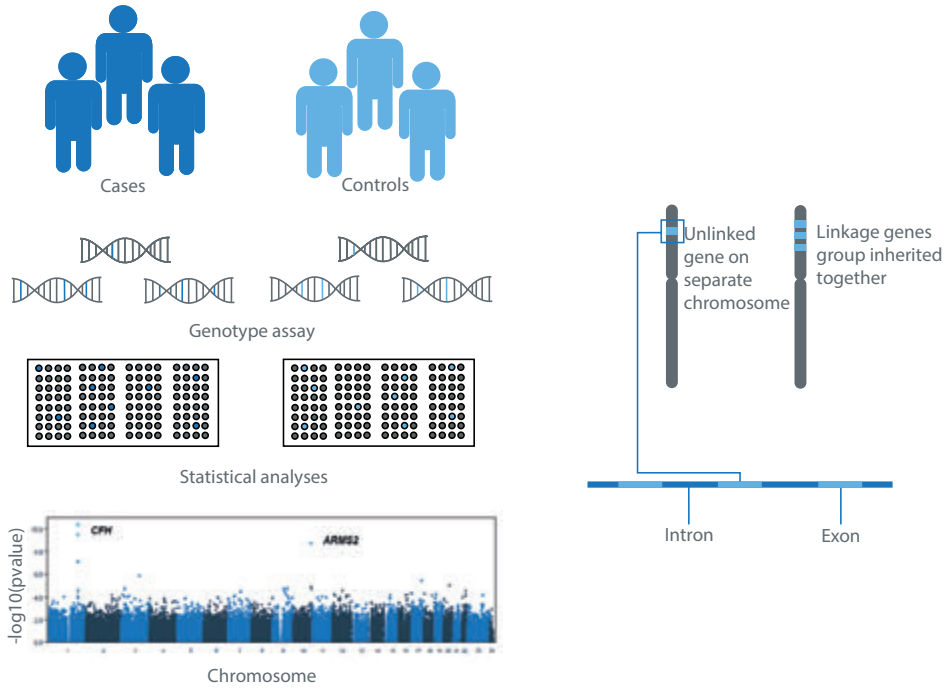

Figure 2. Genetic epidemiology. Genome-wide association studies (GWAS) are based on the principle that genetic variations related to a disease are more common in affected individuals (patients) than in unaffected individuals (controls). Because neighboring loci (i.e., loci located relatively close to each other within the chromosome) are inherited together more often than distant loci, genetic markers distributed throughout the genome can be used to identify disease-associated loci. This so-called "hypothesis-free" approach can reveal candidate genes associated with a given disease. Another hypothesis-free approach is to sequence either the entire exome or the entire genome. In whole-exome sequencing, all of the protein-coding sequences (exons) are sequenced. Whole-exome and whole-genome sequencing are more time-consuming and costly than GWAS, but are more precise and can reveal relatively rare variants that occur at a low frequency within the population. Adapted from[14-16].

In addition to factors that increase risk, other factors have been shown to reduce the risk of developing AMD, including antioxidants and certain nutrients. For example, studies in the late 1980s and early 1990s found that carotenoids—particularly lutein and zeaxanthin—were associated with a reduced risk of developing AMD[21,22]. Since then, a large number of studies investigated the protective effects of micronutrients, food products, and dietary patterns[23,24]. A large randomized controlled trial revealed that supplementation with high-dose antioxidants and zinc reduced the risk of developing late-stage AMD by 25%[(ref. 25)]. Although nutrition can affect the course of the disease, precisely which diet to follow is currently unknown and warrants further study.

Therapy

In wet AMD, cells release vascular endothelial growth factor (VEGF) due to hypoxia. This causes the formation of new blood vessels with increased permeability. The current treatment for wet AMD is intravitreal injections of anti-VEGF compounds. Although this treatment often improves visual acuity, over the long term patients with wet AMD remain at risk for a substantial decline in visual acuity. In contrast, no treatment is available for dry AMD; however, many clinical trials are currently ongoing and focus on inhibitors of the complement cascade[26,27], neuroprotective agents[28], regeneration of the RPE (e.g., ClinicalTrials.gov Identifier: NCT02286089), molecules that inhibit the visual cycle[29,30], anti-inflammatory agents (e.g., ClinicalTrials.gov Identifier: NCT02564978), and antioxidants[31]. Although some of these trials have revealed small beneficial effects, further study is needed; to date, however, most studies have yielded negative results. Because no cure currently exists for AMD, the best approach to retaining good visual acuity lies in prevention by living a healthy lifestyle.

Current perspectives and future directions

AMD is a complex but extremely well-studied disease caused by both genetic and environmental factors; however, the pathophysiology remains poorly understood, and no cure is currently available. In addition, although relatively small studies in Europe have provided snapshots of the prevalence of AMD, an overall picture of the prevalence is currently lacking but is essential for eye-care policymakers. Furthermore, developing a cure for AMD requires additional knowledge with respect to the pathophysiology and progression of AMD, including a better understanding of the role of lipids and nutrition. Moreover, although much is known regarding the genetic factors associated with AMD, the biological consequences of AMD-related variants are currently unknown, requiring in-depth genetic profiling of individuals and an interpretation of the associated risk of developing AMD.

Expanding our knowledge of AMD requires large, harmonized international datasets with sufficient statistical power to identify genetic and/or environmental factors with relatively small effect sizes. Such a dataset would allow researchers to study a homogenous phenotype and obtain precise estimates. The results of these studies will pave the way toward developing new therapeutic strategies and will lead to better advice for individual patients.

AIMS OF THIS THESIS

This thesis addresses several questions related to the genetics and epidemiology of age-related macular degeneration. In **Chapter 2**, we examine the frequency of age-related macular degeneration in Europe and the risk of progression to the other eye. In **Chapter 3**, we investigate how geographic atrophy progresses and whether we can use deep learning to make predictions. **Chapter 4** examines how genetics drives age-related macular degeneration and whether rare gene variants play a role. Lastly, in **Chapter 5** we examine the role of diet and lipid metabolism in age-related macular degeneration.

To answer these questions, we combined several study populations in order to increase the generalizability of our results and increase the statistical power to identify relatively small effects of environmental factors and/or genetic variants. This approach also allowed us to determine the consistency of findings across studies, providing an internal validation. Below is a brief description of the various consortia and study populations used in this thesis.

The *Rotterdam study*[32] is a population-based study consisting of three cohorts that started in 1990, 2000, and 2006. In this study, nearly 15,000 individuals over the age of 45 were recruited in the neighborhood of Ommoord, a suburb of Rotterdam in the Netherlands, and re-examined every five years.

EUGENDA (the European Genetic Database)[33] is a case-control database containing nearly 5,000 individuals who were recruited at the Ophthalmology Department at the Radboud University Medical Center in Nijmegen, the Netherlands, and the Department of Ophthalmology at the University Hospital of Cologne, Germany.

The *European Eye Epidemiology (E3) Consortium*[34] is a collaboration between 31 research groups in 13 European countries. This consortium includes a variety of study types, including population-based studies, case-control studies, and clinical trials. In total, the E3 Consortium contains clinical and imaging data on approximately 170,000 Europeans. (http://www.eye-epi.eu/)

The *EYE-RISK Consortium* is a collaboration between 14 partners throughout Europe, including hospitals, universities, and companies, and is funded by the European Union's Horizon 2020 Research and Innovation Programme. The aim of this consortium is to identify risk factors, molecular mechanisms, and new therapies for age-related macular degeneration. As part of this collaboration, a database was created containing data from approximately 50,000 cases compiled from various studies conducted within the E3 Consortium. (http://www.eyerisk.eu/)

The *Three Continent AMD Consortium*[35] consists of the following four population-based studies: the Rotterdam Study in the Netherlands, the Beaver Dam Eye Study in Madison, Wisconsin, the Blue Mountain Eye Study in Sydney, Australia, and the Los Angeles Latino Eye Study in Los Angeles, California. This consortium, which currently includes a cohort of approximately 30,000 individuals, was formed in 2009 in order to investigate gene–environment interactions, as well as the incidence and progression of age-related macular degeneration.

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2

BURDEN OF AMD

2.1 Prevalence of Age-Related Macular Degeneration in Europe: the Past and the Future

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https://www.aaojournal.org/article/S0161-6420(16)32475-7/fulltext#supplementaryMaterial

ABSTRACT

Purpose: Age-related macular degeneration (AMD) is a frequent complex disorder in elderly of European ancestry. Risk profiles and treatment options have changed considerably over the years, which may have affected disease prevalence and outcome. We determined prevalence of early and late AMD in Europe from 1990 to 2013 using the European Eye Epidemiology (E3) consortium, and made projections for the future.

Design: Meta-analysis of prevalence data.

Participants: 42 080 individuals aged 40 years of age and older participating in 14 population-based cohorts from 10 countries in Europe.

Methods: AMD was diagnosed based on fundus photographs using the Rotterdam Classification. Prevalence of early and late AMD was calculated using random effects meta-analysis stratified for age, birth cohort, gender, geographic region, and time period of the study. Best-corrected visual acuity (BCVA) was compared between late AMD subtypes; geographic atrophy (GA) and choroidal neovascularization (CNV).

Main outcome measures: Prevalence of early and late AMD, BCVA, and number of AMD cases.

Results: Prevalence of early AMD increased from 3.5% (95% confidence interval [CI] 2.1%-5.0%) in those aged 55-59 years to 17.6% [95% CI 13.6%-21.5%] in aged ≥85 years; for Late AMD these figures were 0.1% [95% CI 0.04% - 0.3%] and 9.8% [95% CI 6.3%-13.3%], respectively. We observed a decreasing prevalence of late AMD after 2006, which became most prominent after age 70. Prevalences were similar for gender across all age groups except for late AMD in the oldest age category, and a trend was found showing a higher prevalence of CNV in Northern Europe. After 2006, fewer eyes and fewer ≥80 year old subjects with CNV were visually impaired (P=0.016). Projections of AMD showed an almost doubling of affected persons despite a decreasing prevalence. By 2040, the number of individuals in Europe with early AMD will range between 14.9 and 21.5 million, for late AMD between 3.9 and 4.8 million.

Conclusion: We observed a decreasing prevalence of AMD and an improvement in visual acuity in CNV occurring over the past 2 decades in Europe. Healthier lifestyles and implementation of anti-vascular endothelial growth factor treatment are the most likely explanations. Nevertheless, the numbers of affected subjects will increase considerably in the next 2 decades. AMD continues to remain a significant public health problem among Europeans.

INTRODUCTION

Age-related macular degeneration (AMD) can cause irreversible blindness and is the leading cause of visual impairment in the elderly of European ancestry.[1] Two stages are known for this disease: early AMD, which is characterized by drusen and pigmentary changes, and late AMD, which can be distinguished in 2 subtypes; geographic atrophy (GA) and choroidal neovascularization (CNV).[2]

Worldwide estimates approximated that 30-50 million people are affected by AMD[3, 4], and these numbers are expected to increase over time because of the aging population.[1, 5-9] Although multiple small studies have assessed the prevalence of AMD and its relation to visual decline at various places in Europe[10-12], a clear overview for Europe as a whole is lacking[13]. Comprehensive epidemiologic figures on AMD in Europe would help proper planning for public health and eye care policy makers.

Recent studies report a decrease in AMD-associated blindness and visual impairment[14, 15], which are likely to be attributable to improved diagnostic procedures and hence earlier diagnosis, and the introduction of anti-vascular endothelial growth factor (anti-VEGF) therapy.[14-16] Anti-VEGF therapy for CNV was introduced in 2004 and, since 2006, it has been widely used for clinical care in Europe.[17, 18] However, the impact of anti-VEGF therapy on general visual function of persons with AMD in Europe has not been sufficiently studied.[1, 16]

In this study, we investigated the prevalence of both early and late AMD in Europe using summary data of population-based cohort studies from the European Eye Epidemiology (E3) consortium. We analyzed changes in prevalence over time, compared geographic regions and studied differences between men and women. Moreover, we analyzed the visual acuity of affected individuals before and after the introduction of anti-VEGF therapy and predicted the number of persons with AMD by 2040 in Europe.

METHODS

Study Population

Fourteen population-based cohort studies participating in the E3 consortium contributed to this analysis. This consortium consists of European studies with epidemiologic data on common eye disorders; a detailed description on the included studies has been published elsewhere[16]. For the current analysis, studies with gradable macular fundus photographs (n=42 080 participants) and participants aged 40 years and over provided summary data. Participants were recruited between 1990 and 2013 from the following countries: Estonia,

Table 1. Description of the European Eye Epidemiology Consortium Studies Included in the Meta-analysis

Region		Study	Data collection period	Total Participants (n)	Age Range (yrs)	Median Age (yrs)	Male Gender (%)	European Ethnicity (%)	Crude Prevalence of Early AMD (%)	Crude Prevalence of Late AMD (%)
North	United Kingdom	EPIC	2004-2011	5344	45-85+	60-64	43.1	99.7	-	0.5
	Norway	Tromsø	2007-2008	2631	65-85+	65-69	42.5	91	-	3.5
West	France	ALIENOR-3C	2006-2008	879	70-85+	75-79	37.7	-	16.8	5.6
	Germany	GHS	2007-2012	3839	40-74	50-54	50.2	-	2.3	0.2
	Netherlands	RS-I	1990-1993	6419	55-85+	60-64	40.7	98.9	7.5	1.7
	Netherlands	RS-II	2000-2002	2545	55-85+	55.59	45.4	97.8	6	0.7
	Netherlands	RS-III	2005-2008	3449	45-85+	55-59	43.4	96.4	4.6	0.4
	France	Montrachet-3C	2009-2013	1069	75-85+	80-84	37	100	9.2	2.2
	France	POLA	1995-1997	2196	60-85+	65-69	43.5	-	8.7	1.9
South	Portugal	Lousa	2012-2013	3021	55-85+	60-64	43.9	99.3	15.4	1.3
	Portugal	Mira	2009-2011	2975	55-85+	65-69	43.4	99.7	6.9	0.7
	Greece	Thessaloniki Eye Study	2000-2005	2107	60-85+	65-69	55.6	97.7	-	2.7
	Italy	PAMDI	2005-2006	853	60-85+	65-69	45.8	100	13.5	2.1
Multiple		EUREYE	2000-2002	4753	65-85+	65-69	44.8		12.6	3.3

ALIENOR = Antioxydants, Lipids Essentiels, Nutrition et maladies OculaiRes Study; AMD = age-related macular degeneration; EPIC = European Prospective Investigation into Cancer; EUREYE = European Eye Study; GHS= Gutenberg Health Study; PAMDI = Prevalence of Age-Related Macular Degeneration in Italy; POLA= Pathologies Oculaires Liées à l'Age Study; RS= Rotterdam Study; - = data not available.

France, Germany, Greece, Italy, Northern Ireland, Norway, Netherlands, Spain, Portugal[19,20] and United Kingdom (UK) [16] (Table 1). The composition of AMD in each cohort is shown in Figure 1 (available at www.aaojournal.org). The study was performed in accordance with the Declaration of Helsinki for research involving human subjects and the good epidemiological practice guideline.

Grading of Age-Related Macular Degeneration

Both eyes of each participant were graded and classified separately by experienced graders or clinicians, and the most severe AMD grade of the worst eye was used for classification of the person. To harmonize classification of AMD, studies were graded or reclassified according to the Rotterdam Classification, as previously described.[21] Main outcomes of this study were early AMD (grade 2 or 3 of the Rotterdam Classification) and late AMD (grade 4 of the Rotterdam Classification). Persons with late AMD were stratified as GA and CNV or mixed (both GA and CNV present in one person, either both types in the same eye, or one type per eye), which is henceforth in this article referred to as CNV. The Tromsø Eye Study, the Thessaloniki Eye Study, and the European Prospective Investigation into Cancer and Nutrition (EPIC) study had fundus photograph grading that could not be converted to match the definition of early AMD of the Rotterdam Classification. Therefore, these 3 studies only participated in the late AMD analysis.

Visual Impairment

Visual acuity was measured for each eye separately as best-corrected visual acuity in 2 categories: ≥0.3 and <0.3. When best-corrected visual acuity differed in the 2 eyes, visual acuity of the best eye was used to classify the person. Low vision and blindness were defined as visual acuity <0.3 and further referred to as visually impaired.

Projection of Age-Related Macular Degeneration

The projection of AMD cases in Europe from 2013 to 2040 was calculated using the prevalence data for 5-year age categories obtained from the meta-analysis. Two different scenarios were used to calculate the projection. In the first scenario, it was assumed that the prevalence of both early and late AMD will remain stable until 2040. This scenario accounted for changes in population structure only. The second scenario followed the trend of decreasing prevalence based on data from the meta-analysis of the E3 consortium regarding the period 2006-2013. We calculated the rate of decline, with 2013 as the starting point and 2040 as the end point, and made the assumption that the rate of decline was decelerating and zero at the end point. For each projected year, prevalences were calculated for every 5-year age group, for early AMD from 45 years of age and onward and for late AMD 65 years and onward. The projected prevalences

were then multiplied by the predicted European population estimates obtained from Eurostat for all 28 countries in Europe, and the sum of individuals from all age groups was calculated.[22]

Statistical Analysis

The crude prevalence of early and late AMD were calculated per study for each 5-year age group. A random-effects meta-analysis was performed by weighing the studies according to sample size, for early and late AMD separately for 5-year age groups and for people aged 70 years and older. In case of reported zero prevalence, the Haldane correction was used.[23] In case of 100% prevalence, 0.01 was subtracted to prevent exclusion from the analysis. This analysis was repeated, stratified for the midpoint year of the study recruitment period, before and after the year 2006 and for 10-year birth cohorts. Furthermore, it was repeated for gender, and for geographical area in Europe based on the United Nations Geoscheme.[24] A chi-square test was used to compare time-trends.

In addition, a meta-analysis was performed for eyes with visual impairment owing to late AMD, and per subtype of late AMD. Subsequently, the analysis was stratified for studies conducted before and after 2006, for which the midpoint year of the study recruitment period was used. The number of visually impaired people was calculated before and after 2006. Meta-analysis was performed with Stata software (release 13, version 13.1; StataCorp LP, College Station, TX) using metaprop. Graphical outputs were constructed with GraphPad Prism 7 (for Windows; GraphPad Software, La Jolla CA; www.graphpad.com).

RESULTS

The total study population included in this analysis consisted of 42 080 individuals from 14 studies with a median age of 65-69 years and a slight female predominance (55.8%). The prevalence of all age groups together varied per study between 2.3% and 16.8% for early AMD (total N=2703) and between 0.2% and 5.6% for late AMD (total N=664) (Fig 2A and B available at www.aaojournal.org; to avoid biased estimates only groups larger than 30 individuals are shown; this applied only to the Rotterdam Study 3 age category ≥85 years). Owing to moderate to high heterogeneity (I^2 >= 75% in 73 of 141 analyses), which was not related to time trends, we applied a random-effects model for each meta-analysis. This provided a prevalence of early AMD increasing with age from 3.5% (95% confidence interval [CI] 2.1%-5.0%) at 55-59 years to 17.6% (95% CI 13.6%-21.5%) in persons aged ≥85 (Fig 3A, and Table 2, available at www.aaojournal.org). The prevalence of late AMD rose from virtually zero in the youngest age group to 9.8% (95% CI 6.3%-13.3%) for

those in the highest age group (Fig 3B). Taking together all people aged ≥70 years, the overall prevalence was 13.2% (95%CI 11.2%-15.1%] for early AMD and 3.0% (95%CI 2.2%-3.9%] for late AMD. We investigated prevalence changes over time by dividing the E3 consortium into studies conducted before and after 2006. The prevalence of early AMD before and after 2006 seemed to rise with age in a similar fashion. For late AMD, a trend of decreasing prevalence was observed for the higher age categories after 2006 (Fig 3C and D). Even after exclusion of the 2 cohorts (Rotterdam Study [RS]-II and European Eye Study [EUREYE]) with the highest prevalences in the highest age category before 2006, results remained similar (data not shown).

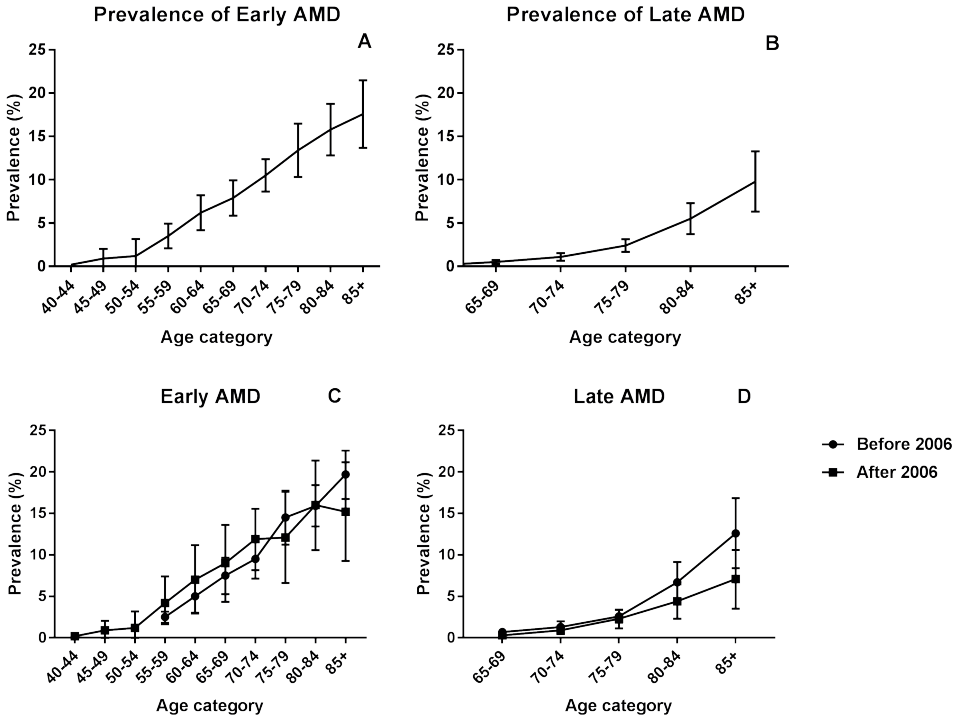

Figure 3. Meta-analysis of (**A**) early and (**B**) late age-related macular degeneration (AMD) in Europe per age category for the participating studies. Meta-analysis of the prevalence of (**C**) early and (**D**) late AMD before and after 2006.

When we analyzed prevalence data as a function of birth cohort, a relatively stable prevalence of early AMD was visible across all birth cohorts, whereas a decreasing prevalence of late AMD was seen for the more recent birth cohorts (Fig 4A and B).

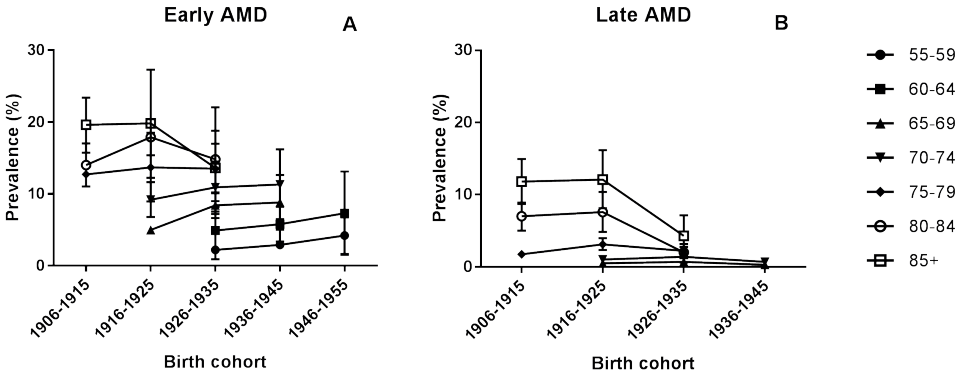

Figure 4. Meta-analysis of early (**A**) and late (**B**) age-related macular degeneration in Europe by 10-year birth cohorts.

Gender and Geographic Region

We studied the relation with gender and found no differences in the prevalence of early and late AMD between men and women except for the age category of 85 years and older for late AMD (Fig 5A and B, available at www.aaojournal.org). This category shows a trend for a higher prevalence in women compared to men, although CIs overlap.

To address differential distribution of AMD in Europe, we stratified studies according to 3 regions defined by the United Nations.[24] In older individuals, we observed a trend toward a higher prevalence of early AMD in the North (16% in ≥70 years; 95% CI 14%-17%) compared to the West (12%; 95% CI 10%-14%) and South (14%; 95% CI 10%-17%) (Fig 6A, available at www.aaojournal.org). Likewise, late AMD had the highest prevalence in the North (4.2%, 95% CI 2%-6%) compared to the West (3.1%; 95% CI 2%-4%) and South (3.1%; 95% CI 2%-4%) (Fig 6B, available at www.aaojournal.org). More detailed analyses showed that a frequency difference was only present for CNV (Fig 6C and D, available at www.aaojournal.org); however, CIs of the regional differences overlapped.

Visual consequences

As most countries implemented anti-VEGF therapy for CNV from 2006 onward, we compared visual impairment from AMD in studies carried out before and after this year. Before 2006, 54.2% of eyes with GA were visually impaired, and 79.8% of eyes suffering from CNV were visually impaired. From 2006 onward, the proportion of visually impaired eyes remained the same for GA (47.6%; P= 0.40), but dropped to 66.2% (P= 0.026) for CNV (Fig 7A). This improvement was also observed for the number of bilaterally visually

impaired persons; 120 of 345 (34.8%) before 2006 to 75 of 259 (28.9%; *P*=0.13) after 2006. The largest drop was seen for people aged 80 years and older; 85 of 175 (48.6%) before 2006 to 46 of 132 (34.8%; *P*=0.016) after 2006 (Fig 7B).

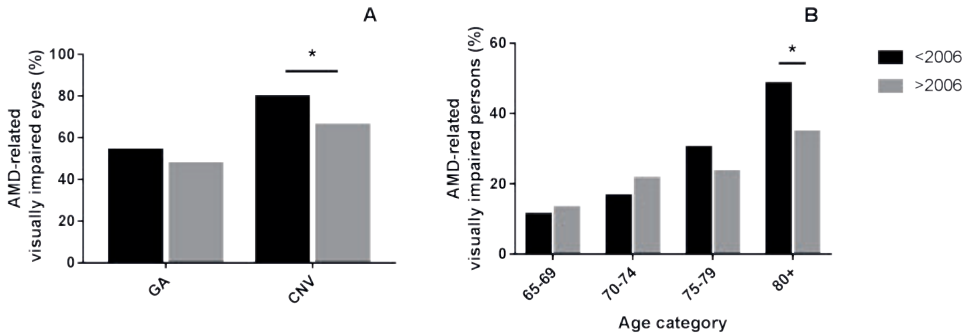

Figure 7. A, Proportion of visually impaired eyes within each subgroup of late age-related macular degeneration (AMD). The proportion of visually impaired eyes remained the same for geographic atrophy (47.6%; *P* = 0.4), but dropped to 66.2% (*P*= 0.026) for choroidal neovascularization after 2006. **B**, Proportion of persons with late AMD with bilateral visual impairment before and after 2006 (*P*=0.016). **P* <0.05.

Projections of Age-Related Macular Degeneration in Europe for 2040

Assuming that the prevalence of early and late AMD will remain stable over time, an increase from 15.0 million in 2013 to 21.5 million for early AMD can be expected by 2040. The number of people with late AMD will almost double during this time period, from 2.7 million in 2013 to 4.8 million in 2040.

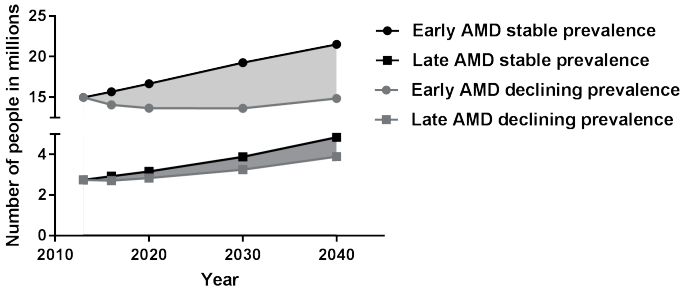

Figure 8. Predicted number of persons with age-related macular degeneration (AMD) in years 2013-2040 as a function of 2 prevalence scenarios.

Assuming a more realistic scenario for which E3 historic data and a decelerating slope were used, we found that the prevalence of early AMD will first decrease and then slightly increase between 2013 and 2040. The model estimated that the number of people with early AMD would remain almost the same; from 15.0 million in 2013 to 14.9 in 2040. This model also displayed that the number of people with late AMD in Europe will increase from 2.7 million in 2013 to 3.9 million by 2040 (Fig 8).

DISCUSSION

Age-Related Macular Degeneration Prevalence and Its Time Trends

Our study provides insight into the prevalence of both early and late AMD in Europe. Based on meta-analyzed data from 14 population-based cohort studies included in the E3 Consortium, the overall prevalence of early and late AMD was 13.2% and 3.0%, respectively, in the age-category ≥70 years. These estimates are comparable to persons of European descent living in other continents.[3, 25]

Our data show a trend toward a slightly decreasing prevalence of AMD in the older age categories. It is unlikely that this is explained by differential mortality in AMD patients before and after 2006, although studies have shown conflicting results on death as a competing risk factor for AMD, and we cannot exclude that this plays a role.[26-28] The decreasing trend in time has also been observed in the Beaver Dam Eye Study, indicating that these trends are not confined to Europe.[29] Decreasing rates have also been observed for other aging disorders such as cardiovascular disease and dementia[30-33], and may to be related to improved lifestyle among the elderly[34-36]; for example, the number of smokers declined by 30.5% from 1990 to 2010 in Europe[37]. Taken together, the decline in prevalence suggests that the increases in the number of AMD patients may not be as substantial as previous prediction studies suggested[38].

Gender and Geographic Regions

Our data showed no difference in the prevalence of early and late AMD with respect to gender. In the oldest age category of 85 years and older, women seemed to have a higher prevalence of late AMD, but detailed analysis showed that this was mostly owing to imprecision of the estimate in men, caused by a lower number of men in this age group. (Fig 9, available at www.aaojournal.org). This has also been observed in other studies.[7,39]

As for regional differences, we noticed that the northern region of Europe showed a slightly higher prevalence of early and late AMD. This trend was the result of a higher prevalence of CNV in the north. Our findings are in concordance with the results

previously published by the Tromsø Eye Study[40] but are in contrast with other studies performed in the north of Europe finding a higher prevalence of GA (EUREYE, Reykjavik Eye Study and Oslo Macular Study).[41-43] Considering the larger sample size and high response rate of the Tromsø Eye Study compared with the other studies, these findings might be more legitimate. No consistent differences were observed for the western and southern regions of Europe.

Visual Consequences

The proportion of eyes affected by CNV that were visually impaired was reduced after the year 2006. Unfortunately, our study lacked actual data on interventions for CNV, but it is likely that the reduction is attributable to the use of anti-VEGF injections, which were introduced as a therapy for CNV in Europe from 2006 onward.[18] This notion is supported by findings from clinical trials[44, 45] and other studies, which show an up to 2-fold decrease in legal blindness due to AMD after 2006.[14, 15, 46, 47] The public campaigns that were initiated after the introduction of anti-VEGF have undoubtedly contributed to the reduction of visual loss, as they made elderly persons more aware of the symptoms and stimulated prompt therapy.[48, 49]

Projections of Age-Related Macular Degeneration in Europe

It is unclear whether the prevalence of AMD will decrease even more in the coming years, but an increase is not likely to be expected. Therefore, we projected the estimated number of AMD-affected persons until the year 2040 based on 2 different scenarios: 1 based on stable prevalence and 1 following the trend of declining prevalences. The results of the first scenario suggests that the absolute number of persons with late AMD will increase by 2.1 million, a 1.5-times increase. A Norwegian study predicted, under the assumption of a stable prevalence, the same relative increase of affected subjects, with a total of 328 000 cases of late AMD in Scandinavia by 2040.[5, 8] A study in the United States calculated a 2.2-times increase in absolute numbers and estimated a total number of affected subjects to be 3.8 million by 2050.[5, 8] Worldwide projections have shown a doubling of late AMD and an increase of 9 million cases by 2040.[3]

The second scenario was based on declining rates, and showed a small increase in the number of people with early AMD, from 14 million in 2016 to 14.9 million by 2040, and a larger relative increase in the number of people with late AMD, from 2.7 million in 2016 to 3.9 million by 2040. Considering the declining rates of smoking and implementation of healthier diet in elderly persons, the second projection may be more legitimate.

Study Limitations

A limitation to this E3 consortium meta-analysis is the heterogeneity across studies regarding study design and inclusion criteria. For example, age at inclusion and method of recruitment varied between studies. Although in every study AMD was classified according to the Rotterdam Classification, studies differed in AMD grading, especially for pigmentary changes and drusen size. Given the heterogeneity, we therefore performed a random-effects meta-analysis for both early and late AMD. Furthermore, patient management and access to healthcare may have differed between study sites, resulting in differences in preventative and treatment options.[50, 51]

When data collection started in 1990, fundus photography was the gold standard for grading AMD. Since 1990, imaging techniques evolved rapidly, greatly improving the diagnosis of AMD features with non-invasive techniques such as optical coherence tomography, autofluorescence, and near-infrared photographs. In addition, multimodal imaging better visualizes edema and subtle changes resulting from CNV, which may not be so apparent when the patient was treated with anti-VEGF therapy.[52,53] Although macular edema due to subretinal neovascularization often coincides with prominent retinal changes such as hemorrhages or hard exudates, our data may have underestimated the true prevalence of CNV.[53]

In summary, this study estimates the prevalence of early and late AMD per age category in Europe over the past two decades. Prevalence of both these forms remained stable or decreased slightly. Nevertheless, we observed a significant reduction in the proportion of visually impaired eyes attributable to CNV after 2006. Unfortunately, due to the aging population, the number of people with AMD will increase during the next decades, indicating a continuous need to develop comprehensive modalities for prevention and treatment of AMD.

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2

BURDEN OF AMD

2.2 Five-year Progression of Unilateral Age-related Macular Degeneration to Bilateral Involvement: The Three Continent AMD Consortium Report

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ABSTRACT

Purpose: To assess the 5-year progression from unilateral to bilateral age-related macular degeneration (AMD) and associated risk factors.

Design: Pooled data analyses of three prospective population-based cohorts, the Blue Mountains Eye Study, Beaver Dam Eye Study and Rotterdam Study.

Methods: Retinal photography and interview with comprehensive questionnaires were conducted at each visit of three studies. AMD was assessed following the modified Wisconsin AMD grading protocol. Progression to bilateral any (early and late) or late AMD was assessed among participants with unilateral involvement only. Factors associated with the progression were assessed using logistic regression models while simultaneously adjusting for other significant risk factors.

Results: In any 5-year duration, 19-28% of unilateral any AMD cases became bilateral and 27-68% of unilateral late AMD became bilateral. Factors associated with the progression to bilateral involvement of any AMD were age (per year increase, adjusted OR 1.07), carrying risk alleles of the *complement factor H* and *age-related maculopathy susceptibility 2* genes (compared with none, OR 1.76 for 1 risk allele, and OR 3.34 for 2+ risk alleles), smoking (compared with non-smokers, OR 1.64 for past and OR 1.67 for current smokers), and the presence of large drusen area or retinal pigmentary abnormalities in the first eye.

Conclusions: One in four to one in five unilateral any AMD cases, and up to one in two unilateral late AMD cases, progressed to bilateral in 5 years. Known AMD risk factors, including smoking, are significantly associated with the progression to bilateral involvement.

INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of blindness in western populations[1]. While the presence of AMD in one eye can be debilitating, vision loss and blindness in both eyes due to bilateral AMD will have severe consequences for the affected individuals [2,3].

Previous studies report the development of late AMD in the second eye to be 20-50% over 5-10 years[4-9]. However, the progression from unilateral early AMD to bilateral early or any (early and late) AMD[10], and its associated risk factors has been less well described.

We aimed to report the 5-year progression of unilateral AMD cases to bilateral involvement in three population-based cohorts, the Three Continent AMD Consortium (3CC). We also aimed to investigate this progression in relation to risk factors and early AMD lesion characteristics.

METHODS

Among the 3CC, we included non-Hispanic white, population-based cohort studies conducted in Australia, the USA and the Netherlands[11,12]. Written informed consent was obtained from each participant at each visit, in all three studies. All studies adhered to the tenets of the Declaration of Helsinki.

Blue Mountains Eye Study

The Blue Mountains Eye Study (BMES) recruited 3654 participants (82.4% of those eligible) aged ≥49 years living in two postcode regions west of Sydney between 1992-1994[13]. Of these participants, 2334, 1952 and 1149 were re-examined after 5, 10 and 15 years, respectively. Examinations were approved by the University of Sydney and the Sydney West Area Health Service Human Research Ethics Committees.

Beaver Dam Eye Study

The Beaver Dam Eye Study (BDES), conducted in Beaver Dam, Wisconsin, examined 4926 participants (83.2% of those eligible) aged 43-86 years from 1988-1990[14]. Of these participants, 3721, 2962, 2375 and 1913 were re-examined after 5, 10, 15, and 20 years, respectively. The University of Wisconsin-Madison approved all study visits in conformity with federal and state laws and compliance with the Health Insurance Portability and Accountability Act.

Rotterdam Study

At baseline (1990-1993), the Rotterdam Study (RS) examined 7983 participants (77.7% participation rate) aged 55+ years, of whom 6419 had ophthalmic examinations and retinal photography performed[15]. Of 6419 participants, 4977, 3637 and 2674 were re-examined at the second (1993-1995), third (1997-1999) and fourth (2002-2004) visits, respectively. The mean follow-up period was 10 years. Only data from the first, third and fourth visits were used. Examinations were approved by the Medical Ethics Committee of the Erasmus Medical Centre and the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study).

Retinal photography

Mydriatic stereoscopic color fundus photographs were taken at each study visit. Zeiss fundus cameras (Carl Zeiss, Oberkochen, Germany) were used in the first three visits of BMES (FF3) and all visits of BDES (FF4), and 30˚ stereoscopic color fundus photographs of the macula and optic disc, and non-stereoscopic photographs of the other retinal fields of both eyes were taken in both studies. Topcon TRV-50VT fundus camera (Topcon Optical Co., Tokyo, Japan) was used in the RS during the first visits, and 35˚ stereoscopic colour fundus photographs of the macula were taken. In the fourth visit, the BMES used a Canon CF-60 DSi with DS Mark II body (Canon, Tokyo, Japan) to take 40˚ digital photographs; and the RS used a Topcon TRC 50EX fundus camera with Sony DXC-950P digital camera (Topcon Optical Co.) to take 35˚ digital photographs.

Photographic grading and definitions of AMD

Retinal photographs of both eyes were initially graded by trained graders of each study following the Wisconsin Age-related Maculopathy Grading System (WARMGS)[16]. All late AMD incident cases detected from each study were adjudicated and confirmed by the retinal specialists of each study team initially, then cross-checked among chief investigators of the three cohorts[17].

A 5-step severity scale for AMD, developed after phenotype harmonization[11] was used to define AMD severity stage. Levels 10, 20, 30, 40 and 50 corresponded to normal, mild early, moderate early, severe early and late AMD (see online supplementary appendix). We grouped levels 20-40 as early AMD. Unilateral any AMD was defined if either early or late AMD were present in one eye only. Unilateral late AMD was defined as late AMD presence in one eye with no late AMD in the fellow eye (regardless of presence of early AMD). Bilateral any and late AMD were defined as presence of any and late AMD in both eyes, respectively. Progression was defined as the transition from unilateral any or late AMD to bilateral.

Total drusen area, measured as a proportion of the WARMGS grid, and the presence of retinal pigment epithelium (RPE) abnormalities were assessed at first detection of unilateral AMD, as prognostic factors for bilateral involvement. Methods used to calculate total drusen area differed across studies thus we derived quintiles of drusen area within each study to obtain comparable measures. Drusen area was categorized as small, intermediate or large, representing participants who had the lowest 20%, the middle 60% and the highest 20% of drusen area in each population accordingly.

Assessment of risk factors

Smoking status was assessed using interviewer-administered questionnaires. In the BMES, participants were classified as 'non-smokers' if they answered 'no' to smoking regularly, 'past smokers' if they quit smoking >1 year prior to the examination, and 'current smokers' if they currently smoked or stopped smoking <1 year before the examination. In the BDES, participants were classified as 'non-smokers' if they had smoked fewer than 100 cigarettes in their lifetime, 'past smokers' if they smoked ≥100 cigarettes but had stopped smoking before the examination or 'current smokers' if they had not stopped smoking[18]. In the RS, smoking status was defined as never, past or current according to participants responses 'no', 'yes, stopped smoking' and 'yes, still smoking', respectively[19].

Mean systolic and diastolic blood pressures were taken from an average of two readings, except for BMES baseline visit, when one measure was taken. Serum total cholesterol levels, high density lipoprotein (HDL) levels and white blood cell count were measured at baseline from non-fasting blood samples in the BDES and RS and fasting blood samples in the BMES[20].

Genotyping methods

We used two major AMD-associated genes to represent AMD genetic susceptibility, the complement factor H (*CFH*; OMIM 134370) and age-related maculopathy susceptibility 2 (*ARMS2*; OMIM 611313). Genotyping methods are described in the online supplementary appendix[17,21,22].

Statistical Analyses

All statistical analyses were performed using SAS V.9.3 (SAS Institute, Cary, North Caroline, USA).

Progression to bilateral AMD was assessed using discrete time survival analysis, focusing solely on the first 5-year interval since initial detection of unilateral cases. Participants were included at first detection of unilateral AMD and assessed for progression to bilateral involvement at the subsequent 5-year visit.

Progression rates were compared across categories of age, genetic risk levels (carrying 0, 1 or 2-4 risk alleles of the *CFH* and *ARMS2* combined) and smoking status, by individual and pooled study samples, using Mantel-Haenzel χ^2 tests for linear trend.

Associations between progression to bilateral involvement and age, sex, smoking status, genetic risk, blood pressure, white blood cell count, total cholesterol and HDL cholesterol levels were assessed in age-adjusted and multivariable-adjusted logistic regression models. Age, drusen area and RPE abnormalities were time-dependent variables corresponding to the visit when unilateral AMD was first detected. All other co-variables were defined at baseline. Final models included age, sex, smoking status, total drusen area, presence of RPE abnormalities that remained statistically significant in the model. Indicators of study site were included in models using pooled-data. Association estimates are presented as adjusted ORs and 95% CIs.

We obtained a receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) to assess how useful the final model might be in predicting progression to bilateral any AMD in 5 years. The AUC is a measure of discrimination and, here indicates the probability that a person with progression will have a higher predicted value in the model than a person without progression.

Due to limited numbers of cases, associations with progression to bilateral late AMD were examined using pooled-data only, and associations between early AMD lesion characteristics and progression to bilateral late AMD could not be assessed.

RESULTS

Participants with unilateral any or late AMD from the BMES, BDES and RS were included (ages 51+, 44+ and 55+ years, respectively). Among 1490 participants (BMES n=335, BDES n=625 and RS n=530) with unilateral any AMD detected at any visit, 94 (28%), 119 (19%) and 126 (24%) progressed to bilateral in the corresponding cohorts, respectively. Of 96 participants (BMES n=25, BDES n=51 and RS n=20) with unilateral late AMD, 17 (68%), 14 (27%) and 11 (55%) progressed to bilateral in the three cohorts respectively.

Progression to bilateral any AMD

Table 1 compares baseline characteristics between those who did and did not progress in the separate and pooled cohorts. Compared with participants who remained unilateral, those who progressed to bilateral were older, and more likely to have 2+ risk alleles from combined *CFH* and *ARMS2* genes.

Table 1. Comparison of baseline characteristics of participants who did and those who did not progress from unilateral to bilateral any AMD, or from unilateral to bilateral late AMD, in the BMES, BDES, RS individually and combined three cohorts.

		5-Year Progression											
		BMES			BDES			RS			Combined		
		No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*
		Unilateral any AMD to bilateral any AMD											
Participants, n (%)		241	94 (28.1)		506	119 (19.0)		404	126 (23.8)		1151	339 (22.8)	
Mean age (SD), years		66.1 (7.5)	72.4 (7.12)	<0.0001	63.6 (9.4)	69.8 (9.0)	<0.0001	68.1 (7.3)	70.9 (7.8)	0.0002	65.7 (8.5)	70.9 (8.1)	<0.0001
Sex (male), %		41.5	46.8	0.4	49.2	42.9	0.2	44.1	40.5	0.5	45.8	43.1	0.4
Smoking Status, %	Never	57.5	49.5	0.3	43.1	40.3	0.9	34.3	29.6	0.6	42.9	38.9	0.3
	Past	28.8	37.6		37.2	39.5		45.0	48.8		38.2	42.4	
	Current	13.7	12.9		19.8	20.2		20.8	21.6		18.9	18.7	
***CFH* (rs1061170), %**	*TT*	44.2	32.1	0.04	40.3	21.9	0.0003	45.9	34.5	0.02	43.0	29.2	<0.0001
	CT	40.9	41.7		47.8	57.1		42.0	44.5		44.5	48.5	
	CC	14.9	26.2		11.9	21.0		12.2	21.0		12.6	22.4	
***ARMS2* (rs10490924), %**	*GG*	66.7	46.8	0.003	62.5	47.1	0.003	65.7	53.8	0.004	64.4	49.5	<0.0001
	GT	31.8	46.8		33.0	42.9		32.2	38.7		32.5	42.3	
	TT	1.5	6.3		4.6	10.1		2.1	7.6		3.1	8.2	
Combined genetic risk category† %	0 risk alleles	27.8	11.4	0.007	23.5	10.9	<0.0001	29.6	18.5	0.003	26.5	13.9	<0.001
	1 risk allele	43.3	45.6		46.3	35.3		43.1	38.7		44.6	39.1	
	2-4 risk alleles	28.9	43.0		30.2	53.8		27.3	42.9		28.9	47.0	
Mean systolic BP (SD), mmHg		142.9 (19.4)	147.6 (21.5)	0.05	130.4 (19.9)	131.9 (18.7)	0.4	140.9 (21.4)	140.5 (20.6)	0.9	136.7 (21.1)	139.4 (21.1)	0.03

Table 1. Continued

		5-Year Progression											
		BMES			BDES			RS			Combined		
		No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*
		Unilateral any AMD to bilateral any AMD											
Mean diastolic BP (SD), mmHg		84.6 (10.2)	83.2 (9.9)	0.3	78.4 (10.1)	76.3 (10.0)	0.04	75.6 (11.7)	71.3 (10.0)	0.0002	78.7 (11.1)	76.4 (11.0)	0.0006
Mean WBCC (SD), x10⁹ cells/L		6.6 (1.8)	6.4 (1.6)	0.4	7.2 (1.9)	7.2 (1.9)	0.8	6.4 (1.8)	6.3 (1.6)	0.5	6.8 (1.9)	6.7 (1.8)	0.2
Mean total cholesterol (SD), mmol/L		6.1 (0.9)	6.3 (1.0)	0.09	6.0 (1.1)	6.1 (1.0)	0.7	6.4 (1.1)	6.2 (1.1)	0.09	6.2 (1.1)	6.2 (1.0)	0.9
Mean HDL cholesterol (SD), mmol/L		1.4 (0.4)	1.5 (0.5)	0.2	1.4 (0.5)	1.3 (0.4)	0.4	1.4 (0.4)	1.4 (0.4)	0.6	1.4 (0.4)	1.4 (0.4)	0.6
		Unilateral late AMD to bilateral late AMD											
Participants (n)		8	17 (68.0)		37	14 (27.5)		9	11 (55.0)		54	42 (43.8)	
Mean Age (SD), years		77.3 (6.2)	76.5 (6.2)	0.8	73.2 (8.0)	79.4 (6.8)	0.01	73.4 (6.8)	75.9 (5.3)	0.4	73.8 (7.6)	77.3 (6.2)	0.02
Sex (male), %		25.0	23.5	0.9	46.0	35.7	0.5	66.7	63.6	0.9	46.3	38.1	0.4
Smoking Status, %	Never	37.5	47.1	0.6	56.8	50.0	0.3	11.1	9.1	0.6	46.3	38.1	0.5
	Past	50.0	29.4		32.4	50.0		66.7	45.5		40.7	40.5	
	Current	12.5	23.5		10.8	0.0		22.2	45.5		13.0	21.4	
***CFH* (rs1061170), %**	*TT*	12.5	31.3	0.6	16.2	14.3	0.9	33.3	9.1	0.3	18.5	19.5	0.6
	CT	50.0	43.8		67.6	64.3		55.6	54.6		63.0	53.7	
	CC	37.5	25.0		16.2	21.4		11.1	36.4		18.5	26.8	

Table 1. Continued

		5-Year Progression											
		BMES			BDES			RS			Combined		
		No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*	No progression	Progression	p Value*
		Unilateral late AMD to bilateral late AMD											
***ARMS2* (rs10490924), %**	*GG*	62.5	31.3	0.2	29.7	21.4	0.03	33.3	27.3	0.6	35.2	26.8	0.2
	GT	37.5	50.0		40.5	78.6		66.7	63.6		44.4	63.4	
	TT	0.0	18.8		29.7	0.0		0.0	9.1		20.4	9.8	
Combined genetic risk category[†] %	0 risk alleles	12.5	6.3	0.8	13.5	0.0	0.3	22.2	9.1	0.4	14.8	4.9	0.3
	1 risk allele	37.5	31.3		16.2	21.4		22.2	9.1		20.4	22.0	
	2-4 risk alleles	50.0	62.5		70.3	78.6		55.6	81.8		64.8	73.2	
Mean systolic BP (SD), mmHg		146.4 (20.3)	147.5 (16.2)	0.9	136.4 (19.8)	136.6 (18.0)	0.97	137.6 (12.2)	144.3 (17.7)	0.4	138.1 (18.9)	143.0 (6.2)	0.2
Mean diastolic BP (SD), mmHg		82.9 (8.5)	83.5 (8.2)	0.9	75.7 (9.3)	71.7 (11.2)	0.2	71.6 (10.2)	78.4 (11.2)	0.2	76.1 (9.7)	78.2 (11.0)	0.3
Mean WBCC (SD), x10⁹ cells/L		6.9 (2.3)	6.8 (1.1)	0.8	7.2 (2.3)	7.1 (2.1)	0.9	6.6 (1.2)	6.9 (1.6)	0.6	7.0 (2.2)	6.9 (1.6)	0.8
Mean total cholesterol (SD), mmol/L		6.6 (1.3)	6.3 (0.9)	0.4	6.1 (1.2)	5.7 (0.8)	0.3	5.9 (1.0)	5.8 (0.9)	0.7	6.1 (1.2)	5.9 (0.9)	0.4
Mean HDL cholesterol (SD), mmol/L		1.5 (0.3)	1.4 (0.5)	0.6	1.4 (0.5)	1.6 (0.4)	0.4	1.4 (0.3)	1.6 (0.8)	0.4	1.4 (0.4)	1.5 (0.5)	0.4

**p Value for association between baseline characteristics and progression of AMD from unilateral to bilateral (categorical factors) and p value for difference in mean baseline level (continuous factors)*
[†]Total number of risk alleles from CFH and ARMS2 combined.
AMD, age-related macular degeneration; ARMS2,age-related maculopathy susceptibility gene 2 (risk allele T); BDES, Beaver Dam Eye Study; BMES,Blue Mountains Eye Study; BP, blood pressure; CFH, complement factor H (risk allele C); HDL, high density lipoprotein; RS, Rotterdam Study; WBCC, white blood cell count..

Table 2 presents proportions of progression to bilateral any AMD by age, genetic risk and smoking status in separate and pooled populations. Progression was associated with increasing age and increased risk alleles of the *CFH* and *ARMS2* genes. There was no significant crude association between smoking status and progression to bilateral any AMD.

Table 3 presents factors associated with progression to bilateral any AMD by individual cohorts. After adjustment, age and the presence of ≥2 risk alleles from the two genes combined were associated with increased risks of progression across three cohorts, while smoking was non-significantly associated with this progression. Large total drusen area (highest compared with lowest quintile) contributed significantly to the risk of progression in each cohort. The presence of RPE abnormalities was significantly associated with increased risk of progression in the BMES and BDES but not the RS.

Table 4 presents factors associated with progression to bilateral any AMD in pooled data. Progression was more commonly observed in the BMES compared with the BDES. Older age, smoking and carrying ≥1 risk allele from the *CFH* and *ARMS2* were significantly associated with an increased risk of progression. Large total drusen area and RPE abnormalities also contributed significantly to an increased risk of progression.

Table 2. Five-year progression from unilateral to bilateral any and late age-related macular degeneration, by age, genotype and smoking status in the BMES, BDES, RS individually and combined three cohorts.

	5-Year Progression from Unilateral to Bilateral AMD															
	Blue Mountains Eye Study				Beaver Dam Eye Study				Rotterdam Study				Combined			
	Any AMD*		Late AMD‡		Any AMD*		Late AMD‡		Any AMD*		Late AMD‡		Any AMD*		Late AMD‡	
Factors	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent
Age (years)																
40-49	-	-	-	-	3/44	6.8	0/0	0.0	-	-	-	-	3/44	6.8	0/0	0.0
50-59	4/47	8.5	0/0	0.0	11/137	8.0	0/1	0.0	11/58	19.0	0/0	0.0	26/242	10.7	0/1	0.0
60-69	24/136	17.7	2/3	66.7	42/241	17.4	1/11	9.1	40/233	17.2	1/3	33.3	106/610	17.4	4/17	23.5
70-79	51/127	40.2	10/14	71.4	46/163	28.2	8/27	29.6	59/197	30.0	7/12	58.3	156/487	32.0	25/53	47.2
80+	15/25	60.0	5/8	62.5	17/40	42.5	5/12	41.7	16/42	38.1	3/5	60.0	48/107	44.9	13/25	52.0
Total	94/335	28.1	17/25	68.0	119/625	19.0	14/51	27.5	126/530	23.8	11/20	55.0	339/1490	22.8	42/96	43.8
p Trend†	<0.0001		0.8		<0.0001		0.07		0.0005		0.5		<0.0001		0.06	
Smoking Status																
Never	46/180	25.6	8/11	72.7	48/266	18.1	7/28	25.0	37/174	21.3	1/2	50.0	131/620	21.1	16/41	39.0
Past	35/102	34.3	5/9	55.6	47/235	20.0	7/19	36.8	61/241	25.3	5/11	45.5	143/578	24.7	17/39	43.6
Current	12/44	27.3	4/5	80.0	24/124	19.4	0/4	0.0	27/110	24.6	5/7	71.4	63/278	22.7	9/16	56.3
p Trend†	0.1		0.4		0.6		0.8		0.4		0.3		0.4		0.2	
CFH (rs1061170)																
TT	27/119	22.7	5/6	83.3	26/230	11.3	2/8	25.0	41/218	18.8	1/4	25.0	94/567	16.6	8/18	44.4
CT	35/120	29.2	7/11	63.6	68/310	21.9	9/34	26.5	53/215	24.7	6/11	54.6	156/645	24.2	22/56	39.3
CC	22/53	41.5	4/7	57.1	25/85	29.4	3/9	33.3	25/72	34.7	4/5	80.0	72/210	34.3	11/21	52.4

Table 2. Continued

	5-Year Progression from Unilateral to Bilateral AMD															
	Blue Mountains Eye Study				Beaver Dam Eye Study				Rotterdam Study				Combined			
	Any AMD*		Late AMD‡		Any AMD*		Late AMD‡		Any AMD*		Late AMD‡		Any AMD*		Late AMD‡	
Factors	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent	No. of cases/ No. at risk	Per cent
p Trend†	0.01		0.3		<0.0001		0.6		0.006		0.1		<0.0001		0.5	
ARMS2 (rs10490924)																
GG	37/169	21.9	5/10	50.0	56/372	15.1	3/14	21.4	64/317	20.2	3/6	50.0	157/858	18.3	11/30	36.7
GT	37/100	37.0	8/11	72.7	51/218	23.4	11/26	42.3	46/124	27.1	7/13	53.9	134/488	27.5	26/50	52.0
TT	5/8	62.5	3/3	100.0	12/35	34.3	0/11	0.0	9/17	52.9	1/1	100.0	26/60	43.3	4/15	26.7
p Trend†	0.0008		0.09		0.0006		0.3		0.003		0.5		<0.0001		0.8	
Combined genetic risk category§																
0 risk alleles	9/63	14.3	1/2	50.0	13/132	9.9	0/5	0.0	22/136	16.2	1/3	33.3	44/331	13.3	2/10	20.0
1 risk allele	36/120	30.0	5/8	62.5	42/276	15.2	3/9	33.3	46/212	21.7	1/3	33.3	124/608	20.4	9/20	45.0
2-4 risk alleles	34/90	37.8	10/14	71.4	64/217	29.5	11/37	29.7	51/156	32.7	9/14	64.3	149/463	32.2	30/65	46.2
p Trend†	0.002		0.5		<0.0001		0.3		0.0008		0.2		<0.0001		0.2	

**Unilateral any AMD progression to bilateral any AMD*

†Unilateral late AMD progression to bilateral late AMD

‡p Trend calculated using Mantel-Haenszel χ^2 test for linear association

§Combined risk dichotomised as 0 or 1 risk allele of CFH or ARMS2 or 2 to 4 risk alleles of CFH and/or ARMS2

AMD, age-related macular degeneration; ARMS2, age-related maculopathy susceptibility 2 (risk allele T); CFH, complement factor H (risk allele C),

Table 3. Associations of AMD risk factors with 5-year progression from unilateral to bilateral any AMD in the BMES, BDES and RS populations.

		BMES		BDES		RS	
Risk Factors		**Age-adjusted OR (95% CI)**	**Multivariable-adjusted OR* (95% CI)**	**Age-adjusted OR (95% CI)**	**Multivariable-adjusted OR* (95% CI)**	**Age-adjusted OR (95% CI)**	**Multivariable-adjusted OR* (95% CI)**
Age per year		**1.12 (1.08 to 1.16)**	**1.15 (1.09 to 1.21)**	**1.08 (1.05 to 1.10)**	**1.07 (1.04 to 1.10)**	**1.05 (1.02 to 1.09)**	**1.04 (1.00 to 1.07)**
Sex (male)		1.54 (0.91 to 2.60)	1.02 (0.49 to 2.12)	0.90 (0.59 to 1.36)	0.76 (0.47 to 1.24)	0.96 (0.63 to 1.46)	0.92 (0.54 to 1.56)
Smoking Status	Never	1.00	1.00	1.00	1.00	1.00	1.00
	Past	1.74 (0.98 to 3.08)	1.43 (0.65 to 3.12)	1.33 (0.83 to 2.12)	1.57 (0.91 to 2.70)	1.51 (0.93 to 2.45)	1.79 (0.98 to 3.29)
	Current	1.61 (0.70 to 3.67)	2.10 (0.73 to 5.98)	1.48 (0.83 to 2.63)	1.47 (0.78 to 2.77)	1.71 (0.93 to 3.13)	1.69 (0.83 to 3.43)
Combined genetic risk category†	0 risk alleles	1.00	1.00	1.00	1.00	1.00	1.00
	1 risk allele	**2.93 (1.22 to 7.05)**	**3.46 (1.33 to 9.02)**	1.71 (0.87 to 3.37)	1.57 (0.77 to 3.20)	1.42 (0.80 to 2.49)	1.52 (0.83 to 2.79)
	2-4 risk alleles	**5.36 (2.16 to 13.30)**	**5.19 (1.93 to 13.93)**	**4.75 (2.43 to 9.27)**	**4.25 (2.11 to 8.54)**	**2.49 (1.40 to 4.40)**	**2.51 (1.35 to 4.67)**
Blood pressure (per 10mmHg)	Systolic	1.03 (0.91 to 1.17)	-	0.99 (0.90 to 1.11)	-	0.95 (0.86 to 1.05)	-
	Diastolic	0.90 (0.70 to 1.16)	0.96 (0.68 to 1.34)	0.97 (0.78 to 1.21)	0.93 (0.73 to 1.18)	**0.72 (0.59 to 0.86)**	**0.71 (0.57 to 0.88)**
WBCC (per SD increase)		0.93 (0.69 to 1.23)	-	1.12 (0.88 to 1.43)	-	0.96 (0.77 to 1.19)	-
Total Cholesterol (per SD increase)		1.25 (0.92 to 1.69)	-	0.95 (0.76 to 1.19)	-	0.88 (0.71 to 1.08)	-
HDL Cholesterol (per SD increase)		1.15 (0.86 to 1.54)	-	0.93 (0.75 to 1.15)	-	1.04 (0.85 to 1.27)	-
Drusen Area‡	Low	1.00	1.00	1.00	1.00	1.00	1.00
	Intermediate	1.32 (0.58 to 2.99)	1.83 (0.75 to 4.49)	1.79 (0.88 to 3.66)	**2.75 (1.26 to 5.99)**	**2.99 (1.52 to 5.90)**	**2.65 (1.32 to 5.31)**
	High	**9.62 (3.77 to 24.55)**	**15.26 (5.08 to 45.83)**	**7.93 (3.69 to 17.04)**	**12.32 (5.10 to 29.79)**	**9.11 (4.27 to 19.43)**	**9.62 (4.29 to 21.57)**
Presence of RPE abnormality		1.18 (0.65 to 2.15)	**2.61 (1.21 to 5.66)**	0.93 (0.61 to 1.42)	**1.73 (1.03 to 2.90)**	1.02 (0.67 to 1.56)	1.49 (0.91 to 2.43)

Bold values indicate significant ORs.

**Adjusted for age, sex, smoking, combined genetic risk score, diastolic blood pressure, drusen area and RPE abnormalities.*

†Total number of risk alleles from the complement factor H (CFH) and age-related maculopathy susceptibility 2 (ARMS2) genes combined (reference: 0 risk alleles)

‡Total drusen area categorized as low, intermediate and high representing the lowest 20% of drusen area, the central 60% and highest 20%, respectively.

AMD, age-related macular degeneration; BDES, Beaver Dam Eye Study; BMES, Blue Mountains Eye Study; HDL, high density lipoprotein; RPE, retinal pigment epithelium; RS, Rotterdam Study; WBCC, white blood cell count.

Table 4. Associations of age-related macular degeneration (AMD) risk factors with 5-year progression from unilateral to bilateral any AMD and late AMD in pooled data of the BMES, BDES and RS

		Bilateral any AMD		Bilateral late AMD	
Risk Factors		**Age-adjusted OR (95% CI)**	**Multivariable-adjusted OR* (95% CI)**	**Age-adjusted OR (95% CI)**	**Multivariable-adjusted OR† (95% CI)**
Study Population (ref: BDES)	BMES	**1.42 (1.03 to 1.97)**	**1.71 (1.16 to 2.54)**	**5.45 (1.86 to 15.90)**	**7.30 (2.05 to 25.96)**
	RS	1.06 (0.79 to 1.42)	1.10 (0.79 to 1.53)	**3.54 (1.16 to 10.80)**	3.43 (0.82 to 14.31)
Age (per year)		**1.08 (1.06 to 1.09)**	**1.07 (1.05 to 1.09)**	**1.08 (1.01 to 1.15)**	**1.13 (1.05 to 1.23)**
Sex (male)		1.06 (0.82 to 1.37)	0.89 (0.65 to 1.22)	0.86 (0.33 to 2.23)	0.81 (0.28 to 2.34)
Smoking Status	Never	1.00	1.00	1.00	1.00
	Past	**1.51 (1.13 to 2.01)**	**1.64 (1.16 to 2.33)**	1.32 (0.46 to 3.77)	1.97 (0.59 to 6.55)
	Current	**1.65 (1.14 to 2.38)**	**1.67 (1.10 to 2.55)**	2.14 (0.47 to 9.76)	2.01 (0.38 to 10.57)
Combined genetic risk category‡	0 risk alleles	1.00	1.00	1.00	1.00
	1 risk allele	**1.72 (1.17 to 2.54)**	**1.76 (1.17 to 2.64)**	3.61 (0.52 to 25.34)	4.91 (0.60 to 40.03)
	2-4 risk alleles	**3.56 (2.42 to 5.25)**	**3.34 (2.21 to 5.04)**	**6.39 (1.04 to 39.09)**	**12.46 (1.52 to 101.97)**
Blood pressure (per 10mmHg)	Systolic	0.99 (0.93 to 1.05)	-	1.05 (0.81 to 1.36)	-
	Diastolic	**0.84 (0.74 to 0.95)**	**0.82 (0.71 to 0.95)**	1.07 (0.67 to 1.70)	-
WBCC (per SD increase)		1.01 (0.87 to 1.17)	-	1.04 (0.65 to 1.64)	-
Total cholesterol (per SD increase)		0.98 (0.86 to 1.12)	-	0.62 (0.36 to 1.04)	**0.47 (0.26 to 0.84)**
HDL cholesterol (per SD increase)		1.02 (0.88 to 1.17)	-	1.19 (0.77 to 1.83)	-
Drusen area§	Low	1.00	1.00	-	-
	Intermediate	**2.04 (1.34 to 3.10)**	**2.32 (1.50 to 3.59)**	-	-
	High	**8.57 (5.42 to 13.56)**	**10.67 (6.45 to 17.67)**	-	-
RPE abnormality presence		0.99 (0.76 to 1.29)	**1.68 (1.23 to 2.29)**	-	-

Bold values indicate significant odds ratios.

**Adjusted for study population, age, sex, smoking, combined genetic risk, diastolic blood pressure, drusen area and RPE abnormalities.*

†Adjusted for study population, age, sex, smoking, combined genetic risk and total cholesterol.

‡Total number of risk alleles from complement factor H (CFH) and age-related maculopathy susceptibility 2 (ARMS2) genes combined (reference: 0 risk alleles).

§Total drusen area categorized as low, intermediate and high representing the lowest 20% of drusen area, the central 60% and highest 20%, respectively.

BDES, Beaver Dam Eye Study; BMES, Blue Mountains Eye Study, HDL, high density lipoprotein; RPE, retinal pigment epithelium; RS, Rotterdam Study; WBCC, white blood cell count.

Figure 1 presents the ROC curve for bilateral any AMD using the multivariable-adjusted model shown in Table 4. The AUC of this model was 0.79, improved by 0.02 from a model without genetic risk categories (0.77) and by 0.11 from an age-sex-adjusted model (0.68).

Supplementary analyses using comprehensive gene-environment risk scores[23] found no additional improvement to the fully-adjusted model in predicting progression to bilateral any AMD (data not shown). There was no meaningful difference in the association between these risk factors and progression to bilateral any AMD after inclusion of secular trend terms in the model, accounting for different detection timing of the unilateral cases (data not shown).

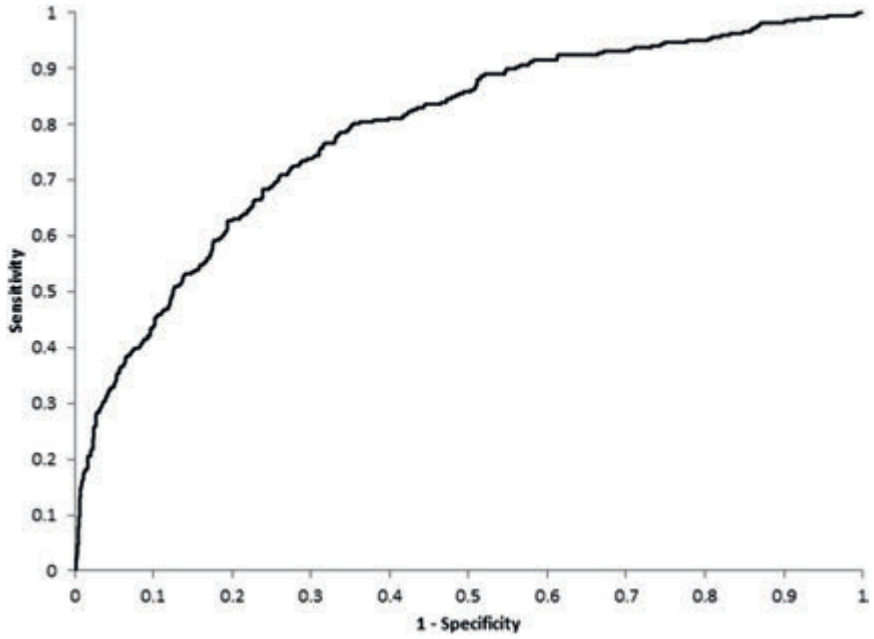

Figure 1. ROC curve indicating the prognostic performance of the model in predicting probabilities of 5-year progression from unilateral to bilateral involvement by any age-related macular degeneration. A ROC curve that follows the left-hand and top axes of the graph (AUC=1) indicates that the model provides perfect discrimination, whereas a diagonal line from the bottom left-hand corner of the graph to the top right-hand corner (AUC=0.5) indicates a model with no discriminative value. Sensitivity (the true positive rate) indicates the proportion of participants with progression who were correctly identified by the model, whereas 1-specificity (the false positive rate) indicates the proportion of participants without progression who were misidentified by the model. AUC, area under the ROC curve; ROC, receiver operating characteristic.

Progression to bilateral late AMD

Compared to participants who remained unilateral late AMD over 5 years, those who progressed to bilateral late AMD were older (Table 1). However, there was no significant trend for age, smoking or genetic crude associations with this progression in separate or pooled cohorts (Table 2).

After adjustment, progression to bilateral late AMD was more commonly observed in the BMES compared to the BDES (Table 4). The presence of ≥2 risk alleles from *CFH* and *ARMS2* genes combined was associated with a high risk of progression. Increased serum total cholesterol was associated with a decreased risk.

DISCUSSION

We found that over a 5-year period, about 19%-28% of unilateral any AMD cases progressed to bilateral, and 27%- 68% of unilateral late AMD cases progressed to bilateral in our three population-based cohorts. In addition to age and AMD genetic risk, smoking and early AMD lesion characteristics were associated with increased risk of progression from unilateral to bilateral involvement in 5 years.

The BDES population includes a younger age spectrum (age 44+ years) compared with the BMES (51+ years) and the RS (55+ years), which may explain why higher proportions of progression were observed in the BMES and RS than the BDES.

The 5-year incidence of late AMD in the fellow eye of unilateral late AMD patients enrolled in a randomized clinical trial was 26%[9] which is similar to that found in the BMES and BDES (29% and 22%, respectively) [4,6]. In the RS 5-year cumulative incidence of late AMD in the fellow eye was 39%[8]. The rates we report currently are greater as the mean age of participants are older than the mean age of the aforementioned samples at baseline, by including additional unilateral AMD cases detected at follow-up visits.

In addition to older age and AMD genetic risk, we documented that past or current smoking was significantly associated with increased risk of progression to bilateral any AMD in pooled analyses. Despite the heterogeneity in definitions, the contribution of smoking to the risk of developing any AMD in the second eye was evident when the sample size increased. The relatively small numbers of participants with unilateral late AMD in each individual population and in pooled data, or the narrow difference in smoking rates between participants with unilateral and bilateral late AMD are likely reasons for the lack of association of smoking with bilateral late AMD found in this report.

The inverse association between increased diastolic blood pressure and reduced risk of progression to bilateral any AMD is not readily explained. The inverse association between increased cholesterol level and reduced risk of progression to bilateral late AMD is not yet understood. Although elevated total cholesterol levels were found to be associated with a reduced incidence of neovascular AMD in a previous study[20], the relationship between total cholesterol levels and AMD risk has been largely inconsistent[12,24,25].

Increasing severity levels of early AMD lesions in one eye were previously reported to be associated with increased incidence of AMD in the fellow eye[10]. We found drusen area to be the strongest predictor for progression to bilateral any AMD within 5 years.

An AUC of 0.79 and 0.77 for bilateral any AMD suggests that both full model and the model without genetic risk categories are satisfactory in distinguishing persons who will progress to bilateral involvement from those who will not. Previous studies found minimal improvement in AUC after including genetic information in prediction models[23,26]. Genetic testing in clinical practice is not supported by ours or other previous findings[27].

We assembled a large number of unilateral any AMD cases from the 3CC. Care has been taken to harmonize AMD grading, confirm late AMD cases across three cohorts[17], and use uniformly the severity scale developed[11] in the 3CC. Limitations include small numbers of unilateral late AMD cases even after pooling, resulting in low power to assess modifiable risk factors. The cohort samples are mostly Caucasians of Northern and Western European descent, and the findings may not be applicable to other ethnic populations.

In summary, 20-25% of unilateral any AMD cases, and up to 50% of unilateral late AMD cases on average, progressed to bilateral in 5 years. Of risk factors associated with the progression to bilateral involvement, only smoking is modifiable. The protective association between cholesterol level and bilateral late AMD warrant further investigation.

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3

GEOGRAPHIC ATROPHY

3.1 Progression of Geographic Atrophy from first diagnosis to life's ending: results from population studies

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ABSTRACT

Importance: Treatments for Geographic Atrophy (GA), a late stage of age-related macular degeneration (AMD), are currently under development. Understanding the natural course is needed for optimal trial design. Although growth rates of GA and visual acuity (VA) at short-term are known from clinical studies, knowledge of growth at long-term, life expectancy, and visual course is lacking.

Objective: To determine long-term progression of GA.

Design: Four cohort studies; Rotterdam Study I, II & III and the Blue Mountain Eye study with up to 25 years follow-up and eye examinations at 5 year intervals.

Setting: Population-based cohorts.

Participants: 171 persons with GA.

Main Outcome Measures: Area of GA was measured pixel by pixel using all available imaging. Growth in and outside ETDRS grid area, growth of the square root transformed area, time until GA reached the central fovea, and time until death were assessed, and best corrected visual acuity, smoking status, macular lesions according to WARMGS grading, and AMD genetic variants were covariates in Spearman, Pearson or Mann-U Whitney test analyses.

Results: 60.7% of eyes with GA was newly diagnosed in our study. Average area of GA at first presentation was 3.96mm^2 (SD 5.03mm^2). Growth rate varied widely between persons (0.02 to 4.05 mm^2/year) with an average of 1.07mm^2/year. Stage of AMD in the other eye correlated with GA growth (r 0.33, p=0.01). Foveal involvement was already present in incident GA in 37%; 74% of eyes developed this after a mean period of 5.6 years (range 3 to 12 years), and foveal involvement did not develop before death in 26%. After first diagnosis, 121/171 (71%) GA patients died after an average period of 6.4 years. Visual function was visually impaired (VA<0.33) in 44% at last visit before death.

Conclusions: Progression of GA is highly variable in the general population. More than one third of incident GA was foveal at first presentation; those with extrafoveal GA became foveal after 5.6 years. Future intervention trials should focus on recruiting those patients who have a high chance of severe visual decline within their life expectancy.

INTRODUCTION

Geographic atrophy (GA), the dry late stage of age-related macular degeneration (AMD), is a growing problem due to the aging population, especially in the European ethnicity. GA is characterized by atrophy of the retinal pigment epithelium (RPE) along with photoreceptor and choriocapillaris loss, which progresses over time. The resulting visual loss can as yet not be prevented,[1,2] but promising therapies are underway. More than 50 trials are currently ongoing or have recently been completed, some with very exciting results [3-5]. The primary end-point that most of these trials use is growth rate of the atrophic lesion during a follow up period varying between 6 months to 6 years[6-11]. More meaningful for patients and clinicians, however, would be the visual prognosis for the rest of the patients' lives[12]. Growth rate over a longer period of time, time until GA reaches the central fovea, and the correlation between progression of GA and life expectancy is currently unclear. The current study employs data from four population-based cohort studies performed on two different continents who have been followed for up to three decades. This provided a unique opportunity to address these questions.

METHODS

Study populations

The participants are from four population based cohorts; the Rotterdam Study (RS) cohorts I, II & III (n=7983, n=3011 and n=3932, respectively), and the Blue Mountain Eye Study (BMES, n=3654)[13,14]. Persons aged 55+ (RSI) and 45+ (RSII&III) years were recruited from Rotterdam, the Netherlands, and residents aged 49+ years from two postcode regions west of Sydney, Australia. Full details in eMethods in Supplement 1. In brief, cohorts had follow-up visits every five years from 1990 (RS) and 1992 (BMES) onwards; total follow-up was up to 25 years for Dutch studies, and 15 years for the Australian cohort. All participants provided written informed consent in accordance with the Declaration of Helsinki to participate in the studies.

Grading and definition of Geographic Atrophy

The diagnosis of GA was based on grading of multimodal images (color fundus photographs CFP; autofluorescence; OCT), of which presence of CFP was the minimum requirement. Exclusion criteria were ungradable GA due to poor image quality, incomplete coverage of the ETDRS grid on CFP. GA was defined as retinal pigment epithelium (RPE) atrophy with a minimal diameter of 175µm and visible choroidal vessels[15] within the ETDRS grid on CFP, as a region of hyper transmission and disruption of the

RPE on OCT[2], and as hypo-auto fluorescence on auto fluorescent imaging. Choroidal neovascularization or treatment for neovascularization prior to GA was not considered GA, nor was RPE atrophy without the presence of any other AMD lesion.

GA was delineated on screen on digital images by four experienced graders from EyeNED Reading Center. Detailed information on GA grading and other AMD features can be found in the eMethods of Supplement 1. Central foveal involvement of GA was considered present when the umbo (foveal dip) showed atrophy of RPE, and peri-foveal area involvement when the edge of the GA was within 250µm[15] from the umbo.

Visual acuity and study covariates

Covariates entered the analyses with the outcome at first diagnosis of GA. Smoking status was categorized as current, former, or never smoker. Follow-up time was calculated from the age at baseline visit. Age of death was obtained from death certificates filled out by the family physician and reports from medical specialists.

Best corrected visual acuity (BCVA) was measured with an ETDRS chart and converted to Snellen equivalent in decimals. BCVA of the eye with GA entered the analysis, this was the eye with the better BCVA when GA was bilateral. Visual impairment was classified into four categories in accordance with the World Health Organization International Classification of diseases ICD-11: blindness <0.05; severe visual impairment <0.1; moderate visual impairment <0.33; and mild visual impairment <0.5. AMD status of the fellow eye was classified by the 3 Continent Classification (3CC): No AMD, mild early AMD, moderate early AMD, severe early or intermediate AMD, or late AMD[16]. Individual genetic and environmental risk scores were calculated as previously described by Buitendijk *et al.*[17] The beta's of the Fritsche *et al.* 2016 paper[18] were used to calculate the genetic risk per participant. When single nucleotide polymorphisms (SNPs) were not available a proxy was used, see eMethods in Supplement 1.

Statistics

Growth of the GA area was calculated as a slope; (area of GA at the follow-up of the study minus area of GA at the start)/years of follow-up. Growth of square root transformed area was calculated with the intention to the effect of baseline area on the rate of growth: (√(area at last visit)- √(baseline area))/years of follow-up. McNemar test (2-sided) for paired proportions was used to evaluate which section in the ETDRS grid was most often affected by GA. Correlations of variables with growth or growth of the square root transformed area were calculated using a Spearman, Pearson or Mann-U Whitney test (all two sided) where appropriate, using the right eye if a participant had bilateral GA. Cumulative incidence was calculated from the incidence rate with the formula: $CI_t = 1 -$

e^{-IR*t} where CI is the cumulative incidence over a *t* period of years, *e* is the constant 2.71828 and IR is the incidence rate. Incidence of new lesions was assumed to have occurred halfway during the follow-up interval. To compare visual impairment between groups, we used Fisher-Freeman-Halton testing because sample sizes in the cross-table were small. A p-value <0.05 was considered statistically significant.

Calculations were made using SPSS (IBM Corp. Released 2012 IBM SPSS Statistics for Windows, Version 25.0 Amonk, NY: IBM Corp). Graphical outputs were constructed with GraphPad Prism 7 (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

Study population

During the entire follow-up of all four cohorts, a total of 242 eyes of 171 participants were identified with GA, of which 147 (60.7%) were incident GA. The 10-year cumulative incidence was 2.8% in RSI, and 3.6% in BMES; the incidence rates were 2.89 and 3.66 per 1000 person years, respectively[17]. The mean age at first diagnosis of GA was 82.6 years (Standard deviation (SD) 7.1 years); 62% were women, see Figure 1 and Table 1. Bilateral GA was present in 71 (42%) cases. Average Dice scores for delineating GA ranged from 0.74-0.99, and was on average 0.83.

Table 1. Characteristics of the four cohort studies

Cohort (start year)	Total nr of people	Average age inclusion (SD)	Women (%)	Current smoker/ previous smoker	Total nr of eyes	Eyes with follow-up	Incident/ prevalent eyes
BMES (1992)	26	81.6yrs (7.4yrs)	19 (73.1%)	3 (11.5%)/ 9 (34.6%)	44	33	32 (72.7%)/ 12 (27.3%)
RSI (1990)	117	83.4 (6.7yrs)	73 (62.4%)	27 (23.1%)/ 39 (33.3%) (6 missing)	163	50	93 (57.1%)/ 70 (42.9%)
RSII (2000)	20	81.0yrs (7.8yrs)	8 (40.0%)	1 (5.0%)/ 17 (85.0%)	25	5	18 (72.0%)/ 7 (28.0%)
RSIII (2006)	8	78.1yrs (9.7yrs)	6 (75%)	1 (12.5%)/ 3 (37.5%)	10	0	4 (40%)/ 6(60%)
Total	171	82.6 (7.1yrs)	106 (62.0%)	32 (18.7%)/ 68 (39.8%) (6 missing)	242	88	147 (60.7%)/ 95(39.3%)

BMES = Blue Mountain Eye Study, RS = Rotterdam Study, SD = standard deviation

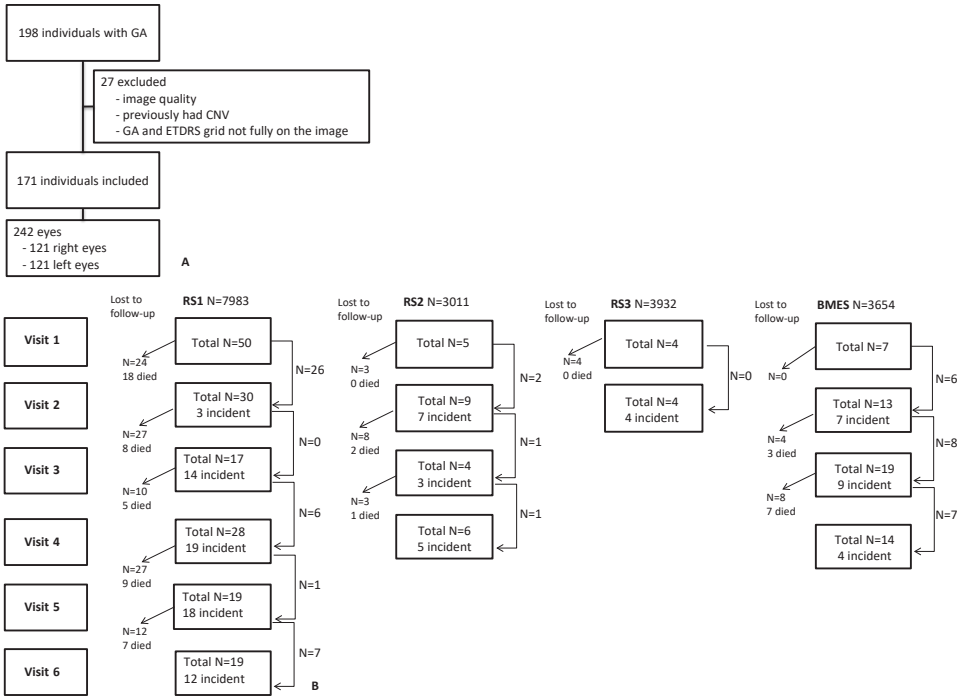

Figure 1. A. Flow-chart of inclusion and **B** follow-up flowchart. RS= Rotterdam study. BMES= Blue Mountain Eye Study. Incident cases are part of the total mentioned in each box. Number of people died is part of the number of people lost to follow up.

Area and growth

Combining all GA, the average area at first presentation was 3.96mm² (SD 5.03mm²), with large variations between individuals (smallest area 0.06 and largest 23.25mm²). The RS II and RS III cohorts, with younger populations, presented with smaller areas: 0.83mm² and 2.39mm². The starting area in prevalent cases was larger with 5.44mm² (ranging from 3.34 to 6.01mm² between cohorts) than the starting area of incident cases 2.64mm² (ranging from 0.39 to 4.97mm² between cohorts). Growth rate was measured in mm²/year and mm/year (Figure1 a and b). The average rate was 1.07mm²/year (SD 0.96mm², median rate 0.76mm²/year with inter-quartile range of 1.38, square root transformation; 0.23mm/year), this was slightly smaller for prevalent cases than incident cases (Table 2). When including the area outside the grid for the 15 eyes where GA extended beyond the grid borders, the average growth rate was 1.28mm²/year (SD 1.37mm²). Rates varied over 200 fold, ranging from 0.02 to 4.05 mm² per year, and up to 6.64mm² per year for the 15 eyes with GA growing outside the ETDRS grid. Baseline lesion size was an important determinant of growth rate (Pearson correlation 0.296, p-value 0.018), but this

lost statistical significance after square root transformation (Pearson correlation 0.020, p-value 0.875, eFigure 1 c and d). We also examined GA growth of bilateral GA and observed a high correlation between eyes (Pearson correlation 0.87 p<0.0001; Pearson correlation of 0.71 (p<0.0003) when including the area outside the ETDRS grid; Figure 2).

Table 2. Area of Geographic Atrophy at first presentation and annual growth

Cohort	Starting area mm² (SD)	Starting area mm² (SD) Prevalent	Starting area mm² (SD) Incident	Average growth mm²/ year (SD)	Average growth mm²/ year (SD) Prevalent	Average growth mm²/ year (SD) Incident
BMES	3.86 (4.61)	3.34 (5.18)	4.97 (5.80)	1.28 (0.87)	0.92 (0.78)	1.49 (0.87)
RS1	4.34 (5.47)	6.01 (6.50)	2.27 (2.74)	0.99 (1.03)	1.07 (1.14)	0.83 (0.80)
RS2	0.83 (1.08)	4.81 (6.02)	0.96 (0.96)	0.53 (0.35)	0.19 (0.24)	0.77 (0.15)
RS3	2.39 (3.68)	3.72 (4.35)	0.39 (0.44)	No follow-up	No follow-up	No follow-up
Total	3.96 (5.03)	5.44 (6.21)	2.64 (3.70)	1.07 (0.96)	0.99 (1.04)	1.16 (0.87)

BMES = Blue Mountain Eye Study, RS = Rotterdam Study, SD = standard deviation

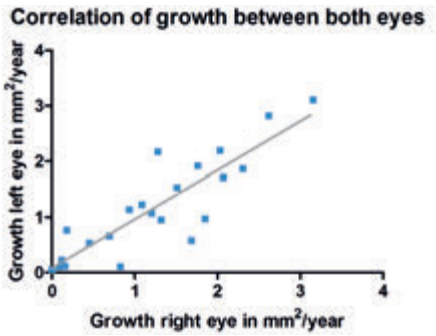

Figure 2. Correlation of GA growth between both eyes of one participant in mm² per year.

Foveal involvement and visual acuity

Of 147 eyes with incident GA, 55 presented with central foveal involvement (37%) at initial presentation. Of these, 76% presented with VA<0.5 and 16% with legal blindness (VA<0.05). Of 42 incident eyes who were examined at subsequent visits, 8 (19.5%) already had central foveal involvement at first presentation; 14 eyes developed central foveal GA within 5 years (33.3%), 8 within 10 years (19.0%), and one eye did so after 12 years. Taken

together, the average time for foveal involvement was 5.6 years, the incidence rate 18.5 per 100-person years. In 11 eyes (26%), GA never reached the central fovea during an average of 5.13 years of follow-up, but 8 of these 11 did reach the peri-foveal area.

We examined the predilection site for the first presentation of GA in the ETDRS grid. First presentation occurred most often in the central circle of the ETDRS grid but outside the central fovea (115 lesions out of 147 incident lesions) (78%), significantly more often than in the superior and temporal inner circle (McNemar test p<0.05), see eFigure 2. GA was more than twice as likely to develop in the inner or central circle than the outer circle. When incident GA was present in the outer circle, all subfields were equally affected (n=43/147 29% vs. n=39/147 27%).

Almost half (48.6%) of the eyes with incident GA presented with BCVA 0.5 or higher. Eyes with central foveal involvement had significantly more often severe visual impairment (BCVA <0.1) and blindness (BCVA <0.05) than eyes without central or peri-foveal involvement (9/31 vs. 0/47 eyes, Fisher-Freeman-Halton exact test p-value=<0.0001). Central foveal GA had worse BCVA than peri-foveal GA (BCVA<0.33 in 23/31 eyes versus 4/17 eyes, Fisher-Freeman-Halton exact test p-value=0.005).

Factors related to growth

We investigated determinants of GA growth, and did not find any differences for age category (younger than 80 years n=44 mean=1.09mm^2 per year, median 0.69mm^2 per year, only incident; mean = 1.14mm^2 vs. 80+ years n=44 mean is 1.05mm^2 per year, median 0.95mm^2 per year, only incident; 1.19mm^2). Sex, presence of hyperpigmentation (n=37), presence of PPA (n=37), drusen area (n=37), smoking (n=63), genetic risk variants in *CFH* (rs1061170, rs1329424, n=57, n=49) and *ARMS2* (rs10490924, n=56) (eFigure 4), or a genetic risk score combining *CFH* (rs1061170 and rs1329424), *ARMS2* (rs10490924) and *C3* (rs2230199) did not show a significant correlation with growth or with the square root transformed growth. Likewise, a combined model of genetic, environmental, and phenotypic factors[17] did not reach statistical significance (Pearson correlation of 0.240, p-value=0.097), eFigure5. The only significant correlation with growth was the AMD status of the fellow eye, GA area grew faster when the fellow eye had a more severe stage (Spearman's rho 0.34, p=0.01).

Survival of GA participants

We particularly addressed life expectancy after diagnosis of GA, since this indicates the burden of visual loss and time available for intervention. 121/171 (71%) participants with GA had died during follow up. Mean age of death was 90.2 years (SD 6.8 years), at a somewhat higher age than persons with CNV or MIXED type of late AMD (88.5 SD

6.4 years) and participants without late AMD (82.9 SD 8.9 years). Participants with incident GA died on average 6.4 years (SD 5.4 years, n=59) after first presentation, ranging from 6 months to 23 years, eFigure 6. The time from incident GA in the second eye to death (n=26) was 5.5 years (SD 4.5 years) ranging from 6 months to 18 years.

We investigated BCVA of GA eyes at the last visit before death; 66% (108/163) had moderate or severe visual impairment (VA<0.33) before death. Among prevalent eyes, 77% (69/89) had moderate or severe visual impairment (VA<0.33) before death; among incident eyes, 53% did so (39/74). Of those with bilateral GA who died during follow-up, 49% had moderate or more severe visual impairment at the last visit before death, Table 3. In the Rotterdam studies, 44% of persons with GA had BCVA 0.33 or less at their last visit at the research center before death, regardless if their other eye was affected by AMD.

Table 3. Visual impairment at last visit in deceased participants with GA

	Blind VA < 0.05	Severe VI VA < 0.1	Moderate VI VA< 0.33	Mild VI VA <0.5
Incident GA[a]	7 (9%)	7 (9%)	25 (32%)	8 (10%)
Prevalent GA[b]	16 (18%)	14 (16%)	39 (43%)	5 (6%)
Bilateral GA	3 (7%)	3 (7%)	16 (35%)	7 (15%)

VI: visual impairment, GA: Geographic Atrophy, VA: visual acuity, [a]n=3 with missing VA, [b]n=1 with missing VA

DISCUSSION

This study investigates the occurrence and natural course of GA in two populations across two continents. Age-specific prevalence and incidence of GA were similar in our cohorts[17]. We found that GA increased in size with 1.07mm^2/year (SD 0.96mm^2/year) ranging from 0.53 to 1.28mm^2/year. The average square root transformation of growth within the ETDRS grid area was 0.23mm/year. For incident eyes the average rate of enlargement in was 1.16mm^2/year versus 0.99mm^2/year for prevalent eyes. First presentation of GA occurred most often in the central circle of the ETDRS grid but outside the fovea, one third presented with atrophy involving the ETDRS outer circle (>1500 µm from the fovea). The incidence rate of GA to reach the central fovea was 18.5 per 100 person years; i.e., 60.4% reached the fovea in 5 years. The average life expectancy of those affected was 6.4 years after the initial presentation; the average age of death 90.2 years, and 44% died with moderate visual impairment or worse.

Our results need to be viewed in light of the strengths and limitations of the study. The long follow-up is a major strength of the study, thus far long follow-up was 5-6 years in most studies[19]. Only the BMES showed results of 15 years of follow-up previously[20]. The long follow-up also enabled us to view GA in the context of life expectancy. The population based cohort design enabled identification of GA cases who were not selected by clinical symptoms and who reflected the entire spectrum of GA. The still functional vision at first presentation makes these participants interesting for interventions, and suggests that screening for GA in an elderly population may facilitate identification of participants eligible for sight-saving therapies. Another strength was the usage of all available imaging per eye to trace the borders of GA simultaneously with software specifically designed for this project, allowing more precise delineation. Among the limitations was the availability of only CFP in the earlier visits of RS and BMES. Another limitation was the low number of participants with incident GA; this hampered the statistical power of the risk associations. In addition, the period between follow-up visits was relatively long, jeopardizing the accuracy of time estimates.

Most studies on progression of GA have focused on patients from clinics. In these studies, growth of GA ranged from 1.14mm^2/year to 3.1mm^2/year as summarized by Fleckenstein *et al*.[19] The growth rate of GA in our study was somewhat lower, but this can be explained by the relatively small lesion area at first presentation. When comparing the square root of GA progression, growth rates were more in line with growth rates reported in literature. Our results were also very similar to those of Holmen *et al*.[21] who also studied incident GA and found an enlargement rate of 0.97mm^2/year. We identified more smaller GA lesions which were not necessarily symptomatic. With respect to time to foveal involvement, AREDS investigators reported that 57% developed this after 4 years[7]. This is very similar to our 5 year cumulative incidence rate of 60.4%. Central fovea as initiation site for GA was also very similar between our studies; 33% in AREDS versus 37% in our study).

The highly correlated growth (0.87) we observed between the two eyes was previously noticed by other research groups who found a correlation coefficient ranging from 0.74 to 0.88[8,22-24]. This high correlation suggests a common mechanism, which may be driven by genetics. Only few studies investigated AMD genetic risk variants for GA progression; they predominantly examined the important SNPs in *ARMS2* (rs10490924) and *CHF* (rs1061170)[7,20,25,26]. Two studies found an increase in GA progression for the *CFH* SNP, and two studies for the *AMRS2* SNP, but other studies did not find any statistically significant results.[20,25,27,28]. We analyzed AMD risk variants as part of a risk score, as well as separate SNPs. We did not find significant effects on GA growth at the univariate level or combined in a genetic and environmental risk score. A consideration is the homogeneous genetic background of GA cases; >95% (104/109) had at least one of the four major risk SNPs, hampering detailed analysis of the genetic effect. A recent genome wide association

study found novel SNPs specifically associated with GA progression: variants in *PRMT6* (rs11184959) and *LSS* (rs2839127)[29]. Jointly, these SNPs explained only 6% of the variation in GA progression. Our study shows that AMD phenotype in the other eye is a much better predictor. Taken together, our results and those of others suggest that the other eye is one of the most important prognostic indicator of progression up until now[7,9,23,24,27,29-31].

We showed that participants with GA generally have functional visual acuity at first presentation, but develop a progressive decline thereafter. GA in and close to the central fovea had a largest impact on visual acuity, as expected and found before [12,24]. Although our examinations at 5 year intervals enabled only a sketchy estimate of the proportion of GA participants who become blind before death, we observed that a high proportion (66%) of GA eyes were severely visually impaired or blind before death, and 49% of patients had bilateral low vision. The time between diagnosis and death varied widely (6 months-23 years) among GA patients, with a median of 5 years. This time period is important as it is the time available for potential interventions. Based on our data, early screening for GA before visual symptoms occur seems most promising to find eligible candidates for trials or future interventions. This improves treatment potential enormously as it increases the chance of preserving functional vision for the longer term.

In conclusion, our natural history study of GA ascertained from a general population showed that 37% of newly diagnosed GA already had atrophy in the fovea, suggesting that a relatively high proportion of GA patients is not very suitable for sight-saving therapy at an early stage. Non-foveal GA reaches the fovea on average within 5.6 years, which implies that this is, on average, the time window for treatment. However, this is highly variable and best predicted by the other eye. Almost half of the patients lose functional vision during their lifetime, making a strong case that the need for successful therapy is high.

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3

GEOGRAPHIC ATROPHY

3.2 A Deep Learning Model for Segmentation of Geographic Atrophy to Study Its Long-Term Natural History

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ABSTRACT

Purpose: To develop and validate a deep learning model for the automatic segmentation of geographic atrophy (GA) using color fundus images (CFIs) and its application to study the growth rate of GA.

Design: Prospective, multicenter, natural history study with up to 15 years of follow-up.

Participants: Four hundred nine CFIs of 238 eyes with GA from the Rotterdam Study (RS) and Blue Mountain Eye Study (BMES) for model development, and 3589 CFIs of 376 eyes from the Age-Related Eye Disease Study (AREDS) for analysis of GA growth rate.

Methods: A deep learning model based on an ensemble of encoder-decoder architectures was implemented and optimized for the segmentation of GA in CFIs. Four experienced graders delineated, in consensus, GA in CFIs from the RS and BMES. These manual delineations were used to evaluate the segmentation model using 5-fold cross-validation. The model was applied further to CFIs from the AREDS to study the growth rate of GA. Linear regression analysis was used to study associations between structural biomarkers at baseline and the GA growth rate. A general estimate of the progression of GA area over time was made by combining growth rates of all eyes with GA from the AREDS set.

Main Outcome Measures: Automatically segmented GA and GA growth rate.

Results: The model obtained an average Dice coefficient of 0.72 ± 0.26 on the BMES and RS set while comparing the automatically segmented GA area to the graders' manual delineations. An intraclass correlation coefficient of 0.83 was reached between the automatically estimated GA area and the graders' consensus measures. Nine automatically calculated structural biomarkers (area, filled area, convex area, convex solidity, eccentricity, roundness, foveal involvement, perimeter and circularity) were significantly associated with growth rate. Combining all growth rates indicated that GA area grows quadratically up to an area of approximately 12 mm^2, after which growth rate stabilizes or decreases.

Conclusion: The deep learning model allowed for fully automatic and robust segmentation of GA on CFIs. These segmentations can be used to extract structural characteristics of GA that predict its growth rate.

INTRODUCTION

Geographic atrophy (GA) occurs in the advanced stage of age-related macular degeneration (AMD). It is characterized by progressive atrophy of the retinal pigment epithelium, overlying photoreceptors, and underlying choriocapillaris.[1] Areas of GA often initially appear extrafoveal, which may result in their causing difficulties in reading or dim-light vision.[2] Over time, the atrophic area may grow, and when it reaches the fovea, visual acuity is severely diminished. Prevalence of GA increases exponentially with age[3] and is highest in people of European ancestry.[4] The number of people affected by GA is expected to increase further in the near future because of the ageing population.[5]

Currently, no approved treatment exists to prevent progression of GA.[6,7] However, clinical trials of several potential therapies are underway.[8] For evaluation of these trials, reliable anatomic endpoints are required, because visual acuity alone provides insufficient insight in the severity of the disease.[9] Growth rate of the atrophic area has been suggested as an important indicator of disease progression.[9-11] However, the speed at which GA progresses varies greatly between patients.[12-14] Therefore, understanding the patterns associated with progression and the variability between patients is important for the design and interpretation of clinical trials.

To assess growth rate, accurate delineation of the GA area is required. However, because manual delineation can be challenging and time consuming,[15,16] automatic segmentation could provide a scalable and reproducible alternative. Deep learning has emerged as a powerful technique for the automatic analysis of medical images.[17] Deep learning models require labeled examples (training data) to tune their internal parameters. The model then learns to extract features that are important for the segmentation task without further need of explicit domain knowledge from experts. It has been successfully applied to color fundus images (CFIs) for classification of severity stages in AMD[18,19] or diabetic retinopathy,[20-22] and recently also for the detection of GA.[23] Although manually labeled examples are still required for training and validation, the model thereafter can be applied to large data sets, opening up new possibilities for studies and possibly reducing the overall effort that is required from experienced graders.

These automatic methods also have the potential to extract structural characteristics of GA efficiently and accurately, as seen in imaging that has been demonstrated to correlate with growth rate. For example, multifocal lesions grow faster than unifocal lesions,[24] and extrafoveal lesions grow faster than foveal lesions.[13] Circular lesions have been demonstrated to grow at a slower rate than more irregularly shaped lesions.[25]. Baseline lesion area has been consistently associated with future growth, with larger lesions growing faster than smaller lesions.[11,13,26,27] However, applying a square root

transformation to the lesion size may remove this dependency.[16,28] Therefore, it has been hypothesized that lesions with approximate circular shape grow at a constant radial speed, thus leading to a quadratic growth of the area.[16,29]

Various imaging methods have been used to assess GA. Color fundus imaging has been used most widely historically, particularly in large epidemiologic studies.[12] More recently, fundus autofluorescence (FAF) and optical coherence tomography (OCT) have also become popular for the study of GA and GA progression.[13,16,27] Several lesion characteristics visible with those methods can be linked to progression of GA. For example, banded or diffuse perilesional patterns on FAF and structural abnormalities at the junctional zone on OCT have been associated with faster GA progression.[13,27,30] Although GA may be detected earlier on FAF images than CFIs,[31] good agreement on quantification of GA area on CFIs between 2 independent reading centers has been demonstrated,[11] and progression rates assessed from both FAF images and CFIs are highly correlated.[13,31] Color fundus imaging has the advantage that it is widely available, often over longer periods, making it suitable for the study of long-term progression of GA.

Previous work on automatic methods for segmentation of GA focuses mainly on OCT[32-34] or FAF.[35] Feeny *et al*.[36] proposed a method based on a random forest classifier in color fundus imaging. In contrast, herein we present a model that is based on deep learning.

The purpose of this study is twofold: (1) to develop and validate a fully automatic model for segmentation of GA on CFIs and (2) to demonstrate its usefulness in a longitudinal setting for the study of GA progression. The performance of the developed model was compared against the work of 4 graders on a challenging dataset to evaluate its robustness. Next, the automatically segmented GA areas provided measures of structural characteristics related to lesion size, location and morphologic features. We investigated the associations between those structural characteristics at baseline and the subsequent growth rate of GA. Finally, we combined GA growth rates across patients to obtain an estimate of average progression of GA area over time.

METHODS

Data

Data for development and evaluation of the deep learning model for GA segmentation were collected from the Blue Mountains Eye Study (BMES)[37] and the Rotterdam Study (RS) cohorts I, II and III.[38] The developed model was applied to CFIs from the Age-Related Eye Disease Study (AREDS)[11] for the assessment of GA growth rate.

The BMES is a population study from the Blue Mountains region in Australia that started between 1992 and 1994 and included 3654 participants 49 years of age or older. For the first 3 visits, 30˚ macula-centered (field 2) CFIs were obtained with a Zeiss fundus camera (Carl Zeiss AG, Oberkochen, Germany). For the fourth visit, 40˚ macula-centered digital CFIs were obtained with a Canon CF-60 DSi with DS Mark II body (Canon, Tokyo, Japan). The BMES was approved by the University of Sydney and the Sydney West Area Health Service Human Research Ethics Committees.

The RS is a population study from a suburb in Rotterdam, The Netherlands. The RS cohort I started in 1990 and included 7983 participants 55 years of age or older. Cohort II started in 2000 and included 3011 participants 55 years of age or older. Cohort III started in 2006 and included 3932 participants 45 years of age or older. The CFIs for the first examinations were obtained with a Topcon TRV-50VT (Topcon Optical Company, Tokyo, Japan), and those from the last 2 examinations were obtained with a Topcon TRC 50EX and a Sony DXC-950P (Sony Electronics Inc., New York, NY) digital camera. All CFIs were 35˚ and macula centered (field 2). The RS was approved by the Medical Ethics Committee of the Erasmus Medical Center and by the Netherlands Ministry of Health, Welfare and Sport.

The AREDS is a long-term, multicenter, prospective study of the clinical course of AMD and cataract. Starting between 1992 and 1998, 11 clinics in the United States enrolled 4757 participants between 55 and 80 years of age. Stereoscopic CFIs (30˚ macula centered) were acquired with a Zeiss FF-series camera (Carl Zeiss AG). The AREDS was approved by an independent institutional review board at each clinical center.

The follow-up interval for RS and BMES was 5 years. The AREDS had follow-ups at 6-month intervals, although the typical interval between available CFIs was 1 year. The BMES, RS and AREDS all adhered to the tenets of the Declaration of Helsinki, and all participants provided informed consent.

A total of 504 CFIs of patients diagnosed with AMD and signs of GA were included from the BMES and RS sets. Twenty-six images with mixed signs of AMD (neovascularization, bleedings, scars) were excluded to disambiguate overlapping areas. Furthermore, no GA was delineated in 43 images because it was either not present or ungradable, and 26 images were excluded because of poor image quality. The remaining 409 images were included for development of the model and evaluation of its performance. This set contained 87 images from the BMES (26 participants, 43 eyes) and 322 images from the RS (149 participants, 195 eyes). The 409 images represent 315 unique visits (some visits had 2 CFIs available).

Images for the study of GA progression were selected from the AREDS set, following the grading available from the database of genotype and phenotype 2014 table. Inclusion

criteria were presence of GA or central GA, and at least 2 years of follow-up. Images with neovascular disease co-occurring with GA were excluded. A total of 3589 images of 376 eyes were included. Most of these images were stereoscopic, so this accounted for 1826 unique acquisitions (eye-visit). Pixel-to-millimeter conversion was fixed for all images, based on the average distance between fovea and center of the optic disc measured in a subset of the images. This distance was assumed to be 4.5mm.[39]

Delineations of GA area were made by 4 graders (3 of them with more than 20 years of experience), using an in-house created software platform for manual annotations (https://www.a-eyeresearch.nl/software/ophthalmology_workstation/).[40] For the RS, additional multimodal imaging (infrared, FAF, OCT or a combination thereof) was available for some of the visits, and the platform allowed images of the same eye (both multimodal and longitudinal) to be aligned manually by identifying corresponding landmarks. The graders could view images of the same eye simultaneously using a synchronized cursor on multiple screens. Geographic atrophy was identified as the absence of the retinal pigment epithelium and increased visibility of the choriocapillaris on CFIs. Additional evidence from other methods was used whenever available. Areas of macular and peripapillary atrophy were delineated as separate classes, but for this study only macular GA was used.

Each grader annotated the entire BMES set, whereas the RS set was divided in such a way that each grader annotated approximately half of the entire set and every image was graded by at least 2 graders. Finally, a consensus grading was made for all images in both sets. During the consensus grading, all graders decided together which of the individual gradings was most accurate and updated this grading, if necessary, until consensus was reached. If 2 CFIs of the same visit were present, both were included for model development, and the delineated GA area was propagated from one image to the other by using the affine transformation calculated from the manual landmarks. For evaluation, only the CFI that was used to make the consensus grading was used. Additionally, for external validation of the model, we randomly selected 100 CFIs from 100 participants in the subset of AREDS images that contain GA (including 32 CFIs with co-occurring neovascular lesions). A single grader delineated the GA area in all of these images, whereas an additional 2 graders delineated GA in 50 of the 100 selected CFIs.

Deep Learning Model

The proposed deep learning model for GA segmentation consisted of an ensemble of several models, each trained with partly overlapping training sets. The network architecture (the topology of connections between internal parameters of the deep learning model) for each model consisted of a deep encoder-decoder structure with residual blocks and shortcut connections, similar to that of De Fauw *et al*,[41] but adapted to work with CFIs.

This architecture, and its variations, can be characterized by a contracting path in which the high-resolution input image is converted to a low-resolution abstract representation, followed by an expanding path in which the original resolution is reconstructed. The contracting and expanding path are connected by shortcut connections. This approach has been shown to be very effective for semantic segmentation in medical imaging for which large contextual information is required.[17,42]

Input to each model was both the original color (RGB) image and a contrast-enhanced version of the same image, both resampled to 512x512 pixels. The contrast-enhanced image was obtained by subtracting a blurred image from the original image.[43] The input was transformed through the many layers of artificial neurons in the contracting and expanding path and ultimately yielded a new image in which the value of every pixel represented a likelihood of being part of an area of GA. A schematic overview of the model can be found in Figure 1. More details about the model and the training procedure used for this study can be found in the Appendix (available at www.aaojournal.org).

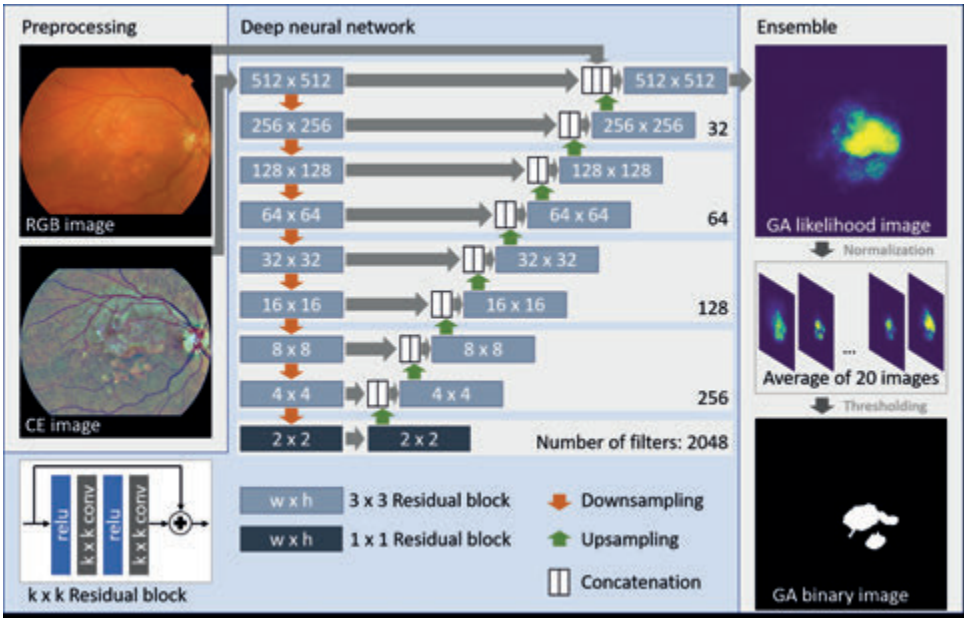

Figure 1. Schematic overview of the model. On the left, the preprocessed input: a 512x512 color image (RGB) and a contrast enhanced (CE) version. In the middle, the model, with the downsampling path (orange arrows), upsampling path (green arrows), and shortcut connections (gray arrows). The ensemble model combines multiple outputs into a single binary image. The geographic atrophy (GA) area in the chosen example is intentionally ambiguous to highlight how the ensemble handles differences in predicted GA between the individual models.

Geographic Atrophy Segmentation

For the development and validation of the model, we applied a 5-fold cross-validation scheme. Data from BMES and RS were merged into 1 dataset and split randomly at patient level into 5 approximately equal folds. In a rotating scheme, 4 folds were used for model training and validation (development set), whereas the remaining fold was used for performance evaluation (test set). Furthermore, 4 separate models were created within each development set. Each model used 3 folds for tuning of the internal parameters (training) and 1 for validation. An ensemble of these 4 models was then evaluated on the respective test set. The output of the ensemble model was obtained by taking the average output of the individual models for every pixel, after correcting for differences in sensitivity between models. This procedure is explained in more detail in the Appendix (available at www.aaojournal.org). Ultimately, an ensemble of the 20 obtained models (4 models developed for each of the 5 rounds) constituted the final model. Performance of this model was validated on the selection of 100 CFIs from the AREDS set.

The performance of the model and the agreement between graders were assessed using the Dice coefficient, which is defined as 2 times the intersection of 2 areas divided by the sum of the individual areas. Hence, a value of 0 represents disjoint areas (no overlap), whereas a value of 1 represents perfect agreement. Dice coefficients were calculated between graders to assess the inter-observer agreement, whereas the areas delineated in the consensus grading were used as reference for the model. Note that the consensus grading was not independent of the individual gradings, and therefore could not be used as a reference to estimate graders' performances. Furthermore, intra-class correlation coefficient of the GA area and of the square root of the GA area were used to measure agreement between graders and the model.

Geographic Atrophy Growth Rate

The final deep learning model (the ensemble of 20 models) was applied to CFIs from AREDS for the analysis of GA progression. It is well documented that GA area increases faster for larger lesions. To remove the dependency of baseline lesion size on growth rate, many researchers apply a square root transformation to the GA area.[28] Similarly, we calculated the square root annual growth in millimeters per year for each eye to assess progression in the AREDS set.[39] This value was obtained from the slope of a linear regression through the square root of the GA area for a selected set of timepoints. The selected set consisted of all available CFIs within a window of 2 years for which the number of available CFIs was highest for the respective eye. The window was limited at 2 years because growth rate and lesion characteristics may change over time.[25] We

calculated the correlation of square root annual growth rate between fellow eyes, and compared growth rate between groups using an unpaired *t* test for unilateral versus bilateral cases, unifocal versus multifocal cases, and foveal versus extrafoveal cases.

To identify structural characteristics or features that may be predictive for growth rate, we built a linear model based on features that were extracted from the segmented GA area at baseline (the first image within the selected window). Candidate features were area, perimeter, convex area, filled area, convex solidity (area divided by convex area), filled area (area divided by filled area), number of lesions, eccentricity, circularity, roundness and foveal involvement. Details on how these features were calculated can be found in the Appendix (available at www.aaojournal.org). Associations between individual features and square root annual growth rate were calculated using univariate linear regression. Because the features were not independent, a multivariate linear model was created to investigate further which features best explain variation in square root annual growth rate. The multivariate model was built using forward selection by iteratively adding the feature that yielded the highest increase in adjusted R^2 value, until it increased no further. When stereoscopic images were available, lesion characteristics were represented by the mean of the 2 calculated values. To obtain a more homogeneous set for the prediction model, we discarded images in which the relative difference in GA area between the left and right stereoscopic image was more than 50%, and included only eyes with at least 2 years of follow-up images.

Finally, we combined all estimates of GA growth in a single figure. Geographic atrophy growth in square millimeters per year (not square root transformed) was estimated as a function of GA area, again using a linear regression for each eye through the GA area in a window of 2 years. This resulted in an estimate of GA growth (the slope of the regression), bounded by a minimum and maximum GA area. The estimated general GA growth for a given GA area was then represented by the mean of all growth estimates for which this GA area fell within the respective area bounds. Confidence intervals were estimated using bootstrapping.

RESULTS

Geographic Atrophy Segmentation

The deep learning model reached a Dice coefficient of 0.72 ± 0.26 (n=315), measured in cross-validation in the BMES and RS data sets, where each test fold was evaluated by the ensemble of 4 models. Dice coefficients between 2 independent graders ranged from 0.72 ± 0.26 to 0.82 ± 0.21 (0.78 ± 0.24 on average). See Table 1 for more details. The intraclass correlation coefficient between the model and the consensus was 0.83 for GA

area and 0.84 for the square root of the GA area. Consistency in those values is visualized further in Figure 2 using Bland-Altman plots. The mean value of the differences between consensus and model did not differ significantly from 0 on the basis of a 1-sample *t* test for either GA area (*P*=0.82) or square root GA area (*P*=0.22). Examples of manually and automatically segmented GA areas can be found in Figure 3.

The average Dice coefficient on the AREDS set was 0.66 ± 0.27 (n=50) for the model, compared with 0.73 ± 0.24 (grader 1) and 0.73 ± 0.27 (grader 2). The intraclass correlation coefficient between the model and the reference grader was 0.77 for GA area and 0.80 for the square root of the GA area (n=100). The mean value of the differences between reference and model did not differ significantly from 0 on the basis of a 1-sample *t* test for either GA area (*P*=0.59) or square root GA area (*P*=0.54). Examples of automatic segmentation results on the AREDS set can be found in Figure S1 (available at www.aaojournal.org).

Table 1. Dice Coefficients between Model and Consensus Grading and between Individual Graders.

	N	Dice coefficient
Model – Consensus	315	0.72 ± 0.26
Grader 1 – Grader 2	146	0.80 ± 0.27
Grader 1 – Grader 3	138	0.78 ± 0.27
Grader 1 – Grader 4	90	0.72 ± 0.26
Grader 2 – Grader 3	91	0.82 ± 0.21
Grader 2 – Grader 4	134	0.78 ± 0.22
Grader 3 – Grader 4	130	0.78 ± 0.19

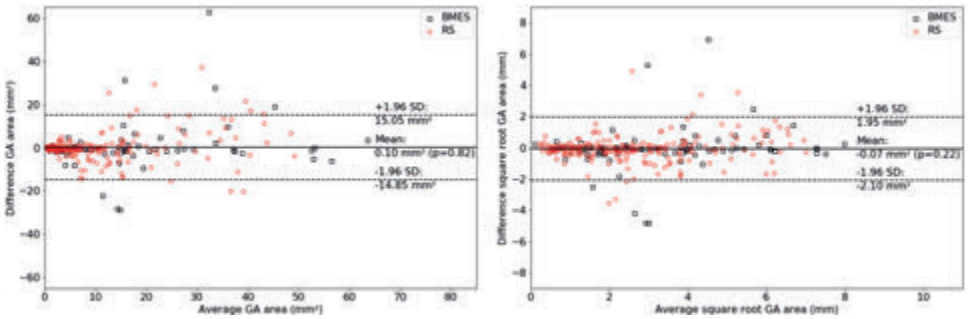

Figure 2. Bland-Altman plot showing (**A**) geographic atrophy area an (**B**) square root GA area. Differences are calculated as the area or the square root area of the consensus grading minus he automatic segmentation. SD = standard deviation.

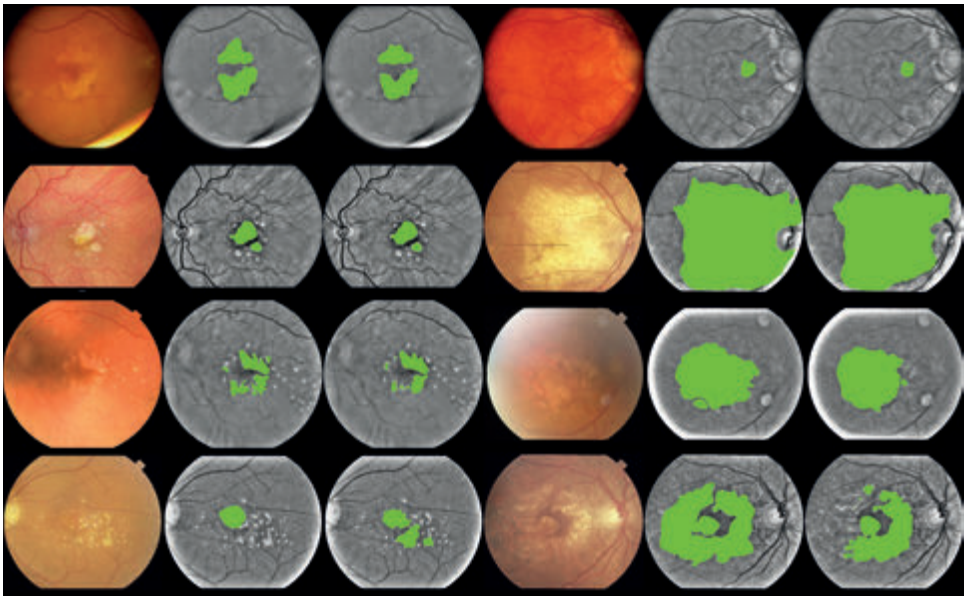

Figure 3. Images showing examples of automatic geographic atrophy (GA) segmentation. The green area corresponds to either the consensus (left) or the model output (right). The top 3 rows show accurate segmentation results for various configurations of GA differing in area, shape and number of lesions and variable image quality and contrast. The bottom row shows examples of inaccurate model output.

Geographic Atrophy Growth Rate

After excluding visits at which the difference between left and right stereoscopic images in the automatically segmented area was more than 50%, 335 of the 376 eyes in the AREDS with at least 2 years of follow-up remained. Square root annual growth of GA for those eyes was 0.25 ± 0.40 mm/year. This value was significantly higher for eyes with small (<5 mm^2) baseline GA area (0.31 ± 0.34mm/year; n=194), compared with eyes with large (≥ 5mm^2) baseline GA area (0.16 ± 0.46mm/year; n=141; P<0.001). Table 2 shows differences in growth rate between groups. We observed that multifocal and extrafoveal lesions grow faster than unifocal or foveal lesions. Patients with bilateral GA showed faster progression than patients with unilateral GA, although this was not significant in our analysis (P=0.58). Growth rates between fellow eyes were correlated (r=0.58; P<0.001). Figure 4 highlights progression of GA for selected individual eyes.

Correlations between baseline lesion characteristic and square root annual growth are summarized in Table 3. Nine out of 11 features were significantly correlated with GA growth rate (after Bonferroni correction). Correlations for the subset with baseline lesions size smaller than 12 mm^2 are analyzed in Table 4. Features included in the multivariate

model were area, circularity, convex area, eccentricity, foveal involvement and number of lesions. The coefficient of determination of this model was 0.18. A visualization that summarizes growth over time for all eyes with GA in the AREDS set can be found in Figure 5. The red dashed line in these graphs represent a quadratic model that best fitted the data for GA area of less than 12 mm^2.

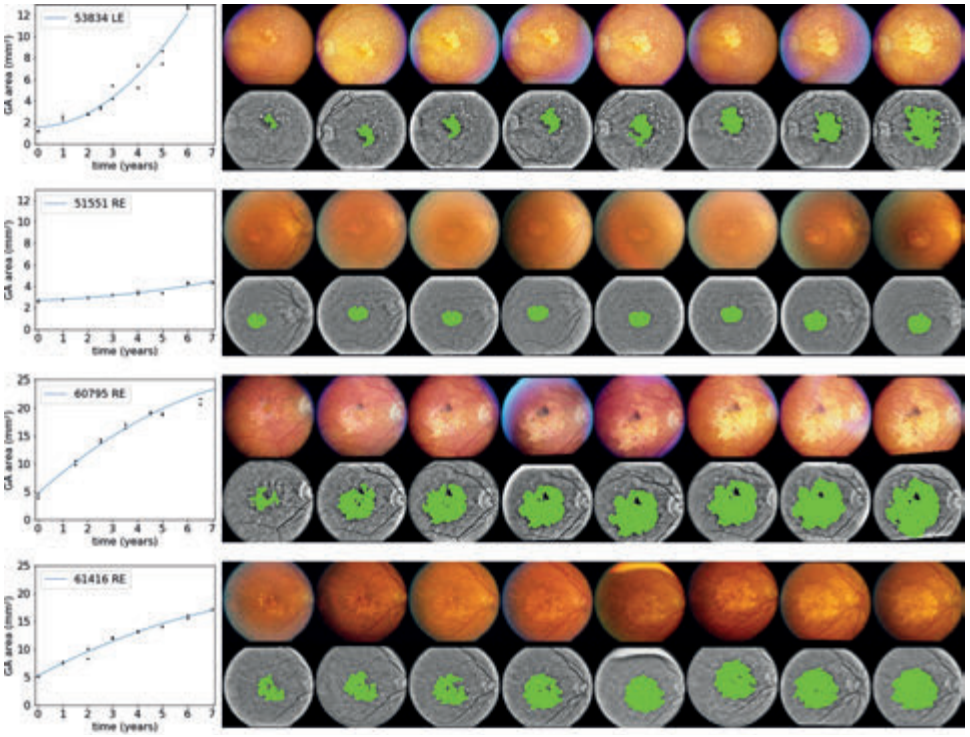

Figure 4. Graphs and images showing progression of geographic atrophy (GA) over time for 4 selected eyes. The graphs represent area measurements over time (2 points per timepoint for the left and right stereoscopic images). The blue line is a quadratic fit through the points. For the top 2 cases, an increment in growth rate can be observed: 53834 left eye (LE) shows a more irregular shape than 51551 right eye (RE) and progressed faster. In the bottom 2 cases, we observe that the growth decreased as the GA area increased.

Table 2. Square Root Annual Growth of the Geographic Atrophy Area.

	Square root annual growth (mm/year)		
	All	**Small (<5mm²)**	**Large (≥5mm²)**
Overall	0.25 ± 0.40 (n=335)	0.31 ± 0.34 (n=194)	0.16 ± 0.46 (n=141)
Unifocal	0.22 ± 0.39 (n=251)	0.28 ± 0.33 (n=142)	0.14 ± 0.44 (n=109)
Multifocal	0.33 ± 0.43 (n=84)	0.39 ± 0.35 (n=52)	0.23 ± 0.52 (n=32)
P value	0.028	0.039	0.339
Foveal	0.21 ± 0.41 (n=258)	0.27 ± 0.31 (n=120)	0.16 ± 0.47 (n=138)
Extrafoveal	0.36 ± 0.36 (n=77)	0.37 ± 0.37 (n=74)	0.14 ± 0.17 (n=3)
P value	0.006	0.066	0.934
Unilateral	0.21 ± 0.32 (n=41)	0.23 ± 0.30 (n=29)	0.15 ± 0.36 (n=12)
Bilateral	0.25 ± 0.41 (n=128)	0.29 ± 0.34 (n=72)	0.19 ± 0.48 (n=56)
P value	0.58	0.446	0.76

Values represent mean ± standard deviation. P values are calculated using an unpaired t test.

Table 3. Correlations between Baseline Lesion Characteristics (Features) and Square Root Annual Growth Rate (in Millimeters per Year).

Feature	***R*² Value**	**Slope**	**Intercept**	***R* Value**	***P* Value**	**Standard Error**
Area	0.101	-0.015	0.351	-0.318	<0.001	0.002
Filled area	0.100	-0.015	0.351	-0.316	<0.001	0.002
Convex area	0.081	-0.012	0.347	-0.285	<0.001	0.002
Convex solidity	0.078	-0.743	0.849	-0.279	<0.001	0.140
Eccentricity	0.073	0.647	-0.167	0.271	<0.001	0.126
Roundness	0.073	-0.697	0.723	-0.270	<0.001	0.136
Foveal involvement	0.050	-2.950	0.370	-0.225	<0.001	0.701
Perimeter	0.029	-0.007	0.336	-0.170	0.002	0.002
Circularity	0.025	-0.282	0.384	-0.159	0.004	0.096
No. of lesions	0.024	0.078	0.130	0.154	0.005*	0.027
Filled solidity	0.001	0.646	-0.396	0.025	0.649*	1.419

*Features are sorted in decreasing order of strength of association. A P value of less than 0.0045 (0.05, Bonferroni corrected) was considered significant. * Not significant*

Table 4. Correlations between Baseline Lesion Characteristics (Features) and Square Root Annual Growth Rate (in Millimeter per year) for Baseline Lesion Size less than 12mm².

Feature	R^2 Value	Slope	Intercept	*R* value	*P* value	Standard error
Convex solidity	0.076	-0.553	0.741	-0.276	<0.001	0.117
Circularity	0.066	-0.365	0.486	-0.258	<0.001	0.083
Roundness	0.060	-0.491	0.628	-0.245	<0.001	0.118
Eccentricity	0.055	0.446	0.005	0.234	<0.001	0.113
Foveal involvement	0.032	-1.858	0.368	-0.179	0.003	0.621
No. of lesions	0.031	0.076	0.188	0.175	0.004	0.026
Perimeter	0.007	0.005	0.252	0.086	0.156*	0.003
Area	0.001	-0.004	0.312	-0.034	0.574*	0.007
Filled area	0.001	-0.004	0.312	-0.034	0.580*	0.006
Convex area	0.001	0.002	0.289	0.026	0.671*	0.005
Filled solidity	0.000	0.091	0.209	0.004	0.948*	1.380

*Features are sorted in decreasing order of strength of association. A P value of less than 0.0045 (0.05, Bonferroni corrected) was considered significant. * Not significant*

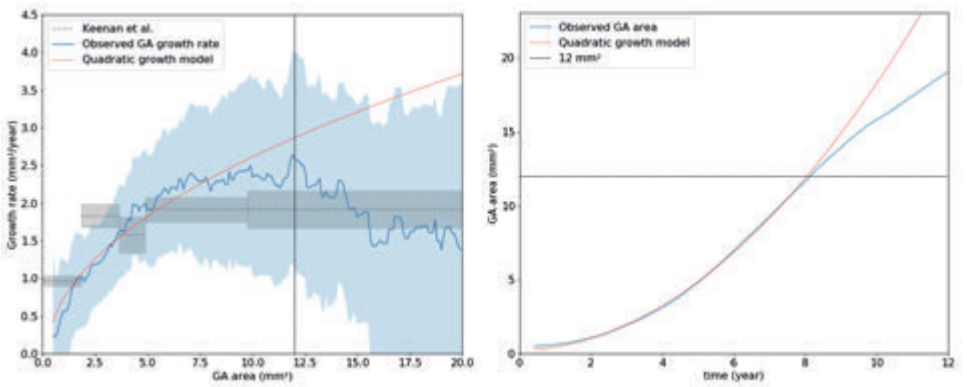

Figure 5. Graphs showing geographic atrophy (GA) growth over time. **A**, Geographic atrophy growth rate (in square millimeters per year) as a function of GA area. The blue line represents growth rates estimated from the segmentations of the deep learning model. The shaded area represents the 95% confidence interval (estimated using bootstrapping). The dashed red line represents the growth rate of a quadratic model, as visualized in (**B**). **B**, Blue line represents the evolution of GA area over time, obtained by numerically integrating the estimated growth rates from (**A**) using a GA area of 0.5 mm² at t=0. The red dashed line represents the best quadratic fit to the plot for GA area of less than 12 mm². Above this area, the observed GA area diverges from the quadratic fit.

DISCUSSION

A deep-learning model for segmentation of GA in CFIs was developed and evaluated. We demonstrated how the automatically obtained segmentations of the model can be used to study growth rate of GA on an independent set and reproduced several previously reported associations with growth rate. The model can also be applied to datasets for which GA measurements are not yet available, providing a fast alternative to manual delineation.

The performance of the deep learning model in terms of Dice coefficient on the BMES and RS set approached that of human experts. The model was able to identify GA even when image quality or contrast were relatively poor, as demonstrated in Figure 3. Nevertheless, some failure cases remained, which was the main reason for the lower average Dice coefficient. We suspect that more training data may solve this issue, because each of the models used only 60% of the data (approximately 245 images) for training, which may not be enough given the inherent difficulty of the problem and the variability in the data. For application to the AREDS set, this problem was circumvented partly by using an ensemble model, which indirectly made use of all training data.

Generalization ability of the model to the AREDS set was assessed on a subset of 50 CFIs. We separately analyzed the performance of the model for cases of pure GA and mixed late AMD, with co-occurring neovascular lesions (Table 5). Performance on the pure GA cases, in terms of Dice coefficient, was comparable with that on the BMES and RS set (0.71 ± 0.26 versus 0.72 ± 0.26). However, performance on mixed cases was significantly worse, as was agreement between graders. Hence, these cases were not included in the analysis of GA growth. We did not observe any bias in the automatic assessment of GA area or square root GA area.

Table 5. Dice Coefficients on the 50 Images Selected from the Age-Related Eye Disease Study Dataset for the Model and 2 Graders Compared with the Reference Grader

	All (n=50)	Pure Geographic Atrophy (n=30)	Mix (n=20)
Model	0.66±0.27	0.71±0.26	0.59±0.27
Grader 2	0.73±0.24	0.78±0.22	0.64±0.25
Grader 3	0.73±0.27	0.81±0.21	0.61±0.31

The obtained mean square root annual growth rate on the AREDS set (0.25 ± 0.40 mm/year) was slightly lower than previously reported values. For example, Domalpally *et al.*[31]

observed 0.30 mm/year, and Keenan *et al.*[44] observed 0.28 mm/year. A reason for this may be the dependence of growth rate on baseline area. When we split the dataset on baseline lesion size, we observed that small lesions have larger square root growth rates (Table 2). This phenomenon was analyzed in more detail in Figure 5. A quadratic curve seemed to fit the observed GA progression very well up to an area of approximately 12 mm^2. For larger areas, the growth rate seemed to stabilize or even decrease, whereas the variability between patients also increased. Similar observations were made by Keenan *et al.*[44], whose reported values are included in Figure 5 for comparison.

The importance of baseline area for assessing growth rate also became apparent in the regression analysis, where area, filled area, and convex area were correlated most strongly with square root annual growth rate. However, when we included only lesions with baseline area of less than 12mm^2 in the regression analysis, no features related to lesion size were significantly associated with square root annual growth rate (Table 4). On an individual level, we also observed a quadratic growth of the area of GA in many cases in the AREDS set, some of them highlighted in Figure 4, in which we fitted a quadratic curve through the GA area over time. Again, the decrease in growth rate for larger lesions was visible (bottom 2 cases in Figure 4).

Of the features that are invariant to lesion size, convex solidity was associated most significantly with square root annual growth rate. Convex solidity is low for irregularly shaped lesions, but also for multifocal lesions. Hence, this feature captures multiple previously reported associations. Of note, the association of square root annual growth rate with circularity was much stronger in the subset of images with baseline area of less than 12mm^2. We observed that the average value for circularity of large lesions (≥ 12mm^2) was significantly lower: 0.40 ± 0.19, versus 0.51 ± 0.23 for small lesions ($P < 0.001$). For large lesions in particular, the model may have produced a segmentation with a very jagged border for lesions with indistinct borders of the atrophic area. This could have led to a relatively large perimeter and hence a lower value for circularity. In those cases, roundness will be a better representation of how well the lesion approaches a circular shape, because it represents the ratio of the area of an enclosing circle and the area of the lesion and hence is less sensitive to irregular borders.[45] Indeed, contrary to circularity, roundness was significantly higher for large lesions (≥ 12mm^2): 0.76 ± 0.10 versus 0.67 ± 0.16 for small lesions ($P< 0.001$).

The multivariate model, which included 6 features related to the baseline shape and size of the GA area, was able to explain 18% of the variation in square root annual growth rate. It is unclear how much of the actual variation is explainable, because there may be factors influencing the growth rate that are not expressed in the image, such as genetics[46], and possibly lifestyle or environmental factors. Moreover, inclusion of follow-up information

or lesion patterns around the border of the GA area may improve the model further, because those have been previously demonstrated to contain additional information and those are not captured by the presented multivariate model.[12] The model also does not take into account nonlinear interactions between features. Finally, errors in the automatically segmented GA may have resulted in inaccuracies, both in the estimation of the features and in the estimation of square root annual growth rate. Therefore, to be expected that in future work, building a model that captures more of the observed variation in growth rate will be achievable.

A limitation of our study is that the conversion from pixels to millimeters may have been inaccurate. This conversion was based on the average distance between fovea and optic disk in a subset of images. Although it is unlikely that this inaccuracy was a source for bias in reported associations with growth rate, reported values for area and growth rate may be slightly larger or smaller in reality.

Furthermore, direct application of the model in a setting where small errors are detrimental, such as clinical trials with GA area or progression rates as the end point, is currently beyond reach. The model may still fail in some cases, and additionally, color fundus imaging may not be the main method for assessment of GA in such a setting, where OCT and FAF are preferred. Nevertheless, the output of the model could still be valuable as a secondary measurement for identification of cases that may need further adjudication.[13,26]

In the future, we will extend the model to other methods, specifically FAF and OCT. This may give more accurate measurements of the atrophic area and hence more reliable assessment of growth rate. In this study, only morphologic features of the atrophic area were considered. A next step would be to include associations between growth rate and other lesions patterns, especially those visible on FAF or OCT. Finally, we are investigating the capabilities of deep learning models to predict directly areas where GA may develop. This will provide predictions of both the extent and location of future GA area.

In conclusion, we have presented and validated a robust segmentation model based on deep learning for GA on CFIs. The model was capable of reproducing known associations between current GA status and future growth. Moreover, we indicated novel structural biomarkers that are predictive for future growth rate, such as solidity, eccentricity or roundness of the lesion. We demonstrated how deep learning can help in the automation of grading, allowing for analysis of larger datasets and helping to understand progression of GA.

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4

GENETICS AND AMD

4.1 Whole-Exome Sequencing in Age-Related Macular Degeneration Identifies Rare Variants in COL8A1, a Component of Bruch's Membrane

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ABSTRACT

Purpose: Genome-wide association studies and targeted sequencing studies of candidate genes have identified common and rare variants that are associated with age-related macular degeneration (AMD). Whole-exome sequencing (WES) studies allow a more comprehensive analysis of rare coding variants across all genes of the genome and will contribute to a better understanding of the underlying disease mechanisms. To date, the number of WES studies in AMD case-control cohorts remains scarce and sample sizes are limited. To scrutinize the role of rare protein-altering variants in AMD cause, we performed the largest WES study in AMD to date in a large European cohort consisting of 1125 AMD patients and 1361 control participants.

Design: Genome-wide case-control association study of WES data.

Participants: One thousand one hundred twenty-five AMD patients and 1361 control participants.

Method: A single variant association test of WES data was performed to detect variants that are associated individually with AMD. The cumulative effect of multiple rare variants with 1 gene was analyzed using a gene-based CMC burden test. Immunohistochemistry was performed to determine the localization of the Col8a1 protein in mouse eyes.

Main outcome measures: Genetic variants associated with AMD.

Results: We detected significantly more rare protein-altering variants in the *COL8A1* gene in patients (22/2250 alleles [1.0%]) than in control participants (11/2722 alleles [0.4%]; P=7.07x10^{-5}). The association of rare variants in the *COL8A1* gene is independent of the common intergenic variant (rs140647181) near the *COL8A1* gene, previously associated with AMD. We demonstrated that the Col8a1 protein localizes at Bruch's membrane.

Conclusions: This study supported a role for protein-altering variants in the *COL8A1* gene in AMD pathogenesis. We demonstrated the presence of Col8a1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis. Protein-altering variants in *COL8A1* may alter the integrity of Bruch's membrane, contributing to the accumulation of drusen and the development of AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among persons older than 50 years in the developed world[1,2]. The disease is characterized by progressive damage to the retinal pigment epithelium and photoreceptors in the macula, ultimately leading to visual impairment and blindness. In the early stages of AMD, a spectrum of changes occur, including hypopigmentations and hyperpigmentations of the retina and the formation of extracellular deposits (drusen) in Bruch's membrane[2]. These drusen increase in size and number during the intermediate stages. Two types of AMD can develop in the end stage of the disease. Geographic atrophy (GA), also referred to as the dry form, is characterized by retinal pigment epithelium cell atrophy, causing photoreceptor cell death. Choroidal neovascularization (CNV), also called the wet form of AMD, is characterized by the formation of new blood vessels, leading to leakage, hemorrhages and sudden loss of vision.

Age-related macular degeneration is a multifactorial disease influenced by a variety of environmental factors, including age, smoking history, and sunlight exposure during working life[3,4]. There is a large genetic component to the cause of AMD, with an estimated heritability between 46% to 71%.[5] Initially, genetic studies in AMD mainly focused on common variants in the population through genome-wide association studies (GWAS) using single nucleotide polymorphism (SNP) microarrays[6–9]. These studies identified genetic variants in or near genes belonging to 4 main pathways, including the complement system, lipoprotein metabolism, angiogenesis, and extracellular matrix remodeling. However, most common genetic variants identified by GWAS are located in noncoding or intergenic regions, and subsequently it is not always apparent which gene near the top-associated SNP is the causative gene.

Involvement of genes in a disease can be established further by identification of protein-altering variants in the coding regions, which are often rare in the population[8]. Thus, several studies focused on the discovery of rare variants by sequencing genes in AMD loci. In these studies, rare variants were identified in complement factor H (*CFH*), complement factor I (*CFI*), complement C3 (*C3*) and complement C9 (*C9*)[10–14] that are associated individually with AMD. Recently, a GWAS performed by the International AMD Genomics Consortium using an exome array enriched with rare variants identified 52 AMD-associated variants at 34 genomic loci. Of these 52 variants, 7 variants were rare and 45 variants were common[8].

Testing the association of individual rare variants can be challenging, because very large sample sizes are needed to obtain sufficient power[15]. Instead of testing each variant individually, gene-based burden tests can evaluate the cumulative effects of multiple

genetic variants within a gene, leading to an increased study power.[16] Sequence analysis of the coding regions of 681 genes within AMD-associated loci in 1676 AMD patients and 745 control participants identified a higher burden of rare variants in *CFI* in patients (7.8%) than in control participants (2.3%)[12,17,18]. Furthermore, evaluation of the cumulative effect of rare protein-altering variants, using exome array data by the International AMD Genomics Consortium, identified a significant burden in 4 AMD-associated genes: *CFH*, *CFI*, tissue inhibitor of metalloproteinases 3 (*TIMP3*) and solute carrier family 16 member 8 (*SLC16A8*)[8]. A limitation of these studies is that either rare variants in a limited set of genes[12] or a limited number of rare variants across the genome[8] were tested.

Whole-exome sequencing (WES) studies allow a more comprehensive analysis of rare protein-altering variants across all genes of the genome[19]. To date, the number of WES studies in AMD case-control cohorts remain few and sample sizes are limited. Whole-exome sequencing of 213 neovascular AMD patients and 1553 healthy control participants from East Asian populations showed association of a variant in ubiquitin protein ligase E3D (*UBE3D*) with AMD[20]. More recently, WES of 39 individuals with bilateral CNV with low genetic risk scores and 36 unaffected control participants with high genetic risk did not detect any genes that reached genome-wide significance[21].

The main goal of the present study was the identification of rare protein-altering variants that are associated with AMD. To achieve this goal, we performed WES in a large European cohort consisting of 1125 patients and 1361 control participants to scrutinize the role of coding variants across the human genome in the cause of AMD.

METHODS

Study Population

A cohort of 2516 individuals of European ancestry (1493 women and 1023 men with a mean age of 79 years) was recruited from the European Genetic Database (www.eugenda.org; n=799) and the Rotterdam Study (n=1717). From the European Genetic Database, 667 AMD patients (488 patients with late AMD) and 132 healthy control participants were evaluated for this study. Inclusion of individuals took place between December 2005 and June 2014. All participants underwent clinical evaluation by a retinal specialist and were graded for AMD according to the Cologne Image Reading Center protocol[22]. Fundus photographs and spectral-domain OCT images were used to classify AMD by the presence of pigmentary changes together with at least 10 small drusen (<63-µm diameter) or the presence of intermediate drusen (63-124-µm diameter) or large drusen (≥125-µm diameter) in the Early Treatment Diabetic Retinopathy Study grid. Furthermore,

late AMD was defined as either AMD with subfoveal GA or CNV in at least 1 eye. Control individuals were included in the study when they exhibited no signs of AMD in either eye and were at least 65 years of age at inclusion.

The design of the Rotterdam Study has been described previously in detail[23,24]. This prospective, population-based follow-up study started in 1990 and has follow-up visits every 5 years. For this analysis, we included a total of 466 AMD patients (74 patients with late AMD) and 1269 control participants from the Rotterdam Study I subcohort 55 years of age and older with WES data. All participants underwent fundus photography of the macula using a 35˚ film fundus camera (Topcon TRV-50VT; Topcon Global Gateway, Tokyo, Japan) after pupillary dilation. For the last 2 follow-up visits, a Topcon digital 35˚ color fundus camera (Topcon TRC 50EX; with a Sony DXC-950P 0.44 megapixel digital camera; Sony Corporation, Minato, Japan) was used. Fundus photographs were graded according to the Rotterdam Classification, which is based on the Wisconsin Age-Related Maculopathy Grading System[25] and the modified International Classification System[26]. Patients were participants with early or late AMD, which is at least soft distinct drusen (≥63-µm diameter), in combination with hypopigmentary or hyperpigmentary changes or soft indistinct drusen (≥ 125-µm diameter) or reticular drusen. Control participants were those older than 65 years with no signs of AMD or those older than 75 years of age with hard or soft distinct drusen (≥63-µm diameter) or pigmentary abnormalities.

In both cohorts, both eyes of all participants were graded separately by experienced graders (T.S.), who were under the supervision of senior retinal specialists (P.T.V.M.dJ., J.R.V., C.C.W.K, and S.F.). The worst affected eye was used to classify the individual. Written informed consent was obtained from all participants. The study was approved by the local ethics committees on research involving human subjects of the participating centers, and all procedures were conducted according to the tenets of the Declaration of Helsinki. The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus Medical Center and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study).

Whole-Exome Sequencing Capture and Variant Calling

Genomic DNA of all participants was isolated from blood samples according to standard procedures. DNA was fragmented into 200 to 400-bp fragments, and the exome library was prepared on a Caliper Sciclone NGS workstation (Caliper Life Science, Hopkinton, MA). The exome was captured with the Nimblegen SeqCap EZ Exome version 2.0 44-Mb kit (Roche Nimblegen, Inc., Madison, WI), covering 329 028 exons and 710 miRNAs. Paired-end sequencing was performed on 2 Illumina HiSeq2000 sequencers using Illumina TruSeq V3 chemistry (Illumina, Inc., San Diego, CA). High-quality reads were mapped to

the UCSC hg19 reference genome using the Burrows-Wheeler alignment tool[27]. Variant calling was performed by Genome Analysis ToolKit (GATK) HaplotypeCaller, following the GATK best practice guidelines (available at https://software.broadinstitute.org/gatk; accessed July 2016). Single nucleotide variants and indels were filtered separately using GATKs Variant-Quality Score Recalibration module. Variants with a variant quality score log-odds (VQSLOD) score lower than -7.2 were removed. Variant annotation was done using annotate variation (ANNOVAR)[28] and an in-house pipeline developed by the Department of Human Genetics of the Radboud University Medical Center[29]. Functional effects of variants were predicted by 3 different prediction algorithms: Sorting Tolerant From Intolerant (SIFT)[30], PolyPhen-2[31], and Combined Annotation Dependent Depletion (CADD)[32] (threshold of deleteriousness for CADD, ≥ 20). In addition, conservation of candidate variants was estimated by PhyloP (threshold for deleteriousness, ≥2.7) and Grantham (threshold for deleteriousness, ≥ 80).

Data Quality Control

Stringent quality control steps were performed with PLINK version 1.07[33] to exclude those positions that had high chances of being false positive results. Variants were removed according to the following criteria: (1) genotypes with a missing rate of more than 5% of individuals and (2) common variants (minor allele frequency, >0.05) that were not in Hardy-Weinberg equilibrium in control participants. After these quality control steps, a total of 744 022 variants were available for analysis. Subject-level quality control was carried out, excluding individuals with a call rate less than 95% or an extreme inbreeding coefficient (cutoff, ± 0.12)[34]. Pairwise identity by descent was calculated to confirm the lack of relatedness among all samples (PI-HAT, <0.25). A multi-dimensional scaling was performed with PLINK version 1.07 to obtain the principal components, which were used to confirm that all individuals were clustered as European samples and to correct for population stratification (Fig S1; available at www.aaojournal.org). After all quality controls, a cohort of 1125 AMD patients and 1361 control participants was selected for association analyses.

Statistical Analyses

A single variant association test was carried out with RAREMETALWORKER (available at http://genome.sph.umich.edu/wiki/RAREMETALWORKER; accessed January 2017) using a linear mixed model. This software performs a score statistics-based rare-variant association analysis, providing single-variant results and a variance-covariance matrix. Linkage disequilibrium relationships between markers within 1 Mb are stored in the covariance matrix to perform the gene-level analyses. Analysis was performed

using an additive model controlling for age, gender, clinic, and the first 4 components. Genome-wide significance levels used for single-variant analysis were defined based on Bonferroni correction ($P \leq 5x10^{-8}$).

By definition, single-variant analyses have limited power to detect rare variant associations, especially for limited sample sizes. Association power was increased by evaluating the accumulated association of multiple rare exonic variants within each gene[35]. Gene-based burden tests were carried out by RAREMETAL[36] using the summary statistics and linkage disequilibrium matrices generated in the single-variant analysis. Three different methods were used: the Combined Multivariate and Collapsing (CMC) counts and Variable Thresholds tests, which are burden tests that assume all alleles to influence the association in the same direction, and the sequence kernel association test (SKAT) test, which evaluates risk and protective alleles to maximize power. A subset of 308 784 rare protein-altering variants (minor allele frequency <0.05) were used in the analysis, to avoid the major presence of non-protein-altering variants (n=435 238) diluting the burden because of deleterious variants. We selected rare variants that alter amino acid residues (nonsynonymous variants), truncate proteins (nonsense and stop-gain variants), or affect RNA splicing (variants affecting the invariate splice donor and splice acceptor sites).

First, we focused on the 34 previously reported AMD loci and we applied a Bonferroni-corrected significance threshold based on the 619 genes located within 500 kb of the top-associated SNP in each of the AMD loci (according to[8]) and carrying at least 1 rare protein-altering variant ($P < 0.05/619 = 8.07x10^{-5}$). Haploview[37] was used to reconstruct the region of interest to validate that the rare Collagen Type VIII Alpha 1 Chain (*COL8A1*) variants belong to different haplotype blocks than the common risk variant rs140647181 identified in a previous single-variant test[8]. In a secondary analysis, we extended the search of rare variant burden to all genes across the genome, applying a Bonferroni-corrected significance threshold of $0.05/17\ 596 = 2.84x10^{-6}$. Quantile-quantile plots of *P* values from single-variant analysis and gene-based tests were generated to discard any batch effect or population substructure.

Characterization of Phenotypic Features of *COL8A1* Variant Carriers

Phenotypic characterization was performed including participants from the Rotterdam Study. Age-related macular degeneration features were based on the eye with the most severe phenotype. Glaucoma-related features were the mean of both eyes at the last visit during follow-up. Refraction was based on the mean spherical equivalent of both eyes at the last visit during follow-up, or the last visit before cataract extraction. Statistical

significance was tested with an independent sample *t* test for continuous variables, a chi-squared or Fisher exact test for dichotomous variables, and a Mann-Whitney *U* test for drusen area because of its nonnormal distribution. All tests performed were 2-sided.

Mouse Retina Staining

Eyes from P60 C57BL/6J wild-type mice were enucleated and embedded in Tissue-Tek O.C.T. Compound (4583, Sakura Finetek, Alphen aan den Rijn, the Netherlands). Seven-micrometer sections were dried for 1 hour at room temperature. Using the hydrophobic PAP pen (Z377821-1EA, Sigma-Aldrich, St. Louis, MO), a circle was drawn surrounding the sections. Retinas were then incubated for 20 minutes in phosphate-buffered saline (PBS) 0.05% Tween (8.22184.0500, Merk millipore, Burlington, MA) and 0.05% Triton X-100 (9002-93-1, Sigma Aldrich) at room temperature. After blocking in 0.1% ovo albumin (A4344,0250, AppliChem GmbH, Darmstadt, Germany), 0.5% fish gelatine (G7041-100G, Sigma-Aldrich) and 5% bovine serum albumin (A7906-100G, Sigma Aldrich) in PBS for 30 minutes, primary antibodies were added and incubated overnight at 4˚C. Primary antibodies used included rabbit polyclonal anticollagen type VIII *α* 1 (1:50; HPA053107, Sigma-Aldrich) and rat monoclonal Laminin β-1 (1:50; MA5-14657, ThermoFisher Scientific, Waltham, MA). Retinas were washed 4x5 minutes in PBS and incubated with the goat antirabbit Alexa 568 (1:500; A11006, Life technologies, Carlsbad, CA) and goat antirat Alexa 488 secondary antibody (1:500; A11006, Life technologies) for 45 minutes at room temperature (dilution 1:500 in blocking solution). Nuclei staining with 4,'6-diamidino-2-phenylindole (1:8000, 0100-20, I.T.K. Diagnostics B.V., Uithoorn, the Netherlands) was combined with the secondary antibody incubation. Sections then were washed 4x5 minutes in PBS, rinsed in MilliQ-purified water, and mounted in Prolong Gold anti-fade reagent (P36930, Life technologies). Imaging was performed using a Zeiss Z1 Imager. All images were obtained at the same intensity. An image with ZEN software was created to obtain TIFF or JPEG files.

RESULTS

Whole Exome Sequencing

We performed WES on 2516 unrelated individuals (1125 patients and 1361 control participants), obtaining an average of 2.8 billion bases per individual and a mean coverage of x63. After variant calling and recalibration, a total of 759 450 variants were identified, being 754 503 single nucleotide variants and 4947 insertions or deletions (indels). Of the complete set of variants, 7.6% (n=57 571) were common variants, and the remaining 92.4% (n=701 879) were classified as rare variants with a minor allele frequency of less than 0.05. Genotype data obtained from WES were checked for concordance with the

genotype data of a customized Illumina exome array[8], available for a subset of the study population (n=1330). Variants genotyped by both WES and exome array (n=80 779) had a concordance rate of more than 99%, demonstrating the high quality of our sequencing data and the high accuracy of our genotype calling.

Single Variant and Gene-Based Association Analyses

We first performed a genome-wide single-variant association analysis for individual common and rare variants using the WES data of 1125 AMD patients and 1361 control participants of European ancestry. Results confirmed association of variants in the *CFH* and Age-Related Maculopathy Susceptibility 2 (*ARMS2*) genes with AMD in this cohort[8]. Two common coding variants in *CFH* (rs1061170 [P=4.24x10^{-11}] and rs1061147 [P=3.30x10^{-10}]) and 1 common variant in *ARMS2* (rs10490924 [P=1.89x10^{-9}]) were associated with AMD above the threshold of genome-wide significance ($P \leq 5x10^{-8}$; see Figs S2 and S3, available at www.aaojournal.org).

Subsequently, we evaluated the burden of rare protein-altering variants in genes at previously identified AMD loci using gene-based burden tests. For this analysis 619 genes were selected that are within 500 kb of the top-associated SNP at 34 AMD loci identified in a recent GWAS[8] (Table S1; available at www.aaojournal.org). A CMC burden test (applying genomic control λ = 0.940) showed a significant burden of rare variants in the *COL8A1* gene (P=7.07x10^{-5}; Fig 1).

We then expanded the burden analysis to protein-altering variants across the genome. The CMC burden test (applying genomic control λ = 1.057) showed a suggestive association in the *KBTBD12* (P=3.50x10^{-6}) and *ZNF787* (P=2.89x10^{-5}) genes, but these associations did not reach the genome-wide significance level (Fig 1). The signal at the *KBTBD12* gene did reach the genome-wide significance threshold when the SKAT test was applied (P=4.45x10^{-7}). In both tests, the association signal was mainly driven by the effect of 1 rare variant (rs148151101; P=1.52x10^{-6}). However, this particular variant in *KBTBD12* was not associated with AMD in an exome array analysis in a cohort of 16 144 AMD patients and 17 832 control participants of European ancestry by the International AMD Genomics Consortium (P=0.387)[8].

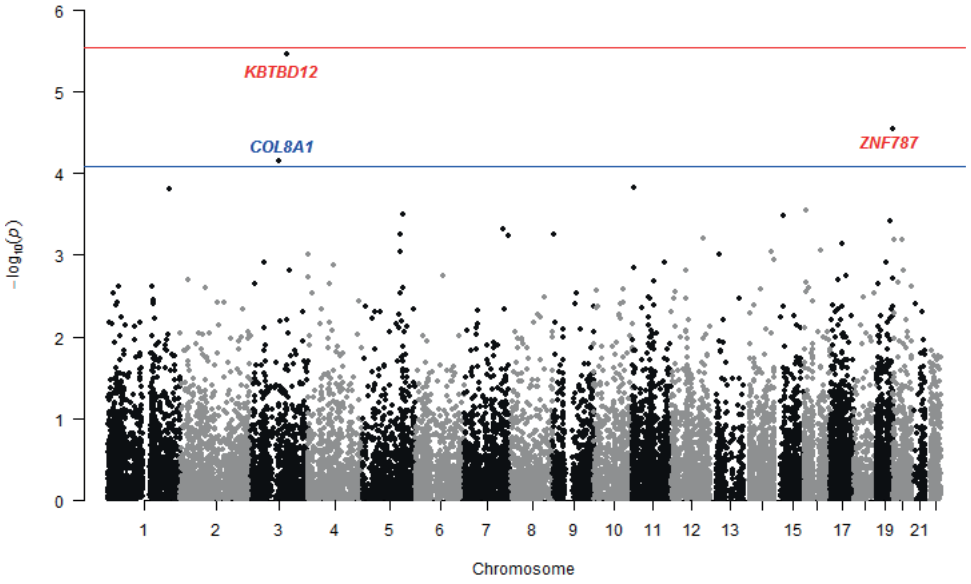

Figure 1. Gene-based burden test for rare variants using whole-exome sequencing data of 1125 AMD age-related macular degeneration (AMD) patients and 1361 control participants of European ancestry. The blue line indicates the significance threshold ($P < 0.05/619 = 8.07 \times 10^{-5}$) for testing 619 genes located in or near AMD-associated loci. The *COL8A1* gene reaches the significance threshold, and is depicted in blue. The red line indicates the genome-wide significant threshold ($P < 0.05/17\,596 = 2.84 \times 10^{-6}$) for genes outside the AMD-associated loci. The *KBTBD12* and *ZNF787* genes do not reach genome-wide significance and are depicted in red. Bonferroni correction was applied to both significance thresholds.

Rare Variant Burden in the *COL8A1* Gene

We next determined whether the rare variant burden in *COL8A1* is independent of the previously identified AMD-associated common variant (rs140647181) near the *COL8A1* gene[8]. This common variant is intergenic, located 560 kb downstream of *DCBLD2* and 177 kb upstream of *COL8A1*. To evaluate the independence between the rare protein-altering variants and the common intergenic variant rs140647181 , we reconstructed the haplotype block structure at the *COL8A1* locus to visualize which regions of the gene are linked closely and are inherited together. Several recombination events between the rare protein-altering variants in the *COL8A1* gene and rs140647181 were observed, meaning that the region containing the rare variants is not inherited together with the region containing the common intergenic variant (Fig 2). These results support that the rare variant burden observed in this study is independent of the common intergenic variant previously associated with AMD.

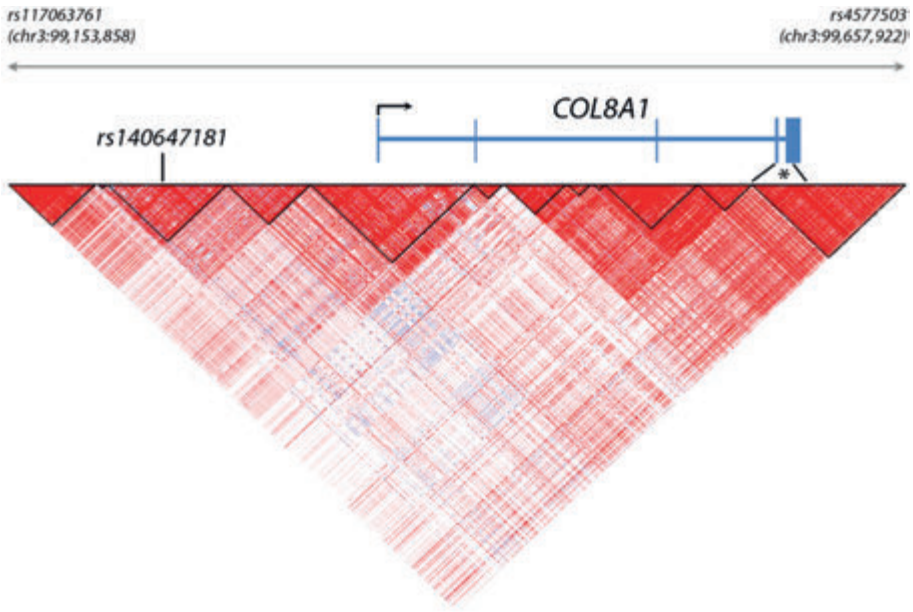

Figure 2. Haploblock structure of the genomic region encompassing the *COL8A1* gene and the age-related macular degeneration-associated common intergenic variant rs140647181. A Haploview plot was generated based on common single nucleotide variants extracted from the 1000 Genomes phase 3 dataset. Red triangles marked with black lines represent genomic regions that are closely linked and are inherited together. This haplotype block distribution shows that the rare protein-altering variants identified in the *COL8A1* gene (indicated with an asterisk) are not located in the same haplotype block as rs140647181, meaning that the rare variants are not inherited together with the common intergenic variant. This supports that the rare variant burden in *COL8A1* is independent of the common intergenic variant rs140647181.

The *COL8A1* burden is explained by 14 rare protein-altering variants spread across the protein (Fig 3), which are found more often in patients (22/2250 alleles [1.0%]) than in control participants (11/2722 alleles [0.4%]; Table 1; Table S2, available at www.aaojournal.org). Six variants, including 1 nonsense variant (p.G414*) and 5 missense variants (p.M70T, p.A96V, p.E520K, p.G711E, p.M744I), were identified only in patients, but not in control participants, and 5 additional variants (p.R225Q, p.A250V, p.R362Q, p.G695D and p.L741F) were found at a higher frequency in patients than in control participants. The nonsense variant p.G414* is predicted to lead to a premature termination in the COL1 domain, or may cause nonsense-mediated decay of the *COL8A1* mRNA. Two missense variants (p.G695D and p.G711E) are predicted to be deleterious with all conservation and pathogenicity tests used and have a CADD score of 20 or more, which classifies them among the top 0.75% most deleterious mutations that are found in the human genome (Table 1). The 2 missense variants p.G695D and p.G711E affect 2 highly conserved amino acid residues in the noncollagenous 1 domain (Fig 3).

Table 1. Rare Protein-Altering Variants Identified in the *COL8A1* Gene in 1125 Age-Related Macular Degeneration Patients and 1361 Controls

Protein change	cDNA Change	Domain	PhyloP*	Grantham*	SIFT (Score)*	PolyPhen2 (Score)*	CADD*	Counts Patients (n=2250)	Counts Controls (n=2722)	Single-Variant *P* Value	Single-Variant Odds Ratio (95% Confidence Interval)	Burden Test *P* Value	Burden Test Odds Ratio (95% Confidence Interval)
V58A	173T→C	NC2	4.317	64	Damaging (0.014)	Poss. damaging (0.646)	19.7	0	1	0.56	0.78232 (0.34-1.79)	7.07×10^{-5}	1.34153 (1.16-1.55)
M70T	209T→C	NC2	4.216	81	Damaging (0.004)	Benign (0.001)	22.5	1	0	0.11	1.9552 (0.85-4.47)		
A96V	287C→T	NC2	1.266	64	Tolerated (1)	Benign (0)	5.6	1	0	0.06	2.18674 (0.96-5)		
P193R	578C→G	COL1	4.028	103	Damaging (0.02)	Poss. damaging (0.463)	22.1	0	1	0.61	0.80691 (0.35-1.84)		
R225Q	674G→A	COL1	6.782	43	Tolerated (0.328)	Benign (0.008)	22.9	1	1	0.48	1.23339 (0.69-2.21)		
A250V	749C→T	COL1	2.257	64	Tolerated (0.338)	Benign (0)	2.5	1	1	0.83	0.93868 (0.52-1.68)		
R362Q	1085G→A	COL1	3.041	43	Tolerated (0.105)	Benign (0.071)	20.5	6	3	0.06	1.3026 (0.99-1.72)		
G414*	1240G→T	COL1	9.803	NA	NA	NA	39	2	0	0.01	2.14633 (1.20-3.85)		
E520K	1558G→A	COL1	9.828	56	Tolerated (0.296)	Poss. damaging (0.945)	21.6	1	0	0.82	1.1015 (0.48-2.52)		
H668Q	2004C→G	NC1	2.728	24	Damaging (0.004)	Prob. damaging (0.989)	25.9	0	1	0.45	0.72856 (0.32-1.66)		
G695D	2084G→A	NC1	9.873	94	Damaging (0.005)	Prob. damaging (0.977)	26.7	4	2	0.08	1.3489 (0.96-1.89)		
G711E	2132G→A	NC1	9.873	98	Damaging (0.014)	Prob. damaging (1)	26.7	1	0	0.08	2.1159 (0.92-4.84)		
L741F	2223G→T	NC1	0.615	22	Tolerated (0.084)	Benign (0.366)	22.2	2	1	0.11	1.47754 (0.92-2.38)		
M744I	2232G→C	NC1	9.477	10	Tolerated (0.186)	Benign (0.001)	24.8	2	0	0.13	1.56374 (0.87-2.81)		

COL1 = triple-helical region; CADD = Combined Annotation Dependent Depletion; NA = not applicable; NC1 = noncollagenous domain 1; NC2 = noncollagenous domain 2; Poss. = possibly; Prob = probably; SIFT = Sorting Tolerant From Intolerant.

** Thresholds for deleteriousness: PhyloP ≥ 2.7, Grantham ≥ 80, SIFT ≤ 0.1, Polyphen ≥ 0.4, CADD ≥ 20.*

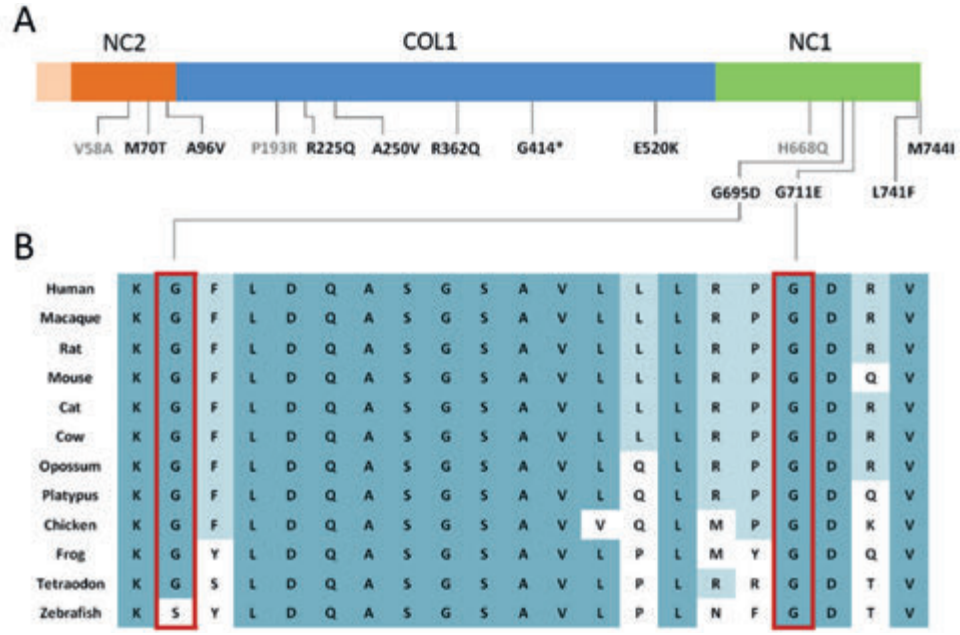

Figure 3. Location and conservation of protein-coding variants in *COL8A1*. **A**, Location of rare protein-altering variants identified in age-related macular degeneration (AMD) patients and control participants in the different *COL8A1* domains: triple-helical region (COL1), noncollagenous domain 1 (NC1) and noncollagenous domain 2 (NC2). Variants detected only in control individuals are depicted in gray. **B**, Alignment of COL8A1 protein sequences of different species. Boxed missense variants identified in AMD patients, predicted to be deleterious in all conservation and pathogenicity tests (Table 1), affect highly conserved glycine residues in the NC1 domain.

Phenotypic Features of *COL8A1* Variant Carriers

We examined the effect of the *COL8A1* variants on the AMD phenotype in participants from the Rotterdam Study only, because it is a population-based cohort study without prior selection on phenotype. This group consists of 16 AMD patients carrying a *COL8A1* variant, 11 individuals carrying a *COL8A1* variant without AMD, and 450 AMD patients without a *COL8A1* variant (Table 2). Features of early AMD were not significantly different between *COL8A1* carriers and noncarriers with AMD, although *COL8A1* carriers had a somewhat higher proportion of hyperpigmentary changes (P=0.062). No statistically significant differences were found for glaucoma-related features such as intraocular pressure and vertical cup-disc ratio. The groups differed significantly in spherical equivalent; with *COL8A1* carriers being more myopic (P=0.005). However, there was no significant difference in the proportion of participants with mild and severe myopia.

Table 2. Comparison of Phenotypic Features between Carriers and Noncarriers of *COL8A1* variants in the Rotterdam Study

Protein Change	*COL8A1* Variant and Age-Related Macular Degeneration (n=16)	*COL8A1* Variant, No Age-Related Macular Degeneration (n=11)	No *COL8A1* Variant and Age-Related Macular Degeneration (n=450)
Age at last visit (yrs)	79.6 (SD, 6.3)	82.5 (SD, 7.9)	80.0 (SD, 6.5)
Spherical equivalent	-0.37 (SD, 1.86)*	1.14 (SD, 1.83)	1.26 (SD, 2.29)*
Mild myopia (-3 to -6D; %)	3/16 (19)	1/11 (9)	23/437 (5)
Severe myopia (≤-6D; %)	0/16 (0)	0/11 (0)	2/437 (0)
Corneal curvature (mm)	7.72 (SD, 0.32)	7.58 (SD, 0.26)	7.70 (SD, 0.26)
IOP (mmHg)	13.8 (SD, 3.0)	14.3 (SD, 2.8)	13.9 (SD, 3.3)
VCDR	0.36 (SD, 0.18)	0.37 (SD, 0.24)	0.32 (SD, 0.18)
Subtype of AMD (no.)	3 GA, 0 CNV, 0 mixed‡, 13 early	-	29 GA, 21 CNV, 21 mixed‡, 379 early
Drusen area >10% (%)	4/16 (25)	0/11 (0)	88/450 (20)
Presence of hyperpigmentation (%)	14/16 (88)	1/11 (9)	287/450 (64)
Presence of reticular drusen (%)	0/16 (0)	0/11 (0)	27/450 (6)
Presence of drusen outside grid (%)	10/16 (63)	7/11 (64)	Not available

*AMD = age-related macular degeneration; CNV = choroidal neovascularization (wet AMD); D = diopters; GA = geographic atrophy (dry AMD); IOP = intraocular pressure; SD = standard deviation; VCDR = vertical cup-to-disc ratio. *P=0.005 independent samples t test (2-tailed) between AMD patients carrying a COL8A1 variant (n=16) and AMD patients without variants in COL8A1 (n=437), t=2.81, degrees of freedom, 451. ‡Geographic atrophy and CNV.*

Localization of *COL8A1* to Bruch's Membrane

Localization of *COL8A1* in the retina has not yet been described in the literature. To assess whether *COL8A1* is localized at Bruch's membrane, the main AMD disease site, we performed immunohistochemistry on retinas of wild-type C57BL/6J adult mice. Mice were selected for these experiments because mouse and human retinas exhibit a common basic architecture[38] and are often used to model human retinal disease, although mice lack a macula, and have a higher photoreceptor cell density and a relatively thicker Bruch's membrane in the central retina[39]. Laminin β-1 was used as a marker for Bruch's membrane[40]. The coimmunostaining of Laminin β-1 and Col8a1 robustly demonstrated that both proteins localize at Bruch's membrane (Fig 4). In addition, Col8a1 showed some expression in the photoreceptor layer, being most evident at the outer plexiform layer, the synaptic region between the photoreceptor cells, and the inner nuclear layer cells. To exclude that the staining was the result of background staining derived from the use

of secondary antibodies, we performed the same procedure without adding primary antibody (Fig S4, available at www.aaojournal.org). This confirmed that the Col8a1 and Laminin β-1 staining observed at Bruch's membrane is the result of the primary antibody.

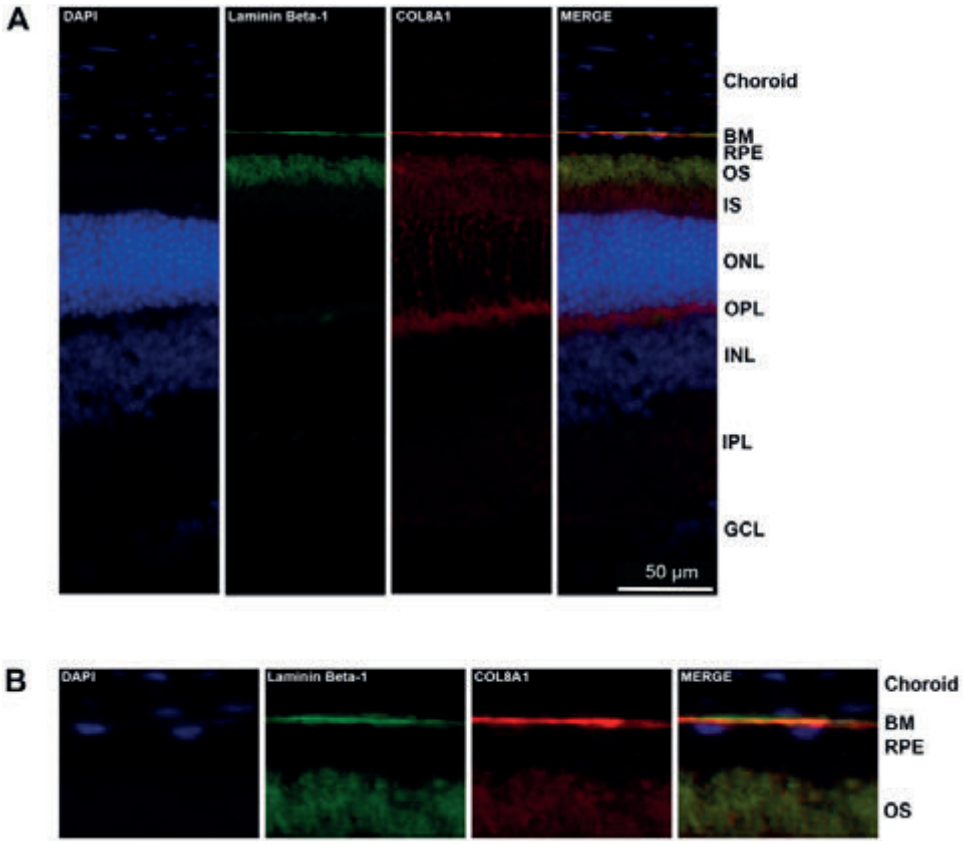

Figure 4. Localization of Col8a1 in mouse retinas. **A**, The localization of Col8a1 (in red) was studied on P90 retinas derived from wild-type C57BL/6J mice. Laminin β-1 (Lamb1; green) was used as a Bruch's membrane marker. Col8a1 colocalizes with Lamb1 at Bruch's membrane. Col8a1 staining also showed a weaker signal in other layers of the retina. **B**, Magnifications of the outer region of the retina, where the colocalization between Lamb1 and Col8a1 can be appreciated. DAPI (4',6-diamidino-2-phenylindole) (blue) was used to stain cell nuclei. BM=Bruch's membrane; GCL=ganglion cell layer; INL=inner nuclear layer, IPL=inner plexiform layer; IS=inner segment; ONL=outer nuclear layer; OPL=outer plexiform layer; OS=outer segment; RPE=retinal pigment epithelium

DISCUSSION

In this study, we aimed to scrutinize the role of rare protein-altering variants in the cause of AMD using WES. By focusing on rare protein-altering variants in the coding regions, we sought to determine the causality of genes in the disease. Because most top SNPs identified in GWAS studies for AMD are in noncoding or intergenic regions[8], it is not always apparent which gene near the top-associated SNP is the causative gene. In this study, WES analysis in 1125 AMD patients and 1361 control participants revealed a significant burden of rare protein-altering variants in the *COL8A1* gene in AMD. The *COL8A1* burden is explained by 14 rare protein-altering variants spread across the protein, which are found more often in patients (22/2250 alleles [1.0%]) than in control participants (11/2722 alleles [0.4%]). The association of rare variants in the *COL8A1* gene is independent of the common AMD-associated intergenic variant rs140647181, located 560 kb downstream of *DCBLD2* and 177 kb upstream of *COL8A1*[8]. No rare-variant burden was observed in the *DCBLD2* gene, nor in other genes at the same AMD locus. Taken together, these findings support that the previously observed association of the common intergenic variant rs140647181 is driven by effects on *COL8A1* rather than by other genes at the locus.

COL8A1 encodes 1 of the 2 *α* chains of collagen type VIII, which is a major component of ocular basement membranes[41]. Several studies have investigated the association between alterations in genes encoding the 2 subunits of collagen VIII (*COL8A1* and *COL8A2*) and ocular abnormalities such as myopic CNV, anterior segment dysgenesis and thin corneal stroma[42-45]. Although several studies postulated a role for *COL8A1* in ocular basement membranes, so far no published data confirmed the localization of *COL8A1* in Bruch's membrane. There are several lines of evidence to support that Bruch's membrane plays a crucial role in AMD. Because of its location, Bruch's membrane is involved intensively in the exchange of numerous biomolecules, nutrients and waste products between the retinal pigment epithelium and the choroidal capillary bed[46]. A disturbed integrity or stability of Bruch's membrane can lead to accumulation of these products in drusen, or can weaken the physical barrier against the invasion of new blood vessels into the retina[47]. In this study, we demonstrate the presence of Col8a1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis[48].

Protein-altering variants in *COL8A1* may lead to structural alterations in Bruch's membrane, which can be responsible for the development of AMD[43]. Interestingly, we describe 14 rare protein-altering variants in *COL8A1*, including 1 nonsense variant (p.G414*), and 2 deleterious missense variants (p.G695D and p.G711E) that affect highly conserved residues in the C-terminal noncollagenous 1 domain. The noncollagenous 1 domain mediates proper folding of the protein and the assembly of collagen VIII and X into polygonal lattices[49-51]. Therefore, these *COL8A1* variants may lead to an aberrantly folded protein,

impairing transport of the protein to Bruch's membrane or altering Bruch's membrane integrity or stability. Consequently, this may contribute to the development of early AMD. In our study, we observed a higher, albeit nonsignificant, proportion of hyperpigmentary changes in AMD patients carrying *COL8A1* variants. Larger patient populations are needed to validate this finding. Previous studies have implicated *COL8A1* in retinal angiogenesis by mediating proliferation and migration of endothelial cells[43], suggesting that *COL8A1* variants could contribute to the development of neovascularization in late AMD. In the Rotterdam Study, we identified 3 *COL8A1* carriers with GA, but no carriers who developed CNV (Table 2). However, in the European Genetic Database cohort, we identified 1 carrier with GA, 3 carriers with CNV, and 2 carriers with the mixed type of AMD with GA and CNV (data not shown). Therefore, we cannot conclude that there is an overrepresentation of CNV in carriers of *COL8A1* variants. Interestingly, *COL8A1* variants seem to contribute to refractive error, although the contribution to severe myopic errors was insignificant. In the Rotterdam Study the refractive error is, on average, emmetropic in AMD cases carrying *COL8A1* variants. Therefore, it is unlikely that myopic thinning of Bruch's membrane contributed to the development of AMD in these carriers.

The findings described herein need to be interpreted in light of several strengths and limitations. We demonstrated that WES with relatively large cohorts is an efficient strategy to detect rare variants in AMD-associated genes. Previous studies that detected rare variants in AMD were focused on predefined gene-sets using targeted sequencing[12] or predefined variant-sets using exome arrays[8], whereas our study performed a comprehensive exome-wide search for rare variants using WES. The main advantage of performing WES is that it enables the identification of all rare variants present in coding regions across the genome, allowing a more comprehensive evaluation of rare variants than other approaches based on a limited set of genes or variants. A burden of rare variants has been described previously in *CFH*, *CFI*, *TIMP3* and *SLC16A8*[8,12], but these findings were not confirmed in our study. This may be because although we had a relatively large cohort, our study may not have had sufficient power to detect these associations. In the study by Fritsche *et al.*[8], a larger cohort was used, consisting of 16 144 AMD patients and 17 832 control participants. However, most of the *COL8A1* variants (11/14) identified by WES in our study were not present on the exome array that was used by Fritsche *et al.*, which may explain why a burden of rare *COL8A1* variants was not observed in that study[8]. In addition, differences in study designs and populations, case definition, geographical origin, statistical tests used, or correction for confounding factors may explain the different results observed among these studies.

In conclusion, we performed an exome-wide sequence analysis of rare protein-altering variants in AMD and we detected a burden of rare variants in the *COL8A1* gene. A common intergenic variant near this gene was associated previously with AMD risk[7,8], but

no protein-altering variants within the gene have been described in AMD so far. This work supports a role for protein-altering variants in the *COL8A1* gene in AMD pathogenesis and suggests that the previously observed association of the common intergenic variant is driven by effects on *COL8A1*. In this study, we demonstrate the presence of Col8a1 in Bruch's membrane, further supporting the role of *COL8A1* variants in AMD pathogenesis. Protein-altering variants in *COL8A1* may alter the integrity of Bruch's membrane, contributing to the accumulation of drusen and the development of AMD. This study showed that WES provides a fruitful approach for gene and variant identification in complex disorders such as AMD. Collaborative efforts among the scientific community are needed to perform even larger exome- or genome-wide sequencing studies[52] that will increase our understanding of the genetic architecture and disease mechanisms of AMD further.

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4

GENETICS AND AMD

4.2 Genetic Risk, Lifestyle, and Age-Related Macular Degeneration in Europe. The EYE-RISK Consortium

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https://www.sciencedirect.com/science/article/pii/S0161642020311192?via%3Dihub

ABSTRACT

Purpose: Age-related macular degeneration (AMD) is a common multifactorial disease in the elderly with a prominent genetic basis. Many risk variants have been identified, but the interpretation remains challenging. We investigated the genetic distribution of AMD-associated risk variants in a large European consortium, calculated attributable and pathway-specific genetic risks, and assessed the influence of lifestyle on genetic outcomes.

Design: Pooled analysis of cross-sectional data from the European Eye Epidemiology Consortium.

Participants: Seventeen thousand one hundred seventy-four individuals, 45 years of age or older participating in 6 population-based cohort studies, 2 clinic-based studies, and 1 case-control study.

Methods: Age-related macular degeneration was diagnosed and graded based on fundus photographs. Data on genetics, lifestyle, and diet were harmonized. Minor allele frequencies and population attributable fraction (PAF) were calculated. A total genetic risk score (GRS) and pathway-specific risk scores (complement, lipid, extra-cellular matrix, other) were constructed based on the dosage of SNPs and conditional β values; a lifestyle score was constructed based on smoking and diet.

Main Outcome Measures: Intermediate and late AMD.

Results: The risk variants with the largest difference between late AMD patients and control participants and the highest PAFs were located in *ARMS2* (rs3750846) and *CHF* (rs570618 and rs10922109). Combining all genetic variants, the total genetic risk score ranged from -3.50 to 4.63 and increased with AMD severity. Of the late AMD patients, 1581 of 1777 (89%) showed a positive total GRS. The complement pathway and *ARMS2* were by far the most prominent genetic pathways contributing to late AMD (positive GRS, 90% of patients with late disease), but risk in 3 pathways was most frequent (35% of patients with late disease). Lifestyle was a strong determinant of the outcome in each genetic risk category; unfavorable lifestyle increased the risk of late AMD at least 2-fold.

Conclusions: Genetic risk variants contribute to late AMD in most patients. However, lifestyle factors have a strong influence on the outcome of genetic risk and should be a strong focus in patient management. Genetic risks in *ARMS2* and the complement pathway are present in most late AMD patients but are mostly combined with risks in other pathways.

INTRODUCTION

Age-related macular degeneration (AMD) is a progressive degenerative disease of the retina and the most important cause of blindness in the Western world. Projections show that in up to 4.8 million Europeans and up to 18.6 million persons worldwide, a blinding stage of AMD will develop by 2040[1, 2]. Age-related macular degeneration is classified into 2 end stages: a more common wet form characterized by choroidal neovascularization (CNV) and a dry form characterized by geographic atrophy (GA) of the retinal pigment epithelium[3]. Only the wet form can be treated with anti-vascular endothelial growth factor agents, but visual decline remains inevitable in the long term[4].

Age-related macular degeneration is a complex genetic disease influenced strongly by a combination of environmental and genetic factors. In particular, smoking and diet are known to increase the risk of AMD considerably. The genetic cause is well established: 52 common, known AMD-associated variants and more than 100 rare variants have been reported[5, 6]. These variants explain most of the disease causes and helped to pinpoint several pathogenic pathways. Of these, the complement cascade seemed to be most important, but the first attempts to target this pathway in intervention trials have achieved limited success[7, 8]. This raises the question whether disease pathways are specific to groups of individuals. If this is the case, intervention trials may be more successful by stratifying patients based on the major disease pathway driving their disease.

In this study, we aimed to investigate the contribution of genetic variants to AMD risk in Europe using data from the large European Eye Epidemiology (E3) Consortium. We aimed to determine the contribution of each disease pathway in AMD and investigated whether lifestyle changes can reduce the risk of late AMD, in particular in individuals with a high genetic risk of AMD.

METHODS

Study Population:

The E3 Consortium is a European collaboration of studies with epidemiologic data on common eye disorders; a detailed description on the consortium can be found elsewhere[9]. All data on AMD were harmonized and collected in the EYE-RISK database (version 6.0). Nine studies from France, Germany, The Netherlands, and Portugal produced data on AMD genotype and phenotype available for analysis and were enrolled as a pooled dataset in the current study. The cohort descriptions of the included studies are listed in the Appendix (available at www.aaojournal.org). The Combined Ophthalmic Research Rotterdam Biobank (CORRBI), Muenster Aging and Retina Study (MARS), and the

European Genetic Database (EUGENDA) were clinic-based studies, and the remaining studies were population based (the Rotterdam Study I, II and III; Antioxidants, Essential Lipids, Nutrition and Ocular Diseases-three cities (Alienor-3C), Maculopathy Optic Nerve nuTRition neurovAsCular and HEarT diseases-three cities (Montrachet-3C); and the Coimbra Eye Study (CES)). Persons 45 years of age and older were included in the analyses; various analyses included only control participants 75 years of age or older. All studies were performed in accordance with the tenets of the Declaration of Helsinki for research involving human subjects and good epidemiologic practice guidelines, and written informed consent was obtained from all participants.

Clinical Examination:

The phenotype of AMD was determined on fundus photographs centered on the macula; individuals received the diagnosis of the worst eye. Age-related macular degeneration features were graded locally by clinicians or experienced graders; classifications were grouped into 3 severity groups. Control participants did not display AMD, aside from only small drusen or only pigment irregularities; persons with early or intermediate AMD showed soft indistinct (large) drusen, reticular drusen, or both, with or without pigmentary irregularities, and further were considered to have intermediate AMD; persons with late AMD had GA or CNV; persons with both end stages were diagnosed as having CNV. Lifestyle factors including smoking and dietary habits were assessed by questionnaire.

Genetic Analyses and Risk Scores

Age-related macular degeneration genetic risk variants were ascertained from the EYE-RISK and E3 database[5, 9]. Studies had used various platforms to determine the 52 known risk variants, such as whole-exome sequencing, exome chip (Illumina HumanExome BeadChip), genomic single-nucleotide polymorphism (SNP) arrays (Illumina 550K [duo] chip or Illumina 610 quad), or TaqMan assays, and a custom-made AMD genotyping platform using single-molecule molecular inversion probes (smMIPs) with next-generation sequencing; for the EYE-RISK genotype assay[10], see cohort descriptions. If variants had been determined by multiple methods that included direct genotyping, we used data from the latter method. When no direct genotyping was available, genotypes were dosages derived from Haplotype Reference Consortium imputation or 1000G. Three (rs71507014, rs67538026, rs142450006) of the 52 known AMD risk variants could not be included in our analysis because genotypes were not available for multiple cohorts.

Genetic risk scores (GRS) were calculated for the 17 174 individuals for whom the five major risk variants (*CFH* rs10922109, *CFH* rs570618, *C2* rs429608, *C3* rs2230199, and *ARMS2* rs3750846) were available. Complete genotype data on minor risk alleles were available in 62.3% persons; 85.1% individuals had 47 of 49 variants. Genetic risk scores

were calculated by multiplying the conditional β value of the AMD risk variant[5] with the allele dosage. Subsequently, all calculations were summed. Pathway-specific GRSs were constructed in the same manner. For the complement GRS, we included all risk variants in the *CFH*, *CFI*, *C9*, *C2*, *TMEM97/VTN*, and *C3* genes. For the lipid GRS, variants in *ABCA1*, *LIPC*, *CETP*, and *APOE* were included. For the extracellular matrix (ECM) GRS, variants in *COL4A3*, *ADAMTS9-AS2*, *COL8A1*, *VEGFA*, and *SYN3/TIMP3* were included. The remaining variants were included in 'other' GRS. The function of *ARMS2* mostly was considered unsettled. However, because recent evidence suggests a role in the complement pathway[11], we analyzed this gene as a stand-alone pathway GRS as well as part of the complement pathway GRS.

Lifestyle Score

Four well-established AMD lifestyle determinants (smoking status and servings of vegetables, fruit and fish per day) were assessed by questionnaire. Smoking status was categorized as no, former, or current smoker. Dietary intakes were analyzed in medium servings per day with a maximum of 1, that is, 120 g of vegetables per day, 120 g of fruit per day, and 100 g of fish per day. β coefficients for associations with late AMD were calculated by multivariate logistic regression, were multiplied by determinant values, and were summed to create a lifestyle risk score. Lifestyle risk scores were stratified into tertiles as an unfavorable, intermediate, or favorable lifestyle.

Statistical analysis

The population-attributable fraction (PAF) was calculated for each variant using the formula of Miettinen *et al.*[12]: PAF = Pc x ((OR−1)/OR), where OR is the odds ratio and Pc is the proportion of exposed patients among the patients. The pooled dataset formed the basis for all analysis. We calculated the discriminative accuracy between late AMD patients and control participants for our model of genetic factors using Saddle Point Signature software version 2.8.3 (Saddle Point Science, Ltd., Worcester Park, United Kingdom) in a batch multivariate regression analysis. Results were cross-validated by the leave-one-out principle. Prediction performance at each iteration was quantified by counting errors of persons assigned to the wrong category (control participants or patients). The dataset was fully balanced between control participants and patients; the regression equations corresponded to a pseudo-dataset, in which the outcome classes were equal in size, but the other statistical features were identical to the true dataset. Missing values were not set to 0, but rather imputed to the mean. Covariates were selected based on error expectation minimization.

Where appropriate, comparisons were made with Pearson chi-square test, Jonckheere-Terpstra test for ordered alternatives, or independent-sample *t* test. Interaction of genetic

and lifestyle risk was assessed by a univariate analysis of variance. Graphical outputs were constructed with GraphPad Prism 5 version 7.00 for Windows software (GraphPad Software, La Jolla, CA). Histograms and a receiver operator characteristic curve were constructed with SPSS Statistics for Windows version 25.0 (IBM Corp).

RESULTS

We identified a total of 17 174 individuals 45 years of age and older with data on genetics and AMD: 13 324 persons without AMD, 2073 persons with intermediate AMD, and 1777 persons with late AMD. Of the persons with late AMD, 309 demonstrated GA, and 1468 demonstrated CNV. Age ranged from 45 to 101 years old with a mean of 68.7 years (standard deviation [SD], 10.4), the proportion of women was 58.5%, the proportion of current smokers was 16.8% (n=2888), and the proportion of former smokers was 39.5% (n=6786). For risk calculations, we aimed to ensure a true phenotype of no AMD and therefore included only control participants 75 years of age or older (n=3167) in these analyses. The proportion of women in this subset (control participants 75 years of age or older and patients with intermediate or late AMD) was 61.3%, the proportion of current smokers was 9% (n=630), and the proportion of former smokers was 36.2% (n=2541).

Single Variants

First, we focused on frequency distributions of the 49 single risk variants in the 3 phenotype groups and ranked variants according to frequency differences between late and no AMD (Fig 1A). Single-nucleotide polymorphisms from the complement pathway and *ARMS2* showed the largest difference in frequency between patients and control participants (rs10922109, rs61818925 and rs570618 [*CFH*]; rs429608 [*C2*]; rs2230199 [*C3*]; and rs3750846 [*ARMS2*]). Among the first 10 variants, 5 variants showed a lower frequency among patients, corresponding to a protective effect on AMD. Next, we calculated the PAF for each single variant. The *ARMS2* variant rs3750846 was associated with a large PAF (0.3) for late AMD, whereas variants in *CFH* exhibited both the largest PAF (0.33 for rs570618) and the largest inverse PAF (-0.37 for rs10922109; Fig 1B). A similar pattern with smaller PAFs was observed for intermediate AMD. Only variant rs11080055 in *TMEM97/VTN* showed a higher PAF for intermediate (0.063) than for late (0.024) AMD. Only 4 late AMD patients (0.2% [4/1777]) did not carry any of the 5 major risk SNPs, compared with 33 control participants (1% [33/3167]).

Genetic Risk Score for Age-Related Macular Degeneration

We subsequently combined all genetic variants in a GRS and assessed its distribution. In the population-based cohort studies (*n*= 13 194), the score ranged from -3.50 to 4.63 (mean, 0.40; SD, 1.24) and showed a normal distribution (Fig 2A). With respect to the distribution

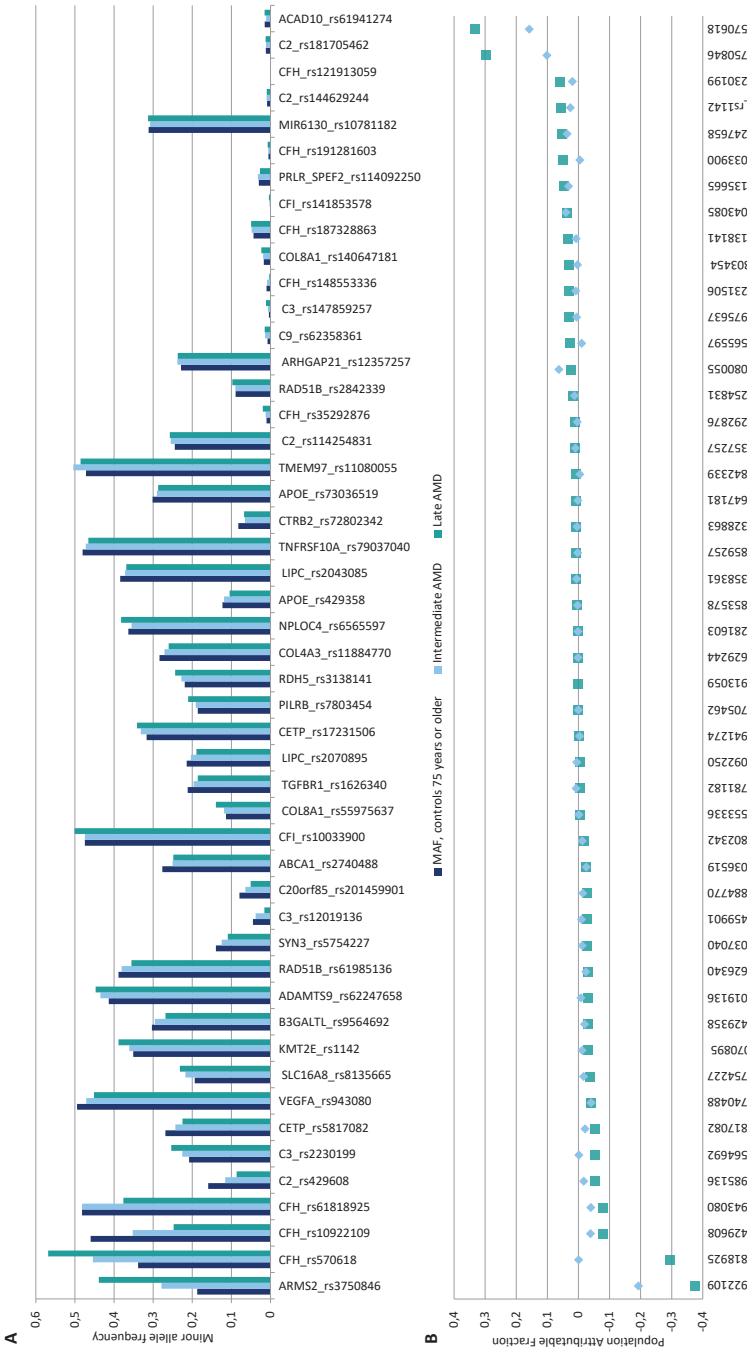

Figure 1A. Minor allele frequency of cases and controls for 49 AMD associated genetic variants. The variants are ranked according to the difference in allele frequencies between late AMD cases and controls, with the most discriminative variants on the left side of the graph. **1B** Population attributable fraction of 49 AMD-associated genetic variants for intermediate (light blue) and late (green) AMD. CFH_rs12913059 is not included for intermediate AMD since it was too rare to make useful calculations.

per phenotype, the GRS in control participants ranged from -3.03 to 3.94 (mean 0.26; SD, 1.16), that in intermediate AMD patients ranged from -3.11 to 4.71 (mean, 0.83; SD, 1.33), and in late AMD patients ranged from -3.00 to 6.23 (mean, 1.64; SD, 1.32; Fig 2B). Although the lowest GRS value was similar for all phenotypes, the entire distribution showed a significant increase with increasing AMD severity (P<0.0001, Jonckheere-Terpstra test for ordered alternatives). When stratifying late AMD into GA and CNV, slightly higher scores were noted for CNV (Fig 2C) that for GA ranged from -2.72 to 4.87 (mean, 1.46; SD, 1.41) and that for CNV ranged from -3.00 to 6.23 (mean, 1.67; SD, 1.30; P=0.01, independent-sample t test). We estimated the discriminative accuracy of a score based on the 49 AMD-associated genetic variants (Figs S3 and S4, available at www.aaojournal.org) for identification of late AMD; the area under the receiver operating characteristic curve was 0.838. We identified a minimal set of variants by using the leave-one-out principle and found an almost identical area under the receiver operating characteristic curve (0.837) when including 27 AMD-associated variants (score is available in the Supplemental Appendix, available at www.aaojournal.org).

Genetic Risk Scores per Pathway

Next, we constructed pathway-specific GRSs for the complement, lipids, ECM, *ARMS2* and 'other' pathways. The complement pathway score ranged from -3.15 to 3.64 in the population-based studies, and 55% of participants scored more than 0 for this pathway. The *ARMS2* score ranged from 0 to 2.15 because only 1 risk variant determines this score. The lipid pathway showed a GRS ranging from -1.44 to 0.49, and the ECM pathway showed a GRS ranging from -0.92 to 1.46, and 36% and 33%, respectively, showed a score of more than 0. The pathway 'other' ranged from -1.06 to 1.45, and 61% showed a positive score.

The distribution of all pathway GRSs in our total study population showed a positive shift with increasing AMD severity (P<0.0001, Jonckheere-Terpstra test for ordered alternatives; Table S1, available at www.aaojournal.org; Fig 5), but the complement and *ARMS2* GRS demonstrated the largest increase for late AMD, especially when combined (shift of mean GRS from 0.39 to 1.59).

Frequency of positive Genetic Risk Score

We studied the proportion of individuals with a positive (>0) GRS for each of the pathways, because this indicates more genetic risk than protection from that particular pathway. Positive GRSs for all pathways were most frequent in late AMD (Fig 6). Positive GRSs for the complement and 'other' pathways were most prevalent in all phenotypes. The largest increase per phenotype severity was found for the complement and *ARMS2* pathways; the proportion of persons with positive GRSs in the complement pathway rose

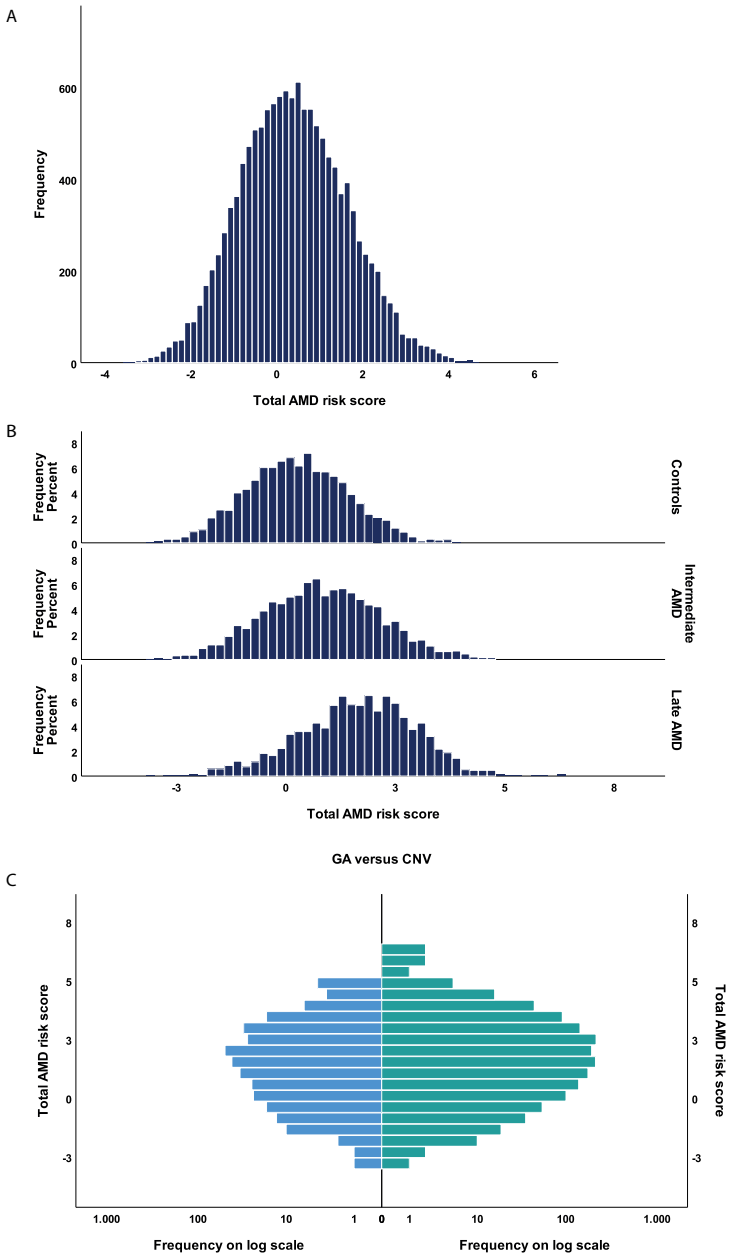

Figure 2. A. Bar graph showing the distribution of the total age-related macular degeneration (AMD) genetic risk score (GRS) in the European population. **B.** Bar graph showing distributions of the total AMD GRS: (top panel) control participants (age, ≥75 years), (middle panel) intermediate AMD, and (bottom panel) late AMD. **C.** Bar graphs showing the distributions of the total AMD GRS: (left panel; light blue) frequency of geographic atrophy (GA) for each total AMD GRS and (right panel; green) frequency of choroidal neovascularization (CNV) for each total AMD GRS, both on a log scale.

from 51% in control participants to 77% (a 26% increase) in late AMD patients, and that for the *ARMS2* pathway rose from 35% in control participants to 65% (30% increase) in late AMD patients ($P<0.0001$ for both, Pearson chi-square 2-sided test).

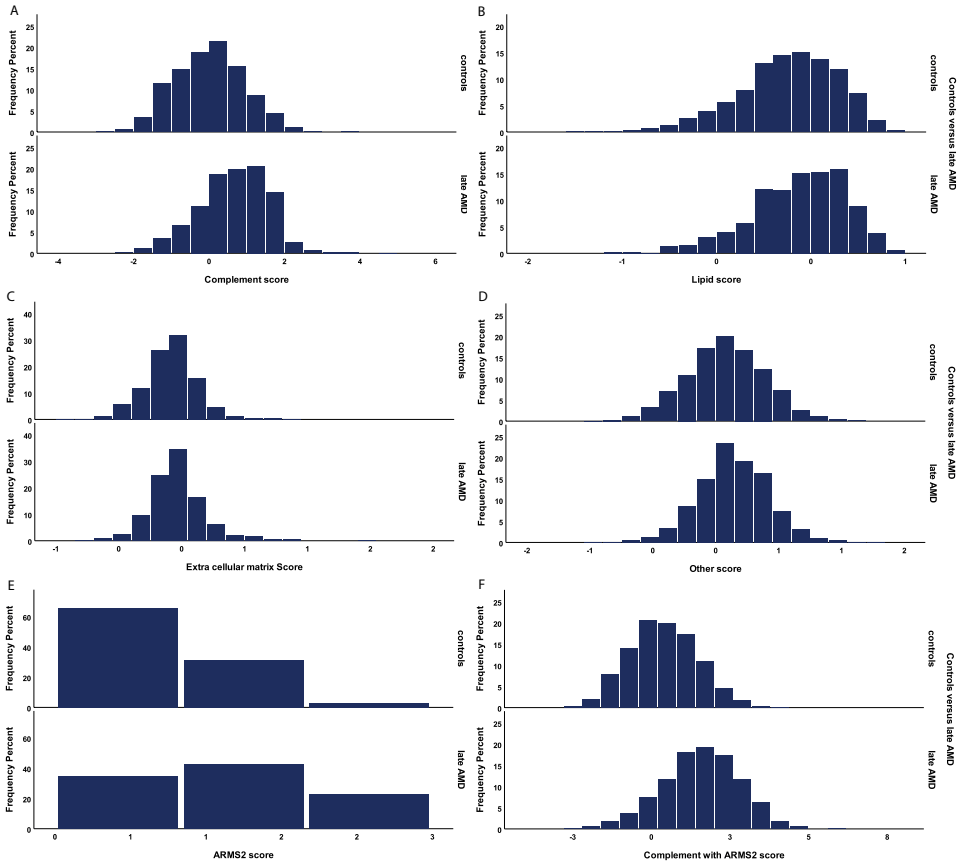

Figure 5. Bar graphs showing the distributions of the genetic risk scores for the complement (A), lipids (B), extracellular matrix (C), ARMS2 (D) and 'other' pathway (E) and the complement with ARMS2 combined pathways (F) in control participants and late age-related macular degeneration (AMD) patients.

Not one pathway GRS was more than 0 in all late AMD patients, but 90% showed a positive GRS for the combination of complement and *ARMS2* pathways. On closer inspection of the remaining 10% (n=152), these late AMD patients did carry risk alleles in these 2 pathways but showed a high frequency of protective variants that resulted in a GRS of less than 0 (Table S2, available at www.aaojournal.org). Subsequently, we examined the risk SNPs in greater detail by investigating the proportion of persons with

at least 1 risk allele per pathway (Fig S7, available at www.aaojournal.org). Ninety-nine percent of persons with late AMD showed a risk SNP in either the complement or 'other' pathway, but this was also the case for control participants. For the *ARMS2*, lipid, and ECM pathways, this was less frequent.

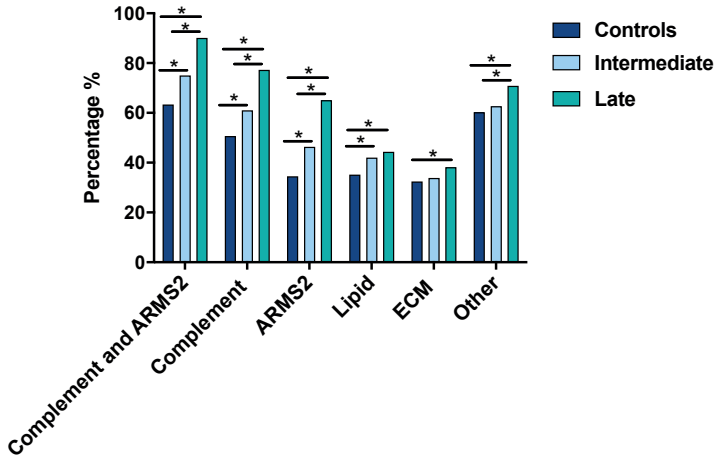

Figure 6. Bar graph showing the percentage of individuals with a positive genetic risk score for each of the pathways. Dark blue, control participants 75 years of age or older; light blue, intermediate age-related macular degeneration (AMD) patients; green, late AMD patients. The asterisk (*) indicated statistical differences in a Pearson chi-square test (2-sided) with P<0.0001; P<0.0028 with Bonferroni correction for multiple testing. ECM = extracellular matrix.

The next question we addressed for each pathway was this: Can late AMD develop without a risk variant in this pathway? For some pathways, this was rare: 0.7% (12/1777) of late AMD patients for the complement pathway and 1.5% (26/1777) of late AMD patients for the 'other' pathway. For the *ARMS2*, lipids, and ECM pathways, these fractions were higher (34.8%, 6.1%, and 19.6%, respectively). When combining the complement and *ARMS2* pathways, only 5 late AMD patients (0.3%) showed no risk allele in this pathway.

Next, we calculated the distribution of pathways with a GRS of more than 0 (Fig 8). Most participants showed 2 to 4 pathways with a GRS of more than 0 (85%). A small proportion (7%) of individuals showed a GRS in only 1 pathway of more than 0, and an even smaller proportion (1% [n=23]) of individuals showed a GRS of 0 or less for all pathways.

Combining Genetics with Lifestyle

Data on lifestyle factors were available for a subset of the study population (n=3525). In these participants, we investigated the AMD lifestyle factors of smoking and dietary

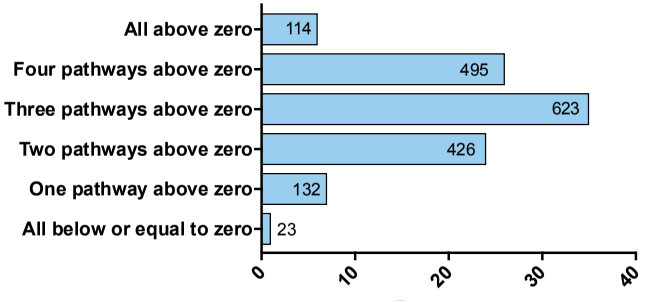

Figure 8. Bar graph showing the number of late age-related macular degeneration patients with positive pathway scores. Numbers inside the bars indicate the frequency

Subgroup	Cases/Controls	Odds Ratio	CI 95%	p-value
Low genetic risk				
Favorable lifestyle	27/292	1 reference		
Intermediate lifestyle	46/250	1.99	1.30-3.30	0.007
Unfavorable lifestyle	37/198	2.02	1.19-3.43	0.009
Intermediate genetic risk				
Favorable lifestyle	51/207	2.67	1.62-4.39	<0.0001
Intermediate lifestyle	84/167	5.44	3.39-8.73	<0.0001
Unfavorable lifestyle	95/170	6.04	3.79-9.65	<0.0001
High genetic risk				
Favorable lifestyle	124/90	14.90	9.23-24.05	<0.0001
Intermediate lifestyle	198/84	25.94	15.94-40.77	<0.0001
Unfavorable lifestyle	230/71	35.03	21.77-56.37	<0.0001

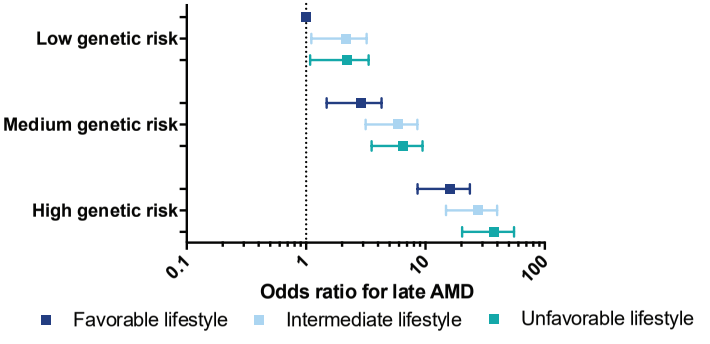

Figure 9. Table and graph showing the odds ratio of risk for late age-related macular degeneration (AMD) stratified by genetic risk score (GRS) and lifestyle risk. CI = Confidence interval.

intake of vegetables, fruit, and fish. Patients more often were current smokers (OR, 1.39) and consumed fewer vegetables (OR, 0.40), less fruit (OR, 0.35), and less fish (OR, 0.17; $P<0.0001$ for all; Table S3, available at www.aaojournal.org). We composed a lifestyle score based on these variables and stratified the score into tertiles: favorable, intermediate, and unfavorable lifestyle. For each GRS category (also tertiles), we observed that the more unfavorable the lifestyle, the higher the risk of late AMD. Lifestyle increased the risk 2 to 2.3 times depending on the genetic risk. In the highest genetic risk group, the OR increased from 14.9 to 35.0 in individuals with an unfavorable lifestyle (Fig 9).

DISCUSSION

This study provides a comprehensive interpretation of AMD genetic risk in the European population. The risk allele most discriminative between late AMD patients and control participants was located in *ARMS2*, closely followed by a risk-increasing and a protective allele in *CFH*. We observed a normal distribution of AMD-associated GRS, with variants increasing disease risk, but also a significant number offering protection against AMD. Patients with late AMD showed higher GRSs than control participants. Mathematically, we showed that the genetic contribution of the complement pathway and *ARMS2* to late AMD was at least 90%. However, most patients carried genetic risk in multiple pathways, signifying the complex cause of AMD. All persons benefitted from a healthy lifestyle, but those with a high GRS showed the strongest risk reduction. This highlights the possibilities to counteracting predicted disease outcomes with lifestyle choices.

Our results need to be seen in light of the strengths and limitations of this study. An important strength was the very large number of Europeans included in this study. From the E3 Consortium, we included 9 studies with genetic data, that is, population studies from The Netherlands, France, and Portugal, as well as case-control studies from The Netherlands and Germany. Data were harmonized and entered into a single database, which allowed us to perform in-depth analyses on combinations of phenotype, genotype, and lifestyle factors in the pooled dataset. Grouping genes into pathways and calculating pathway-specific genetic susceptibility enabled us to study molecular drivers and personalized risks. A limitation of our study was the incompleteness of data on several determinants in some studies. We focused on 49 genetic variants that were associated individually with AMD[5], of which only few were rare. Hence, we cannot elaborate on risks provided by most of the currently known rare variants. The studies providing the greater part of patients were case-control studies without follow-up data, and therefore we were restricted to cross-sectional analyses.

A positive GRS indicated more causative genetic risk than protection by genetic variants. Because this was present in 63% of the population (2546/4044), we conclude that genetic

susceptibility to AMD is highly prevalent. Among cases with late AMD, the proportion of a positive GRS rose to 89% (1581/1777). We investigated this in greater detail and found that the 5 major risk alleles were absent in only 66 persons (1%), indicating that 99% of the study population carried at least 1 major risk allele. By contrast, on average, 2.5 major risk alleles were present among late AMD patients and were absent in only 0.2% (4/1777). A set of 27 risk variants was enough to reach discriminative accuracy 0.84 for late AMD versus no AMD. Adding more variants did not improve this further, and the area under the receiver operating characteristic curve was in line with previous studies[13, 14]. It should be emphasized that such high discrimination based solely on genetic variants is exceptional for a complex disorder, although this is still challenging at mean GRS levels.

Considering individual pathways, 19 of 52 common AMD risk variants are in the complement pathway[5]. Previous studies have reported already that common variants in the complement pathway explain 57% of the heritable risk of AMD[15], and our study underscores the high attribution of this pathway to the overall GRS. Comparing the risk of the most important *CFH* SNP (rs570618 in high linkage disequilibrium (LD) 0.991 with rs1061170, Y402H) with an Asian population, we and others observed only a slightly higher OR of late AMD in Europeans (2.47 vs. 2.09)[16] but very different allele frequencies (minor allele frequency, 0.34 vs. 0.049) [17]. With respect to function, the complement pathway is part of the innate immune system, and numerous studies have shown that imbalance of this cascade at the protein level is important for AMD pathogenesis. Genetically, this system harbors strong causative as well as highly protective risk alleles (Fig 1), which mathematically can add up to a GRS of 0. Whether this also reflects a neutral risk at the tissue level is unclear, because persons with late AMD and a negative GRS for the complement pathway still carried risk-increasing alleles in this pathway. Nevertheless, the risk-reducing effect of these protective alleles are of high biological interest, and investigation into the functional consequences may provide leads for future therapy.

The rs3750846 (or its proxy, rs10490924, A69S) variant in the *ARMS2* locus carried the highest risk of late AMD and the second highest attribution to overall AMD occurrence in our study (Fig 1). In East Asia, this allele is twice as common (minor allele frequency, 0.40 in East Asians vs. 0.19 in Europeans), but the risk of late AMD for carriers seems comparable (OR, 2.94 in India vs. OR, 3.06 in Europe)[18, 19]. The function of *ARMS2* is the subject of ongoing research. Recently, Micklisch *et al*. showed *in vitro* that *ARMS2* functions as a surface complement regulator by binding to the cell membrane of apoptotic and necrotic cells and that it subsequently binds properdin and activates complement[11]. This provides evidence that *ARMS2* can be an initiator of complement. We considered 2 different scenarios for the pathway of *ARMS2*: a function in the complement pathway and an independent function. When regarded as a complement gene, the vast majority (90%) of late AMD patients showed an increased genetic risk in this pathway,

making complement the main driver of late AMD. As a stand-alone function, *ARMS2* also provided a significant contribution because it was present in two thirds of late AMD patients.

Variants in the lipid and ECM pathways showed smaller effects and attribution to overall late AMD. Variants in genes with other functions (the 'other' pathway) also showed smaller effects, but the 16 variants combined were rather frequent and predisposed considerably to late AMD.

We further investigated the impact of the most important lifestyle factors, smoking and diet, in relationship to genetic risk. As expected, persons with AMD showed a lower intake of vegetables, fish, and fruit and higher rates of smoking (Table S3)[20-26]. Together, a more unfavorable lifestyle almost doubled the risk of late AMD. This occurred in all genetic risk strata, but the OR increase was most prominent in those at high genetic risk. These findings confirm previous reports from the Rotterdam Study[27, 28] and Age-Related Eye Disease Study, which demonstrated interaction between single nutrients and *CFH* and *ARMS* and a protective role of diet in those with a high GRS[29]. The current study analyzed a more comprehensive set of risk variants and found that a healthy diet and not smoking also were beneficial in persons with low genetic risk. Oxidative stress is the most recognized molecular effect of smoking in the pathogenesis of AMD[30], and antioxidants are the most important contribution to a healthy diet. Oxidative stress with abundant reactive oxygen species, peroxidation of lipids, proteins, RNA, and DNA in the retina can lead to cytotoxic effects and inflammation, enhancing the development of AMD[31]. Unfortunately, a healthy diet consisting of sufficient fruits, vegetables, and fatty fish is consumed by only a minority of the elderly[28], and smoking is still twice as high among those with late AMD (Table S3). This asks for more rigorous measures for prevention, and training of doctors in behavioral change techniques may be part of this.

In conclusion, this large European consortium showed that genetic risk of AMD is highly prevalent in the population at large and that risk variants in the complement pathway are by far the lead drivers of late AMD. Nevertheless, late AMD is mostly a result of multiple genetic pathways and lifestyle choices. The frequency and risk estimates provided by this study can lay the foundation for future intervention studies that are tailored to pathways.

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5

LIPIDS AND AMD

5.1 Increased High Density Lipoprotein Levels Associated with Age-Related Macular Degeneration. Evidence from the EYE-RISK and E3 Consortia

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ABSTRACT

Purpose: Genetic and epidemiologic studies have shown that lipid genes and high density lipoproteins (HDLs) are implicated in age-related macular degeneration (AMD). We studied circulating lipid levels in relationship to AMD in a large European dataset.

Design: Pooled analysis of cross-sectional data.

Participants: Individuals (N=30 953) aged 50 years or older participating in the European Eye Epidemiology (E3) consortium and 1530 individuals from the Rotterdam Study with lipid subfraction data.

Methods: AMD features were graded per eye on fundus photographs using the Rotterdam classification. Routine blood lipid measurements, genetics, medication, and potential confounders were extracted from the E3 database. In a subgroup of the Rotterdam Study, lipid subfractions were identified by the Nightingale biomarker platform. Random-intercepts mixed-effects models incorporating confounders and study site as a random-effect were used to estimate associations.

Main Outcome Measures: AMD features and stage; lipid measurements.

Results: HDL was associated with an increased risk of AMD (odds ratio [OR], 1.21 per 1-mmol/l increase; 95% confidence interval [CI], 1.14-1.29), whereas triglycerides were associated with a decreased risk (OR, 0.94 per 1-mmol/l increase; 95%CI, 0.91-0.97). Both were associated with drusen size. Higher HDL raised the odds of larger drusen, whereas higher triglycerides decreases the odds. LDL cholesterol reached statistical significance only in the association with early AMD (P=0.045). Regarding lipid sub fractions, the concentration of extra-large HDL particles showed the most prominent association with AMD (OR, 1.24; 95%CI, 1.10-1.40). The cholesteryl ester transfer protein risk variant (rs17231506) for AMD was in line with increased HDL levels (P=7.7x10^{-7}), but lipase C risk variants (rs2043085, rs2070895) were associated in an opposite way (P=1.0x10^{-6} and P=1.6x10^{-4}).

Conclusions: Our study suggested that HDL cholesterol is associated with increased risk of AMD and that triglycerides are negatively associated. Both show the strongest association with early AMD and drusen. Extra-large HDL subfractions seem to be drivers in the relationship with AMD, and variants in lipid genes play a more ambiguous role in this association. Whether systemic lipids directly influence AMD or represent lipid metabolism in the retina remains to be answered.

INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of blindness in the developed world, with 10.4 million persons diagnosed worldwide in 2015[1]. It is a multifactorial disease affecting the elderly, which involves genetics and lifestyle factors. The diagnosis of AMD is based on imaging of the retina, with drusen as the hallmark of early disease. Chorioretinal neovascularization and atrophy of the retinal pigment epithelium (RPE) are indicative of late disease. The number of drusen and total drusen area are prominent predictors of progression of the early stages of AMD[2, 3].

Drusen are lipid-rich, protein-containing deposits that accumulate between the RPE and Bruch's membrane. The accumulation of drusen resembles the formation of atherosclerotic plaques[4] seen in cardiovascular disease, with a similar composition of proteins and protein complexes, such as apolipoprotein E, cholesterol esters, and complement proteins.[5, 6] The lipid load in drusen is as high as 40%[7] and is thought to be derived partly from the systemic circulation. This triggered many studies evaluating the relationship between serum or plasma lipids and AMD.[8-12] Some found associations with various serum or plasma lipid levels and drusen or AMD[11-18], but results mainly were weak and inconsistent. Because a biological explanation is lacking, the relationship remains unsettled yet intriguing.

Genetically, lipid metabolism also is involved in AMD. Genetic associations have been established for 4 genes encoding components of the high-density lipoprotein (HDL) metabolism: Adenosine triphosphate-binding cassette transporter A1 (*ABCA1)*, cholesteryl ester transfer protein (*CETP)*, apolipoprotein E (*APOE),* and lipase C, hepatic type (*LIPC)*[19-25]. ABCA1 encodes a cellular cholesterol efflux pump leading to formation of nascent HDL. Apolipoprotein E, encoded by the *APOE* gene, facilitates cholesterol uptake by HDL. CETP exchanges cholesteryl esters and triglycerides between HDL and other lipoproteins and thereby, influences HDL particle size.[26] Finally, hepatic lipase encoded by the *LIPC* gene hydrolyzes triglycerides and phospholipids in lipoproteins[27] and thereby, partly converts very low-density lipoproteins (VLDLs) and intermediate density lipoproteins to low density lipoproteins (LDL)[20] and plays a role in altering the HDL contents.

The European Eye Epidemiology (E3) consortium within the European EYE-RISK project enabled us to investigate the relationships between systemic lipids levels, lipid genes, and AMD using a very large data set. With nuclear magnetic resonance (NMR) spectroscopy, we studied these relationships in greater detail to investigate which particles drive potential associations.

METHODS

Study Population

Routine Blood Lipid Measurements Fourteen studies from France, Germany, Italy, The Netherlands, Norway, Portugal and the United Kingdom participating in the E3 consortium enrolled in the current study (Supplemental cohort descriptions, available at www.aaojournal.org). The E3 consists of European studies with epidemiologic data on common eye disorders; a detailed description on the studies included in the consortium has been published elsewhere.[28] All studies with gradable macular fundus photographs (*n*=30 953 participants) 50 years of age and older contributed their data to the EYE-RISK database version 4.0. Studies were population-based cohort studies except for Creteil and the European Genetic Database (EUGENDA), which are clinic-based studies. Routine blood lipid measurements and AMD outcomes of the same visit were used for this analysis; for TwinsUK, the closest visit to capturing of the retinal fundus photographs was used. All studies were performed in accordance with the Declaration of Helsinki for research involving human subjects and the good epidemiologic practice guideline. All participants gave fully informed consent and the study was approved by all local institutional review boards of each study site.

Detailed Lipid Analyses. The population-based Rotterdam Study (RS) I provided data on lipid subfractions that were determined at visit 4. Descriptive statistics of this cohort are shown in Table S1. (available at www.aaojournal.org).

Clinical Examination:

Age-related macular degeneration features were graded per eye on fundus photographs by experienced graders or clinicians; the most severe AMD grade classified the AMD status of the person. When needed, photographs were regraded by expert graders from Moorfields Eye Hospital and the RS to harmonize the outcome. Age-related macular degeneration status was determined for all included studies using the Rotterdam Classification as described previously.[29] In brief, grade 0 or 1 are considered no AMD; grades 2 and 3 with soft indistinct drusen, reticular drusen or distinct drusen with pigmentary changes are considered as early AMD, and grade 4 with geographic atrophy or choroidal neovascularization as late AMD. The area of the Early Treatment Diabetic Retinopathy Study grid covered by drusen was estimated in RS I visit 4 per grid circle, and was calculated using previously defined harmonization criteria[30]. Medication use and lifestyle factors including smoking habits were assessed by questionnaire; lipid measurements and other clinical determinants such as hypertension, body mass index (BMI), and diabetes mellitus were examined at each individual research center (Supplement cohort descriptions available at www.aaojournal.org). Fasting blood draws

were taken in all studies except for the EUGENDA study, Muenster aging and retina study (MARS), and the Tromsø Eye Study, which drew blood samples in a nonfasting scenario. Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were measured in plasma (Pathologies Oculaires Liées à l'Age Study [POLA], Prevalence of Age-Related Macular Degeneration in Italy (PAMDI study), Montrachet-3 city (3C) study, and Creteil) or in serum (remaining studies) using standard operating procedures. When LDL was not measured and triglycerides were less than 4.52mmol/l, a proxy was calculated using the Friedewald formula[31]: LDL cholesterol = Total cholesterol – HDL cholesterol – (total triglyceride/2.19); only positive values entered the analysis.

Nuclear Magnetic Resonance Metabolomics Analysis

Lipid subfractions were measured with the Nightingale's NMR-based biomarker platform in fasting ethylenediaminetetraacetic acid plasma samples (Nightingale Ltd., Helsinki, Finland). These measurements cover multiple metabolic pathways, including lipoprotein lipids and subclasses, fatty acids, amino acids, and glycolysis-related metabolites. The NMR-based metabolic profiling has been described previously in detail[32] and has been used in multiple large-scale epidemiologic and genetic studies[33-36].

Genetic Analyses

The Alienor-3 city (3C) study and Montrachet-3 city (3C) study participants were genotyped with the Illumina Human 610-Quad BeadChip (Illumina, Inc., San Diego, CA) and imputed with the 1000 Genomes Phase I integrated variant set (March2012) using Shapeit software version 2.r727 (https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html) for pre-phasing and Impute2 software version 2.3 (https://mathgen.stats.ox.ac.uk/impute/impute_v2.html) for imputation. The RS I, II and III participants were genotyped using the Illumina 550K, 550k due/610K Illumina arrays (Illumina, Inc., San Diego, CA). The genotypes were imputed with the 1000 Genomes (phase 1 version 3) reference panel using the Markov chain haplotyping/minimac software[37-39]. The EUGENDA participants were genotyped with a custom-designed Illumina HumanCoreExome array (Illumina, Inc., San Diego, CA) within the International AMD Genetics Consortium. Details regarding the design of this array, as well as annotation, imputation, and quality control of the genotypic data, have been described previously [19]. All cohorts applied similar quality control procedures to genotype data before analysis, and imputation quality was r^2>0.3.

A total AMD genetic risk score was calculated using 33 out of the 52 known AMD risk variants[19] available in the EYE-RISK database version 4.0 (Table S19, available at www.aaojournal.org). Genetic allele dosage was annotated as 0 for noncarriers, 1 for heterozygotes, and 2 for homozygotes. The genetic risk score was composed by calculating the sum of the βs of independent risk variants. The score was standardized

and added as a covariate in a linear regression analysis with AMD as the dependent variable. The linear regression was corrected for age, sex, lipid lowering drugs, and study site. The effect of individual lipid-related single nucleotide polymorphisms on each lipid level or lipid subfraction was assessed in a mixed-effects regression correcting for age, gender, lipid lowering drug use, plasma or serum, and fasting state and using study site as a random effects term. The *P* value threshold for these analyses was 0.05/60 = 0.00083 ($8.3x10^{-4}$) after Bonferroni correction.

Statistical Analysis

The outcome variable was presence of early or late AMD versus no AMD. Differences in baseline characteristics were evaluated with a Wald test using a logistic regression analysis, adjusting for age, gender, and study site. Analyses were conducted on complete data. Odds ratios (ORs) for the routine blood lipid measurements were calculated using random-intercepts mixed-effects logistic regression models, including study site as a random effect term to allow for variability between study sites. The study site-specific fixed-effects estimates were transformed to their marginal counterparts as described by Heagerty and Zeger[40].

Association of HDL cholesterol with AMD characteristics (presence of various drusen sizes, hyperpigmentation, or hypopigmentation) was calculated in a univariate logistic regression analysis for the worse eye, defined as the eye with the most severe lesions of each AMD characteristic, correcting for age, gender, lipid-lowering drugs use and study site. The linear regression for HDL cholesterol and drusen area was calculated in the RS I visit 4 only.

For the analysis on lipid subfractions, all subfractions were +1 log transformed and scaled to make comparable measurements. Association magnitudes were reported in units of standard deviation or OR change per 1-standard deviation increase in each metabolite, as previously suggested by others[34, 35]. To account for the correlation between lipid subfractions, the eigenvalues were calculated as proposed by Li and Ji[41] on the SNPSpD online interface[42]. Bonferroni correction was applied to correct for multiple testing using the eigenvalues to calculate the *P*-value threshold (*P*=0.001087). To test for differences between AMD stage and the mean of the lipid subfractions, a Welch test was performed on the total of all age categories. The Welch test was chosen because homogeneity of variance was violated among the AMD severity classes. The post hoc Games-Howell test was used to investigate differences between the mean of the no-AMD and late-AMD groups.

Mixed-effects logistic regression models were performed with R package lme4[43], and mixed-effects regression models with nmle[44] (R Core Team, Vienna, Austria); Welch-

tests and genetic risk scores were carried out with SPSS software for Windows version 24.0 (IBM Corp., Amonk, NY). Graphical outputs were constructed with GraphPad Prism for Windows version 7 (GraphPad Software, La Jolla, CA).

RESULTS

We identified a total of 4730 individuals with early AMD, 2441 with late AMD, and 23 782 nonaffected persons. The baseline characteristics of these participants are summarized in Table 2. Age-related macular degeneration patients and controls differed in age, gender, BMI, lipid-lowering drug use, and smoking, in accordance with the known AMD risk profile.

Table 3 shows the association between lipid levels and AMD adjusted for age, gender, lipid-lowering drug usage, BMI, smoking, plasma or serum, fasting state and study site in the E3 consortium. Analyses for late AMD were also corrected for diabetes. Total cholesterol was not associated with any of the AMD outcomes. Higher HDL cholesterol was associated with an increased risk of any AMD, and risk estimates were slightly higher for early AMD (OR, 1.34 per 1-mmol/l increase) than for late AMD (OR, 1.12 per 1-mmol/l increase), but had overlapping confidence intervals (CIs). Low-density lipoprotein cholesterol and triglycerides were associated inversely with early AMD (OR, 0.96 and 0.88 per 1-mmol/l increase, respectively) and any AMD (OR, 0.98 and 0.94 per 1-mmol/l increase, respectively), but effect sizes were smaller than for HDL cholesterol. Sensitivity analysis on fasting and nonfasting sampling methods and gender showed no interaction, or it showed interaction but with similar point estimates of the ORs, which made sampling effect or confounding unlikely. However, a sensitivity analysis on plasma or serum sampling methods did show a change in direction of effect of triglycerides measured in plasma, although the change was not statistically significant (Tables S4-S10, available at www.aaojournal.org). To investigate whether observed associations were the result of the preferential survival of elderly persons without cardiovascular disease, we repeated the analyses in various age strata (Tables S11-S13, available at www.aaojournal.org). Even in those aged 65 years of age or younger with AMD, HDL cholesterol was associated significantly with increased risk of AMD (OR, 1.19 per 1-mmol/l increase; P=0.02). Low-density lipoprotein cholesterol was associated inversely with AMD (OR, 0.93 per 1-mmol/l; P=0.02); associations with triglycerides became insignificant.

Table 2. Baseline Data and Results of Logistic Regression Analysis of the 14 European Studies.

		Controls (n=23 782)	Cases (n=7171)	*P* Value	Odds Ratio	95% Confidence Interval
AMD	Early		n=4730			
	Late		n= 2441			
Gender (% female)	Early		61.7 (n=2918)	**<0.0001**	1.21	1.13 - 1.29
	Late		59.4 (n=1449)	0.74	0.98	0.88 – 1.10
	Any	57.5 (n=13680)	60.9 (n=4367)	**<0.0001**	1.15	1.09 - 1.23
Age (yrs)	Early		72.7 (SD, 8.4)	**<0.0001**	1.06	1.06 – 1.06
	Late		76.9 (SD, 8.1)	**<0.0001**	1.12	1.11 – 1.13
	Any	68.1 (SD, 8.7)	74.1 (SD, 8.5)	**<0.0001**	1.08	1.07 - 1.08
BMI (kg/m²)	Early		26.6 (SD, 4.2)	**0.008**	0.99	0.98 – 1.00
	Late		26.4 (SD, 4.0)	**<0.0001**	1.03	1.02 – 1.05
	Any	27.0 (SD, 4.3)	26.5 (SD, 4.1)	0.543	1.00	0.99 – 1.05
Smoking (%) **Former** **Current**	Early		40.4 (n=1843) 8.7 (n=399)	0.77 0.14	1.01 1.10	0.94 – 1.09 0.97 – 1.24
Former **Current**	Late		44.8 (n=917) 12.3 (n=253)	**<0.0001** **<0.0001**	1.51 3.29	1.31 - 1.75 2.66 – 4.07
Former **Current**	Any	41.3 (n=9530) 12.8 (n=2947)	41.7 (n=2760) 9.9 (n=652)	**0.02** **<0.0001**	1.09 1.37	1.01 - 1.17 1.22 - 1.53
Hypertension (%)	Early		48.7 (n=2153)	0.30	1.04	0.97– 1.12
	Late		45.3 (n=977)	0.84	1.01	0.90 – 1.14
	Any	49.0 (n=11010)	47.6 (n=3130)	0.43	1.03	0.96 - 1.10
Diabetes (%)	Early		9.7 (n=435)	0.35	0.95	0.84 – 1.06
	Late		13.2 (n=284)	**0.002**	1.33	1.11 – 1.58
	Any	10.7 (n=2408)	10.8 (n=719)	0.70	1.02	0.92 - 1.13
Lipid-lowering drugs (%)	Early		24.7 (n=1084)	**0.006**	0.89	0.83 – 0.97
	Late		22.5 (n=459)	0.86	0.99	0.85 – 1.14
	Any	24.5 (n=5492)	24.0 (n=1543)	**0.004**	0.90	0.83 - 0.97

AMD = age-related macular degeneration; BMI= body mass index; SD = standard deviation.
Odds ratios are corrected for age, gender, and study site. Numbers in bold indicate statistically significant values.

Table 3. Mixed-Effects Logistic Regression Associations of Blood Lipids with Age-Related Macular Degeneration

Lipid		Controls	Cases	Odds Ratio for 1-mmol/l Increase	95% Confidence Interval	*P* Value
Total Cholesterol	Early AMD	5.60 (4.90-6.30) n=20 555	5.58 (4.80-6.30) n=3907	0.98	0.95-1.01	0.24
	Late AMD	5.60 (4.90-6.30) n=20 234	5.66 (4.90-6.50) n=1620	1.03	0.997-1.07	0.07
	Any AMD	5.60 (4.90-6.30) n=20 555	5.60 (4.80-6.34) n=5538	1.00	0.97-1.02	0.67
HDL cholesterol	Early AMD	1.40 (1.16-1.69) n=19 931	1.50 (1.23-1.80) n=3802	1.34	1.22-1.48	**6.48×10^{-10}**
	Late AMD	1.40 (1.16-1.69) n=19 662	1.47 (1.20-1.79) n=1626	1.12	1.002-1.24	0.044
	Any AMD	1.40 (1.16–1.69) n=19 931	1.50 (1.22-1.80) n=5439	1.21	1.14-1.29	**1.35×10^{-9}**
LDL cholesterol	Early AMD	3.49 (2.84-4.14) n=19 590	3.37 (2.71-4.02) n=3746	0.96	0.92-0.999	0.045
	Late AMD	3.49 (2.85-4.14) n=19 334	3.45 (2.79-4.14) n=1580	1.01	0.97-1.06	0.51
	Any AMD	3.49 (2.84-4.14) n=19 590	3.39 (2.74-4.07) n=5337	0.98	0.95-1.01	0.13
Triglycerides	Early AMD	1.34 (1.00-1.87) n=19 539	1.30 (0.97-1.80) n=3768	0.88	0.84-0.92	**2.44×10^{-7}**
	Late AMD	1.34 (1.00-1.87) n=19 474	1.43 (1.01-2.01) n=1601	1.01	0.97-1.06	0.57
	Any AMD	1.34 (1.00-1.87) n=19 539	1.32 (0.98-1.86) n=5374	0.94	0.91-0.97	2.35×10^{-5}

Data are median (tenty-fifth-seventy-fifth percentile). Odds ratio estimates and 95% confidence intervals of lipid on early, late or any AMD after adjusting for age, gender, lipid-lowering drug usage, body mass index, smoking, plasma or serum, fasting state and study site. Late AMD was also corrected for diabetes. P=0.0042 is Bonferroni statistically significant. Numbers in boldface indicate statistically significant values.
AMD = age-related macular degeneration; HDL = high-density lipoprotein; LDL = low-density lipoprotein.

Routine Blood Lipids Measurements and Early Age-Related Macular Degeneration Phenotype

Because the association between HDL cholesterol and AMD was most pronounced in those with early AMD, we performed more detailed analyses using the various early AMD features as outcomes. Effects of HDL cholesterol and triglycerides became larger with increasing drusen size (Fig 1). Likewise, higher HDL levels were associated with greater drusen area (β, 0.014; *P*=0.001). Higher triglyceride levels were associated with smaller drusen area. Correcting for smoking did not change these results (data not shown). Lipids were not statistically significantly associated with pigmentary changes, and total cholesterol and LDL cholesterol were not associated with any early AMD characteristic (Tables S14 and S15, available at www.aaojournal.org).

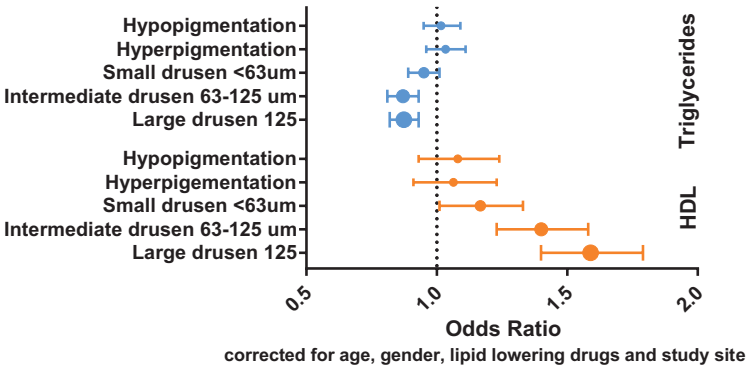

Figure 1. Graph showing the association of high-density lipoprotein (HDL) cholesterol and triglycerides with age-related macular degeneration characteristics.

Lipid Subfractions in the Rotterdam Study

To explore whether the association between HDL cholesterol, triglycerides, and AMD was driven by specific lipid subfractions, we examined lipid subfractions with NMR in RS I (Fig 2; Table S16, available at www.aaojournal.org). The concentration of extra-large HDL particles was associated most significantly with any AMD, particularly the subfractions of phospholipids and total lipids within extra-large HDL particles. These sub fractions are highly correlated (Pearson correlation, >0.97). Next, total cholesterol and free cholesterol in small VLDLs were associated significantly, as well as the ratio of apolipoprotein B-to-apolipoprotein A1, with a Pearson correlation ranging between 0.93 and 0.87. No other metabolites were associated significantly with AMD. Correcting for smoking did not

change these significant results (data not shown). The apolipoprotein B-to-apolipoprotein A1 ratio is a surrogate for the LDL-to-HDL ratio with a small ratio suggesting a high level of HDLs compared with LDLs.

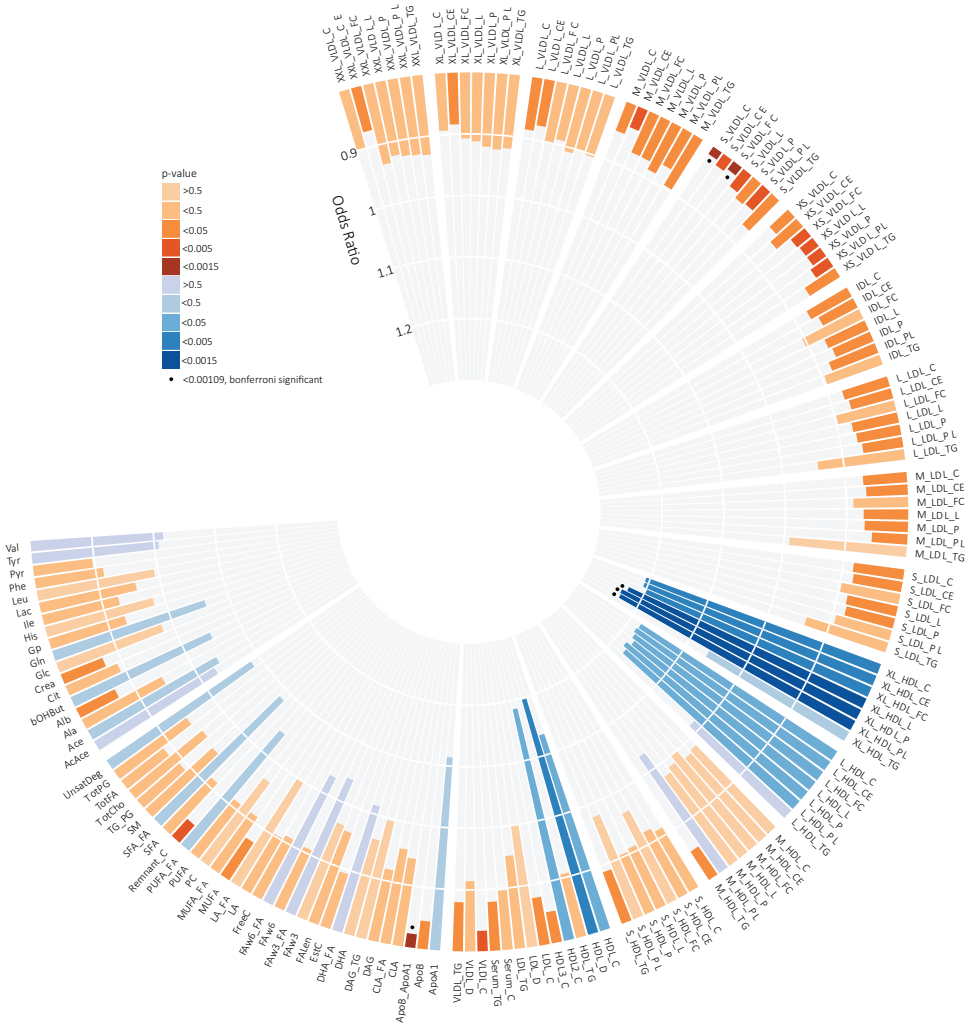

Figure 2. Graph showing the association of metabolic variables and age-related macular degeneration (AMD). Each bar represents the association with AMD, the size of the bar is the odds ratio, the color refers to effect direction and significance. Dots indicate Bonferroni statistically significant metabolic variables corrected for age, gender, and lipid-lowering drugs. Labels describe the properties measured in each lipid subfraction: C= total cholesterol; CE = cholesterol esters; FC= free cholesterol; L=total lipids; P=concentration of particles; PL= phospholipids; and TG=triglycerides. List of abbreviations available in the Supplementary materials, available online at www.aaojournal.org

These associations show a dose-dependent relationship with AMD stages from the Rotterdam Classification (Fig 3). To test if the mean of the lipid subfractions per AMD stage differed statistically, we performed a Welch test, which was significant for each of the 6 subfractions. The subfractions related to HDL also showed a statistically significant difference in the Games-Howell post hoc test comparing the mean of those with no AMD to those with late AMD (*P*=0.01 for the concentration of extra-large HDL, *P*=0.02 for phospholipids in extra-large HDL, *P*=0.01 for total lipids in extra-large HDL). The Games-Howell post-hoc test was not significant in the other 3 subfractions, likely because of the small group size and variance in late AMD. (Fig S4, available at www.aaojournal.org, shows dose dependency per age category).

We also performed the analyses stratified for lipid-lowering drug use, which was reported by fewer cases (17.0%) than controls (24.2%) in the RS (*P*=0.02). Significance was found only in those not taking lipid-lowering drugs, and point estimates were highly similar to the overall group (Tables S17 and S18, available at www.aaojournal.org).

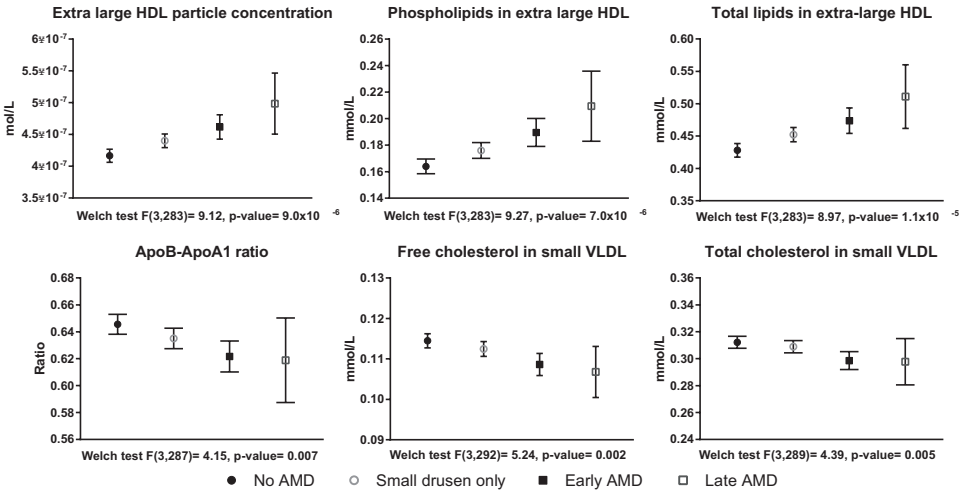

Figure 3. Box-and-whisker plots showing the stage-dependent relationship of the 6 associated lipid subfractions with age-related macular degeneration (AMD). Error bars indicate 95% confidence intervals of the mean. Apo= apolipoprotein; HDL= high-density lipoprotein; VLDL= very low-density lipoprotein.

Lipid Genes, Lipid Subfractions, and Age-Related Macular Degeneration

Because genetic variants are an important cause of AMD, we investigated the relationship among genes, lipids, and AMD. First, we investigated whether a genetic risk score with 33 single nucleotides polymorphisms covering all major AMD genes influenced lipid levels in the E3-consortium and found that with increasing genetic risk also came an increase of HDL cholesterol (*P*=0.03, Table S19 available at www.aaojournal.org). Subsequently, we focused on the individual AMD lipid genes. In E3, the *CETP* variant rs17231506 was associated positively with HDL cholesterol levels and associated negatively with LDL cholesterol, whereas both *LIPC* variants rs2043085 and rs2070895 were associated inversely with HDL cholesterol. In addition, the *APOE* variant rs429358 was associated with decreased levels of total cholesterol, triglycerides, and LDL cholesterol but with increased levels of HDL cholesterol. The *APOE* variant rs73036519 showed no significant effect on the routine lipid measurements or on the lipid subfractions. The *ABCA1* variant rs2740488 influenced only total cholesterol and HDL cholesterol (Table 20). When restricting the analysis to lipid subfractions in the RS (Table 21), we found similar results for the *CETP* variant and the *LIPC* variants with all extra-large HDL subfractions.

Table 20. Mixed-Effects Linear Regression Model Estimating the Effect of Single-Nucleotide Polymorphisms on Routine Lipid Measurements

Lipid	***CETP*** **rs17231506, Risk Allele T, Reference allele C**	***LIPC*** **rs2043085, Risk Allele C, Reference Allele T**	***LIPC*** **rs2070895, Risk Allele G, Reference Allele A**	***APOE*** **rs429358, Risk Allele T, Reference Allele C**	***APOE*** **rs73036519, Risk Allele G, Reference Allele C**	***ABCA1*** **rs2740488, Risk Allele A, Reference Allele C**
Total cholesterol	0.03 (*P*=0.07)	-0.019 (*P*=0.16)	-0.04 (*P*=0.008)	**-0.18 (*P*< 0.0001)**	0.01 (*P*=0.55)	**0.06 (*P*=0.0002)**
HDL cholesterol	**0.08 (*P*< 0.0001)**	**-0.04 (*P*< 0.0001)**	**-0.05 (*P*< 0.0001)**	**0.03 (*P*< 0.0001)**	-0.007 (*P*=0.24)	**0.03 (*P*=0.0001)**
LDL cholesterol	**-0.05 (*P*=0.0001)**	0.02 (*P*=0.09)	0.004 (*P*=0.77)	**-0.19 (*P*< 0.0001)**	0.01 (*P*=0.67)	0.04 (*P*=0.01)
Triglycerides	-0.025 (*P*=0.04)	0.001 (*P*=0.93)	-0.006 (*P*=0.66)	**-0.06 (*P*=0.0004)**	0.03 (*P*=0.03)	0.004 (*P*=0.78)

HDL= high-density lipoprotein; LDL = low density lipoprotein.

βs are corrected for age, gender, lipid-lowering drugs, plasma or serum, fasting state, and study site. βs indicate the effect of the risk allele versus the reference allele. Bonferroni: 0.05/60 = 0.00083 ($8.3x10^{-4}$). Numbers in boldface indicate statistically significant values.

Table 21. Linear Regression Model Estimating the Effect of Single-Nucleotide Polymorphisms on Lipid Subfraction.

Lipid	*CETP* rs17231506, Risk Allele T, Reference Allele C	*LIPC* rs2043085, Risk allele C, Reference Allele T	*LIPC* rs2070895, Risk Allele G, Reference Allele A	*APOE* rs429358, Risk Allele T, Reference Allele C	*APOE* rs73036519, Risk Allele G, Reference Allele C	*ABCA1* rs2740488, Risk Allele A, Reference Allele C
Percentage extra-large HDL	**0.15 (*P*=7.68x10^{-7})**	**-0.14 (*P*=1.00x10^{-6})**	**-0.13 (*P*= 1.55x10^{-4})**	-0.07 (*P*= 0.112)	0.04 (*P*=0.167)	0.05 (*P*=0.164)
Phospholipids in extra-large HDL	**0.15 (*P*=3.51x19^{-7})**	**-0.13 (*P*=4.0x10^{-6})**	**-0.13 (*P*= 1.04x10^{-4})**	-0.03 (*P*= 0.479)	0.04 (*P*=0.233)	0.03 (*P*=0.349)
Total cholesterol in small VLDL	-0.09 (*P*=0.003)	-0.09 (*P*=0.003)	-0.08 (*P*=0.02)	-0.05 (*P*=0.294)	-0.02 (*P*=0.631)	0.08 (*P*=0.027)
Ratio ApoB-to-ApoA1	-0.10 (*P*=0.002)	0.004 (*P*=0.897)	-0.003 (*P*=0.936)	-0.09 (*P*=0.037)	-0.04 (*P*=0.250)	0.02 (*P*=0.521)
Total lipids in extra-large HDL	**0.15 (*P*=4.06x10^{-7})**	**-0.14 (*P*=8.59x10^{-7})**	**-0.12 (*P*=3.13x10^{-4})**	-0.08 (*P*=0.056)	0.04 (*P*=0.213)	0.05 (*P*=0.138)
Free cholesterol in small VLDL	-0.08 (*P*=0.01)	-0.08 (*P*=0.005)	-0.09 (*P*=0.009)	0.01 (*P*=0.784)	-0.03 (*P*=0.434)	0.07 (*P*=0.058)

Apo= apolipoprotein; HDL= high-density lipoprotein; LDL= low-density lipoprotein; VLDL=very low-density lipoprotein. βs are corrected for age, gender, and lipid-lowering drug use; subfractions are log+1 and standardized. βs indicate the effect of the risk allele versus the reference allele. Numbers in boldface indicate statistically significant values.

DISCUSSION

Based on pooled data of 30 953 participants from Western Europe, we have showed that high circulating HDL cholesterol levels and low triglyceride levels are associated significantly with AMD. The magnitude of the effect was higher for early than for late AMD, and associations were related to drusen size and area. By focusing on lipid subfractions, we revealed that extra-large HDL particles, small VLDL particles, and the apolipoprotein B-to-apolipoprotein A1 ratio, a surrogate for the LDL-to-HDL ratio, were dose-dependent drivers of this association. Age-related macular degeneration risk variants in lipid genes did not provide a clear explanation; in particular, the variants in *LIPC* that increase the risk of AMD decreased HDL cholesterol in the systemic circulation.

Our results should be interpreted in light of the strengths and limitations of the study. The combined efforts of 2 European consortia enabled us to create a very large database providing the statistical power to resolve conflicting findings from previous studies. The detailed NMR lipid analysis in a subset created the opportunity to find the metabolic profile behind the lipid associations. A weakness of the consortia was the use of different

protocols for blood sampling, definition of confounders, and AMD phenotyping. We addressed this issue by performing a stratified analysis on sampling methods and found that only the associations for triglycerides changed direction of effect for plasma, albeit nonsignificantly. We harmonized all confounders as well as the criteria for early and late AMD, and corrected for study site in the mixed-effect models.

Many previous studies did not find a statistically significant association between lipids and AMD, but studies with the larger sample sizes often found a positive association with HDL cholesterol and an inverse association with triglycerides[13]. The current pooled study showed that the levels of these lipids were within physiologic range in both cases and controls and that absolute differences were small in millimoles per liter. However, our data suggest that an increase of HDL from the twenty-fifth percentile to the seventy-fifth percentile coincides with an AMD risk increase of approximately 20%. Selective survival does not seem to explain our findings because the association was present already in the youngest age group (≤ 65 years). The exact clinical interpretation remains to be defined. Nevertheless, the findings contribute to the understanding of AMD pathogenesis.

Animal research has provided some key insights in retinal lipid metabolism. Studies in rodents show that most lipids in the retina are synthesized locally and up to a quarter are derived from the systemic circulation[45] Another study in mice shows that a high-fat diet increases cholesterol in the retina but not as much as in the circulation. These results suggest that transport from the systemic circulation to the retina does take place, albeit modestly. Although LDLs deliver cholesterol most efficiently from the systemic circulation to the retina, HDL cholesterol, with apolipoprotein A-I as its major lipid component[46], does this as well via scavenger receptors[26, 47, 48]. The RPE processes the internalized lipids and subsequently secretes them again on the apical side via ABCA1 transporters into the interphotoreceptor matrix. Thereafter, lecithin-cholesterol acyltransferase, located at the surface of nascent HDL[49], converts free cholesterol into esterified cholesterol,[50] which is present in nascent HDL. In this way, lecithin-cholesterol acyltransferase transforms nascent HDL into larger, mature HDL, while LIPC hydrolyzes phospholipids in the HDL lipoprotein[23, 51]. As suggested by Tserentsoodol *et al.*[26], because of the absence of LDL in the retina, it is possible that CETP has a role in transferring esterified cholesterol between lipoproteins or photoreceptor membranes. In the interphotoreceptor matrix, HDL functions as a transport vehicle between the RPE and the photoreceptors supporting the high synthesis and degradation of the lipid-rich photoreceptor discs[26]. The RPE maintains the lipid balance by transporting lipoproteins back to Bruchs membrane[52]. The lipid contents of these lipoproteins resemble those of LDL lipoproteins rather than of HDL, because it has a high abundance of esterified cholesterol and both apolipoprotein A and B[53]. It has been proposed that this large amount of esterified cholesterol acts as a barrier for lipid transport through an aging retina, thereby facilitating the formation of

deposits[54]. Another mechanism proposed to form deposits is through the impairment of ABCA1 transporter of macrophages, which impairs the efflux of free cholesterol out of the macrophage. This results in senescent macrophages with high levels of cholesterol in the retina of mice[55].

Interestingly, lipoproteins seem to be related closely to the complement system, the major pathway in AMD pathogenesis. Proteomic studies have shown that HDL lipoproteins can contain essential complement components, such as C1, C2, C3, C5 and factor B[56-58]. One study showed that complement factor H (CFH) and lipoproteins have competitive binding in the sub-RPE extracellular matrix, and when CFH is low, lipoproteins can accumulate under the RPE[59]. By contrast, HDL also can carry complement regulators such as FH1, CFHR4 and CFHR5[60, 61]. Apolipoprotein A-I attached to HDL can bind clusterin, a complement lysis inhibitor that stops the complement cascade just before the C5b-9 complex is inserted into the target[62]. These findings suggest that HDL is involved in proinflammatory as well as complement-inhibitory tasks. Higher HDL levels may cause imbalance of the physiologic homeostasis[8, 63]. Taken together, this plethora of biological leads supports the contention that HDL may play a role in the initiation of AMD. More comprehensive research into lipid metabolism in the retina is warranted.

In our study, we found elevated levels of HDL cholesterol in the circulation and decreased levels of triglycerides in persons with AMD. In more detailed analysis, we observed a higher concentration of extra-large HDL particles with higher total lipid and phospholipid contents, which are under genetic control of *CETP* and *LIPC*. The high phospholipid content of extra-large HDL very likely is related to the larger particle size, because phospholipids comprise the outer shell of the lipoprotein. *CETP* may exert its effect on AMD partly through systemic HDL, in line with previous Mendelian randomization studies[24, 66]. The opposing effects that we found for *LIPC* are less easily explained, but have been observed by others[23, 24]. This finding suggests that systemic HDL may be a biomarker rather than directly causally related to AMD. In a larger study, Kettunen *et al.*[33] found more genetic effects on lipid subfractions; variants in *CETP* and *APOE* also had a decreasing effect on the small VLDL subfractions, whereas a variant in *ABCA1* increased extra-large HDL. Our smaller sample size hampered the replication of these findings.

Where do these lipid associations fit in the chronology of AMD development? The more pronounced risk for early AMD and increasing ORs of HDL cholesterol for the larger size drusen suggest that lipids play an important role at the early phase of disease. Hypothetically, intervention at this phase would be most promising in preventing blindness. We did not find statistical significance for any lipid subfraction in only those using lipid-lowering drugs, possibly because there is no effect, but probably due to the lower power in this subgroup. Evidence from other studies indicates that statins increase

HDL levels slightly[67] but reduce extra-large HDL[68] and that HDL protein composition may change as well[69]. Most epidemiologic studies do not find any effect of lipid lowering drugs on AMD[8, 70-72]; however, one study observed a slower progression of AMD in persons with a certain *CFH* risk variant[73]. Large randomized controlled trials with long term follow-up are needed to clarify the relation between lipid-lowering drugs and AMD.

In conclusion, this study showed that HDL cholesterol and triglycerides levels are particularly associated with early AMD, mostly through the association with drusen. Extra-large HDL subfractions seem to be drivers of this association. Whether systemic lipids directly influence lipid metabolism in the retina or whether these lipids mirror pathologic features in the retina is a question that remains to be answered.

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5

LIPIDS AND AMD

5.2 Mediterranean Diet and Incidence of Advanced Age-Related Macular Degeneration: The EYE-RISK Consortium

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ABSTRACT

Purpose: To investigate associations of adherence to the Mediterranean diet (MeDi) with incidence of advanced age-related macular degeneration (AMD; the symptomatic form of AMD) in 2 European population-based prospective cohorts.

Design Prospective cohort study of the Rotterdam study I (RS-I) and the Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires (Alienor) Study populations.

Participants: Four thousand four hundred forty-six participants 55 years of age or older from the RS-I (The Netherlands) and 550 French adults 73 years of age or older from the Alienor Study with complete ophthalmologic and dietary data were included in the present study.

Methods: Examinations were performed approximately every 5 years over a 21-year period (1990-2011) in RS-I and every 2 years over a 4-year period (2006-2012) in the Alienor Study. Adherence to the MeDi was evaluated using a 9-component score based on intake of vegetables, fruits, legumes, cereals, fish, meat, dairy products, alcohol and the monounsaturated-to-saturated fatty acids ratio. Associations of incidence of AMD with MeDi were estimated using multivariate Cox proportional hazard models.

Main Outcomes Measures: Incidence of advanced AMD based on retinal fundus photographs.

Results: Among the 4996 included participants, 155 demonstrated advanced incident AMD (117 from the RS-I and 38 from the Alienor Study). The mean follow-up time was 9.9 years (range, 0.6-21.7 years) in the RS-I and 4.1 years (range, 2.5-5.0 years) in the Alienor Study. Pooling data for both the RS-I and Alienor Study, participants with a high (range, 6-9) MeDi score showed a significantly reduced risk for incident advanced AMD compared with participants with a low (range, 0-3) MeDi score in the fully adjusted Cox model (hazard ratio, 0.59; 95% confidence interval, 0.37-0.95; P=0.04 for trend).

Conclusions: Pooling data from the RS-I and Alienor Study, higher adherence to the MeDi was associated with a 41% reduced risk of incident advanced AMD. These findings support the role of a diet rich in healthful nutrient-rich foods such as fruits, vegetables, legumes and fish in the prevention of AMD.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in industrialized countries[1]. This degenerative disease affects the central part of the retina, which is crucial for daily living tasks such as reading, driving, and recognition of faces. Worldwide, 196 million people will be affected by AMD in 2020, increasing to 288 million in 2040[2]. Advanced forms of the disease (neovascular or atrophic AMD) associated with a deep visual impairment generally are preceded by asymptomatic early stages. Although no treatment currently is available for atrophic AMD, effective treatments are available for the neovascular form[3, 4]. These treatments also incur major costs to society, with an estimated $2.3 billion of Medicare claims in 2013[5]. The risk of AMD developing is determined jointly by age, individual genetic background, and lifestyle[1, 6]. Prevention strategies based on the modifiable risk factors of AMD may help to decrease the major medical and social burden associated with AMD.

Epidemiological studies have observed a reduced risk of AMD associated with high consumption of antioxidants (lutein and zeaxanthin[7-12], fruits and vegetables rich in these nutrients) and omega 3 polyunsaturated fatty acids,[8,9, 13-15] provided by fish and nuts[13, 14, 16, 17]. However, a single nutrient or food approach cannot capture the synergistic effects of food and nutrients consumed in combination in the diet. The Mediterranean diet (MeDi) is characterized by high consumption of plant foods and fish, low consumption of meat and dairy products, olive oil as the primary fat source, and a moderate consumption of wine[18]. Adherence to the MeDi has been linked to lower rates of mortality,[19] chronic diseases, stroke,[20] cognitive decline,[21] and recently diabetic retinopathy.[22] Regarding AMD, very few studies are available to date.[23-27] In 3 population-based studies, the MeDi was associated with a lower prevalence of early AMD,[23] neovascular AMD,[25] and any AMD[26, 27], although dietary modifications resulting from AMD cannot be excluded in these cross-sectional studies. In a post hoc analysis of a randomized clinical trial, the MeDi was associated with a lower incidence of advanced AMD,[24] but the selected nature of the sample limits its generalizability. We therefore investigated the associations between MeDi and incidence of advanced AMD in a large sample from 2 population-based prospective studies.

METHODS

Study Population

The EYE-RISK project (http://www.eyerisk.eu/) aims to identify risk factors, molecular mechanisms, and therapeutic approaches for AMD. It uses epidemiologic data describing clinical phenotype, molecular genetics, lifestyle, nutrition and in-depth retinal imaging derived from existing European epidemiologic cohorts to provide major insights

needed for prevention and therapy of AMD. Within the EYE-RISK consortium, a unique harmonized database of individual data from 16 European epidemiologic studies was constructed[28]. Two prospective studies with appropriate data for the present analyses were available: the Rotterdam Study I (RS-I)[29] and the Antioxydants, Lipides Essentiels, Nutrition et maladies Oculaires (Alienor) Study.[30] For both studies, all participants provided written informed consent in accordance with the Declaration of Helsinki to participate in the study.

The Rotterdam Study I

At baseline, 7983 eligible persons 55 years of age or older were interviewed and examined. Ophthalmologic examinations were carried out and fundus photographs were obtained at each round starting from 1990 through 1993. Follow-up rounds were completed from 1993 through 1995, from 1997 through 1999, from 2002 through 2004, and from 2009 through 2011. After pharmacologic mydriasis, 35˚ stereoscopic color fundus photographs of the macula (Topcon TRV-50VT; Topcon Optical Co., Tokyo, Japan) were obtained in each of the first 3 visits, and 35˚ digital images (Topcon TRC 50EX) were obtained for the fourth and fifth visits[31].

The Rotterdam Study was approved by the Medical Ethics Committee of the Erasmus Medical Center (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO; license number, 1071272-159521-PG). The Rotterdam Study has been entered into the Netherlands National Trial Register (www.trialregister.nl) and into the World Health Organization International Clinical Trials Registry Platform (www.who.int/ictrp/network/primary/en/) under shared catalogue number NTR6831.

The Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires Study

At baseline (2006-2008), 963 participants 73 years of age or older were interviewed and underwent an ophthalmological examination[30]. Of these, 624 and 614 were re-examined at the second (2009-2010) and third (2011-2012) visits, respectively. The study was approved by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III) in May 2006 (http://www.alienor-study.com/langue-english-1.html).

The eye examinations took place in the Department of Ophthalmology of the University Hospital of Bordeaux. Two 45˚ nonmydriatic color retinal photographs were obtained using a high-resolution digital nonmydriatic retinograph (Topcon TRC-NW6S)[30]. At the third visit (2011-2012), for participants who were not able to come to the hospital, the eye examination took place at home and 40˚ retinal photographs were obtained using a digital nonmydriatic portable retinograph (Smartscope, Optomed, Oulu, Finland).

Age-Related Macular Degeneration Classification

Retinal photographs of both eyes were graded by trained graders of each study and were interpreted according to a modification of the Wisconsin Age-Related Maculopathy Grading System[32] for RS-I and according to the International Classification[33] for the Alienor Study. All advanced AMD cases were adjudicated and confirmed by retina specialists of the corresponding study. Phenotype harmonization was performed within the EYE RISK Consortium[28].

Incidence

At each visit, each participant was classified according to the worst eye into one of the following exclusive groups: no AMD, early AMD, or advanced AMD. Advanced AMD was defined by the presence of neovascular or atrophic AMD. Neovascular AMD included serous or hemorrhagic detachment of the retinal pigment epithelium or sensory retina, subretinal, or sub-retinal pigment epithelium hemorrhages, and fibrous scar tissue. Geographic atrophy was defined as a discrete area of retinal depigmentation 175µm in diameter or larger characterized by a sharp border and the presence of visible choroidal vessels. Early AMD (in the absence of advanced AMD) was defined by the presence of (1) soft indistinct (≥125µm, decreasing density from the center outward and fuzzy edges) or reticular drusen only or soft distinct drusen (≥63µm, with uniform density and sharp edges) and pigmentary abnormalities, or by (2) soft indistinct large drusen (≥125µm, decreasing density from the center outward and fuzzy edges) or reticular drusen and pigmentary abnormalities (corresponding to grades 2 and 3 of the Rotterdam Classification). No AMD was defined by the absence of early AMD and advanced AMD.

Incidence of advanced AMD was defined as the participant progressing from no or early AMD at baseline to advanced AMD (either neovascular or atrophic AMD) at any point during the study period. The date of occurrence of advanced AMD was calculated as the midpoint of the interval between the last visit without advanced AMD and the first visit with advanced AMD. Follow-up ended at the date of occurrence of advanced AMD, or the date of the last gradable photograph. Participants with advanced AMD or no gradable eyes at baseline were excluded from the analysis.

For the purpose of AMD subtype analysis, neovascular AMD comprised all participants demonstrating some neovascular lesions, with or without coexisting atrophy. Atrophic AMD was defined as pure geographic atrophy (in the absence of neovascular AMD).

Dietary Assessment

In RS-I, participants completed a checklist at home and underwent a face-to-face interview conducted by a trained dietician at the research center using a 170-item

validated semiquantitative food frequency questionnaire (FFQ)[34]. The food items were converted into quantities consumed daily (grams per day). By using the computerized Dutch Food Composition Table, these dietary data were converted to total energy intake (TEI; in kilocalories per day) and nutrient intakes (in grams per day)[34].

In the Alienor Study, participants were visited at home by a specifically trained dietician who administered a 40-item validated FFQ and a 24-hour dietary recall[35, 36]. The food items were converted into number of servings per day. The 24-hour recall was used to estimate nutrient intake (in grams per day) and TEI (in kilocalories per day) and to compute the monounsaturated fatty acids (MUFAs)-to-saturated fatty acids (SFAs) ratio.

Adherence to the MeDi was assessed using the MeDi score developed by Trichopoulou *et al*.[37]. This score included 9 components; vegetables, fruits, legumes, cereals, fish, meat, dairy products, alcohol and the MUFAs-to-SFAs ratio. The MeDi score was applied to both studies. The daily intake of each food or beverage group was calculated as quantity in grams per day in the RS-I and as the number of servings per day in the Alienor Study. Participants with unreliable TEI were excluded (valid TEI range: women, 600–3200 kcal; men, 600–4200 kcal). For each component hypothesized to benefit health (vegetables, fruits, legumes, cereals, fish, and MUFAs-to-SFAs ratio), 1 point was given if intake was higher than the gender-specific median values and 0 otherwise. For components presumed to be detrimental to health (meat and dairy products), 1 point was given if intake was less than the gender-specific median values and 0 otherwise. For alcohol, 1 point was given for moderate consumption and 0 otherwise (moderate consumption: women, 1-10 g/day; men, 5-15 g/day). Gender-specific medians were calculated separately for each study. The total MeDi score was computed by adding the scores (0 or 1 point) for each component per participant. Scores ranged from 0 (nonadherence) to 9 (perfect adherence). Participants were classified according to 3 categories of the MeDi score: low (0–3), medium (4–5), or high (6–9).

Covariates

Age (years), gender, education (primary, secondary, higher), smoking (never smoker, smoker <20 pack-years, smoker ≥20 pack-years; pack-year = packs [20 cigarettes] smoked per day for number of years of smoking), and multivitamin and mineral supplement use (yes or no) were measured using self-reported questionnaires at baseline[30, 38] for each study. Vascular risk factors included body mass index (weight in kilograms per height [in square meters]), diabetes (treated or self-reported), hypertension (blood diastolic blood pressure ≥90mmHg or systolic blood pressure ≥140 mmHg, treated, or self-reported), and hypercholesterolemia (treated or self-reported). Complement Factor H (*CFH*) Y402H (rs1061170) and Age-Related Maculopathy Susceptibly 2 (*ARMS2*) A69S (rs10490924), the 2 main AMD-related single nucleotide polymorphisms, were assessed in each study[39, 40].

Statistical Analysis

Participants excluded from analyses were compared with those included using a logistic regression model adjusted for age and gender for each characteristic separately. The same method was used to compare characteristics of participants included between the 2 cohorts.

The associations of MeDi score with incidence of advanced AMD were analyzed using Cox proportional hazards models with delayed entry and age as a time scale, which allows for a better adjustment for age than the classical Cox models based on time from entry in the study[41]. Model I was unadjusted and model 2 was adjusted for gender, AMD grade at baseline (no or early AMD), TEI (continuous), education, body mass index, smoking, multivitamin or mineral supplement use, diabetes, and hypercholesterolemia.

Variables retained in model 2 were factors associated incidence of AMD, with MeDi score, or both after adjustment for age and gender ($P<0.10$). For the pooled analysis, including data from both studies, models were adjusted further for the study (fixed study effect).

Low MeDi score was designated as the reference group. The *P* value for trend was calculated by using the median value of the MeDi score for each category. In all Cox models, the proportional hazard assumptions were tested.

Participants from the RS-I and Alienor Study were different regarding some characteristics. To estimate the potential effect of these differences, interactions between study and each covariate were assessed and none were significant. Thus, because the proportional hazard assumptions were satisfied and there were no interactions, to account for differences between the 2 studies, all models combining both studies were adjusted for a fixed effect.

Secondary Analyses

We also assessed whether associations of MeDi score with incident advanced AMD may be the result of individual dietary components by examining associations between the individual components of the MeDi score and advanced AMD. Each component was introduced independently into model 2. In secondary analyses, *CFH* Y402H and *ARMS2* A69S polymorphisms were added to model 2. Interactions between *CFH* Y402H and *ARMS2* A69S polymorphisms and MeDi score also were analyzed. Interaction terms for the number of risk alleles and MeDi score were assessed separately for each genetic variant using model 2. All *P* values representing a 2-tailed test of significance with α of 0.05 and SAS software version 9.4 (SAS Institute Inc. Cary, NC) were used for all analyses.

RESULTS

Of the 7146 participants at risk of demonstrating advanced AMD, 1337 had no follow-up data for both eyes. In addition, 813 were excluded because of missing or unreliable dietary data (Fig 1). Overall 4996 participants (4446 from RS-I and 550 from Alienor Study) free of advanced AMD at baseline with complete and reliable dietary data together with follow-up information were included in our analyses. Among the 4996 included participants, 155 demonstrated advanced incident AMD (117 from RS-I and 38 from the Alienor Study). The mean follow-up time was 9.9 years (range, 0.6-21.7 years) in RS-I and 4.1 years (range, 2.5-5.0 years) in the Alienor Study.

In both studies, participants included in the analyses tended to be younger than those excluded (Table 1). In RS-I, after adjustment for age and gender, included participants tended to be women, to have a higher education, to have a history of smoking and diabetes, to have a lower TEI, to have a higher MeDi score, and less often to carry a *CFH* Y402H CC genotype than excluded participants. In the Alienor Study, included participants were more likely to have a higher education than excluded participants.

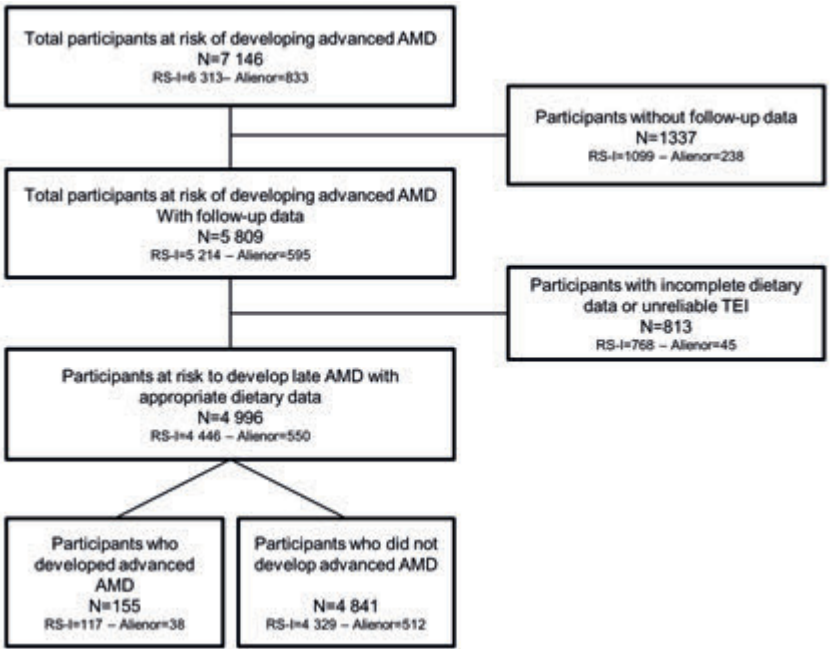

Figure 1. Flowchart showing selection of participants for analyses. AMD = age-related macular degeneration; Alienor = Antioxydants, Lipids Essentiels, Nutrition at Maladies Oculaires; RS-I = Rotterdam Study I; TEI = total energy intake.

Table 1. Baseline Characteritics of the Rotterdam Study I and the Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires (Alienor) Study According to Participants Included and Excluded from Analyses

Characteristics	Rotterdam Study I			Alienor Study			*P* Value (Rotterdam study I vs. Alienor Study for Included Participants)‡
	Included* (*n*=4446)	Excluded† (*n*=1 867)	*P* Value‡	Included* (*n*=550)	Excluded† (*n*=283)	*P* Value‡	
Age (yrs), mean (SD)	66.9 (7.3)	73.4 (10.0)	<0.0001	79.2 (4.2)	81.1 (4.3)	<0.0001	<0.0001
Gender			0.006			0.61	0.08
Male	1813 (40.8)	766 (41.0)		209 (38.0)	108 (38.2)		
Female	2633 (59.2)	1101 (59.0)		341 (62.0)	175 (61.8)		
Education	n=4426	n=1808	<0.0001		n=283	0.03	<0.0001
Primary	2200 (49.7)	1151 (63.6)		301 (54.7)	180 (63.6)		
Secondary	1823 (41.2)	529 (29.3)		130 (23.7)	56 (19.8)		
Higher	403 (9.1)	128 (7.1)		119 (21.6)	47 (16.6)		
Smoking (pack-years)	n=4131	n=1644	0.009	n=547	n=278	0.77	<0.0001
Never smoker	1501 (36.3)	667 (40.6)		356 (65.1)	179 (64.4)		
<20	1173 (28.4)	413 (25.1)		95 (17.4)	51 (18.3)		
≥20	1457 (35.3)	564 (34.3)		96 (17.5)	48 (17.3)		
Multivitamin/mineral supplement use	n=4442	n=1867	0.11		n=281	0.32	0.05
No	4070 (91.7)	1660 (88.9)		475 (86.4)	237 (84.3)		
Yes	370 (8.3)	207 (11.1)		75 (13.6)	44 (16.7)		
Body mass index (kg/m^2), mean (SD)	n=4426	n=1775	0.26	n=542	n=274	0.87	0.17

Table 1. Continued

Characteristics	Rotterdam Study I			Alienor Study			*P* Value (Rotterdam study I vs. Alienor Study for Included Participants)‡
	Included* (*n*=4446)	Excluded† (*n*=1 867)	*P* Value‡	Included* (*n*=550)	Excluded† (*n*=283)	*P* Value‡	
	26.3 (3.6)	26.2 (4.0)		26.0 (4.0)	25.9 (4.1)		
Diabetes	n=4444		0.003		n=281	0.33	0.63
No	3941 (88.7)	1693 (90.7)		489 (88.9)	243 (86.5)		
Yes	503 (11.3)	174 (9.3)		61 (11.1)	38 (13.5)		
Hypertension			0.28		n=281	0.48	<0.0001
No	1883 (42.3)	593 (31.8)		86 (15.6)	46 (16.4)		
Yes	2563 (57.7)	1274 (68.2)		464 (84.4)	235 (83.6)		
Hypercholesterolemia	n=4442		0.18		N=281	0.76	<0.0001
No	4317 (97.2)	1837 (98.4)		275 (50.0)	146 (52.0)		
Yes	125 (2.8)	30 (1.6)		275 (50.0)	135 (48.0)		
CFH (rs1061170)	n=3972	n=1581	0.02	n=450	n=235	0.81	0.03
TT	1649 (41.5)	632 (40.0)		212 (47.1)	101 (43.0)		
CT	1801 (45.3)	709 (44.8)		181 (40.2)	110 (46.8)		
CC	522 (13.2)	240 (15.2)		57 (12.7)	24 (10.2)		
ARMS2 (rs10490924)	n=3971	n=1582	0.16	n=450	n=235	0.12	0.11
GG	2490 (62.7)	1028 (65.0)		309 (68.7)	145 (61.7)		
GT	1339 (33.7)	500 (31.6)		126 (28.0)	85 (36.2)		
TT	142 (3.6)	54 (3.4)		15 (3.3)	5 (2.1)		

Table 1. Continued

Characteristics	Rotterdam Study I			Alienor Study			*P* Value (Rotterdam study I vs. Alienor Study for Included Participants)‡
	Included* (*n*=4446)	Excluded† (*n*=1 867)	*P* Value‡	Included* (*n*=550)	Excluded† (*n*=283)	*P* Value‡	
Total energy intake (kcal), mean (SD)		n=687	0.0002		n=242	0.90	<0.0001
	1968 (484)	2016 (609)		1719 (530)	1704 (549)		
Mediterranean diet score		n=398	0.04§		n=209	0.70§	<0.0001
Low (0-3)	1376 (31.0)	153 (38.4)		171 (31.1)	58 (27.8)		
Medium (4-5)	2123 (47.7)	181 (45.5)		236 (42.9)	100 (47.8)		
High (6-9)	947 (21.3)	64 (16.1)		143 (26.0)	51 (24.4)		
AMD grade at baseline			0.11			0.05	0.001
No AMD	4179 (94.0)	1654 (88.6)		444 (80.7)	241 (85.2)		
Early AMD	267 (6.0)	213 (11.4)		106 (19.3)	42 (14.8)		

AMD= age-related macular degeneration; ARMS2= Age-Related Maculopathy Susceptibility Protein 2; CFH = complement factor H; SD =standard deviation. Data are no. (%) unless otherwise indicated.

**Participants included in 1 or more analyses for incidence of advanced AMD.*

†Participants excluded from all analyses.

‡P value from logistic regression adjusted for age and gender.

§P value from logistic regression adjusted for age, gender, and total energy intake

Participants from the RS-I, tented to be younger, to have a lower education, to have a history of smoking, to have less hypertension, to have less hypercholesterolemia, to have a higher TEI, to have a lower adherence to the MeDi score, and to have less early AMD than participants from the Alienor Study. Also, the presence of the *CFH* Y402H polymorphism differed slightly between the 2 studies.

Participants from the Alienor Study tented to have a higher median of consumption of vegetables, cereals and fish, whereas participants from the RS-I tended to have a higher median of consumption of dairy products. Consumption of fruits, legumes, meat and the MUFAs-to-SFAs ratio were similar (Table S1, available at www.aaojournal.org).

For both studies, the incidence of advanced AMD was lower among participants who showed a high adherence to the MeDi score (Fig 2). The effect of MeDi is more notable among older people (85 years of age or older) at higher risk of AMD, but because the proportional hazard assumption is respected, associations are considered similar among the different age groups.

In the unadjusted model, similar estimations were obtained in both studies, with a hazard ratio (HR) of 0.56 (95% confidence interval [CI], 0.33-0.96) in the RS-I and an HR of 0.48 (95% CI, 0.18-1.26) in the Alienor Study for participants with a high MeDi score, by comparison with those with a low MeDi score (Table 2). When pooling both studies, a high MeDi score was associated significantly with a lower risk for incident advanced AMD (HR, 0.53; 95% CI, 0.33-0.84; *P* = 0.009 for trend). These associations remained similar and significant after further adjustment for gender, TEI, AMD grade at baseline, education, body mass index, smoking, supplement use of multivitamins or minerals, diabetes, and hypercholesterolemia (HR, 0.59; 95% CI, 0.37-0.95; *P*=0.04 for trend).

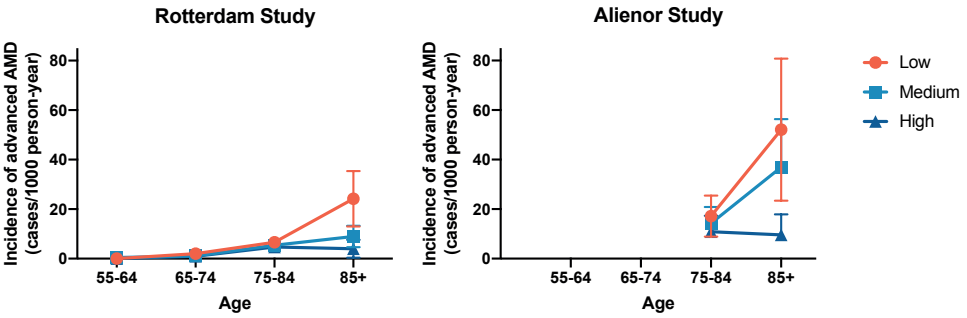

Figure 2. Graphs showing the incidence of advanced age-related macular degeneration (AMD) according to adherence to Mediterranean diet (MeDi) score. Alienor = Antioydants, Lipides Essentiels, Nutrition et Maladies Oculaires.

Table 2. Association between Mediterranean Diet Score and Incidence of Advanced Age-Related Macular Degeneration

	No. at Risk for Advanced Age-Related Macular Degeneration	No. of Incident Cases	Mediterranean Diet Score			
			Low 0-3	*Medium 4-5*	*High 6-9*	P Value for Trend*
Model I[†]						
Rotterdam I	4446	117				
HR (95% CI)[‡]			Reference	0.69 (0.46-1.03)	0.56 (0.33-0.96)	*0.036*
Alienor	550	38				
HR (95% CI)[‡]			Reference	0.80 (0.39-1.63)	0.48 (0.18-1.26)	*0.16*
Overall	4996	155				
HR (95% CI)[§]			Reference	0.71 (0.50-1.00)	0.53 (0.33-0.84)	*0.009*
Model 2[‖]						
Rotterdam I	4104	108				
HR (95% CI)[‡]			Reference	0.70 (0.46-1.06)	0.69 (0.40-1.20)	*0.19*
Alienor	539	38				
HR (95% CI)[‡]			Reference	0.83 (0.38-1.80)	0.52 (0.19-1.40)	*0.23*
Overall	4643	146				
HR (95% CI)[§]			Reference	0.70 (0.49-1.01)	0.59 (0.37-0.95)	*0.04*

Alienor = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; CI = confidence interval; HR = hazard ratio.
** P value for trend calculated using the median value for each Mediterranean diet score category.*
[†] Model 1, unadjusted model.
[‡] Estimated using Cox proportional hazard model.
[§] Estimated using Cox proportional hazard model with additional adjustment for study.
[‖] Model 2, adjusted for gender, total energy intake, age-related macular degeneration grade at baseline, education, body mass index, smoking, supplement use of multivitamins or minerals, and presence of diabetes and hypercholesterolemia

In secondary analyses, we further adjusted for *CFH* Y402H and *ARMS2* genes and the HR remained unchanged (data not shown). Interaction terms between MeDi and *CFH* Y402H and *ARMS2* genes were not statistically significant (P=0.89 and P=0.18 for interaction, respectively; data not shown).

Adherence to MeDi score was not associated significantly with the risk for incident neovascular AMD in the RS-I, in the Alienor Study, or in the pooled analysis (Table 3). It was associated significantly with the risk for incident atrophic AMD in the RS-I (HR, 0.41; P=0.046 for trend), but the association did not reach significance in the Alienor Study (HR,

0.52; *P*=0.52 for trend). In the pooled data analysis, a higher MeDi score was associated significantly with a reduced risk for incident atrophic AMD (HR, 0.42; 95% CI, 0.20-0.90; *P*=0.04 for trend).

We assessed whether the benefit of high adherence to the MeDi score was the result of a specific component. Using the gender specific median as cutoffs, no component was associated significantly with incidence of advanced AMD (Table S2, available at www.aaojournal.org).

Table 3. Association between Mediterranean Diet Score and Incidence of Advanced Neovascular and Atrophic Age-Related Macular Degeneration

			Mediterranean Diet Score categories			
	No. at Risk for Advanced Age-Related Macular Degeneration	**No. of Incident Cases**	***Low (0-3)***	***Medium (4-5)***	***High (6-9)***	***P* Value for Trend***
Neovascular AMD						
Rotterdam I	4104	68				
HR (95% CI)†			Reference	0.87 (0.51-1.51)	1.03 (0.53-1.99)	*0.91*
Alienor	538	18				
HR (95% CI)†			Reference	0.80 (0.25-2.63)	0.75 (0.20-2.91)	*0.65*
Overall	4642	86				
HR (95% CI)‡			Reference	0.78 (0.48-1.27)	0.88 (0.49-1.57)	*0.64*
Atrophic AMD						
Rotterdam I	4104	52				
HR (95% CI)†			Reference	0.61 (0.34-1.10)	0.41 (0.16-1.03)	*0.046*
Alienor	538	21				
HR (95% CI)†			Reference	1.08 (0.38-3.06)	0.52 (0.13-2.12)	*0.52*
Overall	4642	73				
HR (95% CI)‡			Reference	0.70 (0.42-1.15)	0.42 (0.20-0.90)	*0.04*

Alienor = Antioxydants, Lipides Essentiels, Nutrition et Maladies Oculaires; AMD = age-related macular degeneration; CI = confidence interval; HR = hazard ratio.

** P value for trend is calculated using the median value for each Mediterranean diet score category.*

† Cox proportional hazard model adjusted for gender, total energy intake, AMD grade at baseline, education, body mass index, smoking, supplement use of multivitamins or minerals, diabetes and hypercholesterolemia.

‡ Cox proportional hazard adjusted for gender, total energy intake, AMD grade at baseline, study, education, body mass index, smoking, supplement use of multivitamins or minerals, diabetes and hypercholesterolemia.

DISCUSSION

High adherence to the MeDi was associated with a 41% reduced risk of incident advanced AMD in the pooled analysis. None of the 9 components, including vegetable, fruit, legume, cereal, fish, meat, dairy products, and alcohol consumption and MUFAs-to-SFAs ratio were associated significantly with incidence of advanced AMD, highlighting the importance of assessing dietary patterns rather than single components. In our studies, a high adherence to the MeDi was associated significantly with a reduced risk of incident atrophic AMD. A similar association was observed for neovascular AMD but did not reach statistical significance.

By evaluating the individual and the pooled associations of the adherence to the MeDi and incidence of advanced AMD in 2 well-established and harmonized European population-based prospective cohorts, this study expands on prior studies, mainly cross-sectional, case-control, and clinical trials on this topic. Visual impairment resulting from AMD could influence dietary practices; prospective studies, by assessing diet prior the onset of the disease, limit reverse causation. Thus, a prospective design is more accurate and less biased than a cross-sectional or case-control design to evaluate the association between diet and AMD. In addition, although using a prospective design, clinical trials are limited by the selected nature of the sample. Results from population-based studies are more generalizable.

Our results are partially consistent with previous cross-sectional studies: the Carotenoids in Age-Related Eye Disease Study (CAREDS) reported a lower prevalence of early AMD in American women with high adherence to the MeDi[23], the Coimbra Study demonstrated a lower prevalence of any AMD in Portuguese participants who showed a high adherence to the MeDi[26, 27], and the European Eye Study showed a lower prevalence of neovascular AMD in participants with a high MeDi score, whereas atrophic AMD was not associated with MeDi score[25]. Our findings confirm the post hoc analyses of the Age-Related Eye Disease Study (AREDS) clinical trial. In this sample of American participants 55 to 80 years of age, a high MeDi score was associated with a 26% lower risk of progression to advanced AMD[24]. The AREDS study also showed the fish and vegetable components were associated with a lower risk of progression to advanced AMD[24]. Our study were in the same direction, but did not reach the statistical significance when gender-specific median cutoffs were used. No significant interactions were observed between MeDi score and *CFH* Y402H and *ARMS2* genes. Our findings report a significant association with advanced AMD. Regarding subtypes, only atrophic AMD was associated significantly with MeDi score. For neovascular AMD, even if the association was not statistically significant, the HRs were similar to those for atrophic AMD. These differences could be explained by a low number of incident cases. In the European Eye Study, the only study to show

separate results for the 2 advanced forms of AMD, the association was significant with neovascular AMD. Although in our studies this association with neovascular AMD was not statistically significant, HRs were similar to those for the European Eye Study.

Our results thus support public health efforts to emphasize adherence to the MeDi for everyone. The biological basis for the potential benefits of the MeDi is associated with a decrease in oxidative stress and inflammation, which are also involved in the pathophysiology of AMD[42, 43].

The Prevención con Dieta Mediterránea (PREDIMED) study, a clinical trial among persons at high cardiovascular risk, showed that adhering to a MeDi reduced the incidence of major cardiovascular events[20]. Median consumptions were similar to the goals suggested by PREDIMED for vegetables (≥2 servings/day), fish (≥3 servings/week), and meat (<1 serving/day) in the Alienor Study and for meat in the RS-I. For both studies, the medians of fruit and legume consumption were less than the goals of PREDIMED (≤3 serving/day) as well as the medians of vegetable and fish consumption in the RS-I. Although the medians in our study were lower for consumption of vegetables and fruits, the association with the MeDi score was significant, suggesting the importance of a global approach to prevent the development of AMD.

By showing a prospective association between AMD and MeDi, an energy-unrestricted diet mainly composed of nutrient-rich food, our study confirmed the importance of dietary quality focused on healthful foods and dietary patterns rather than single nutrients or low-energy diet for AMD.

In observational studies, residual confounding is always a concern. In the present study, results were similar in the basic model (unadjusted model) and the fully-adjusted model (adjusted for gender, TEI, AMD grade at baseline, cardiovascular risk factors, educational level, and dietary supplement use), suggesting that our results are not highly confounded. In the fully adjusted model, the association between MeDi and incidence of AMD seems to be weaker in the RS-I. This could be explained by a lower statistical power resulting from a low incidence of participants demonstrating advanced AMD combined with the increasing number of covariates compared with the unadjusted model. In addition, our findings are based on prospective follow-up, thereby limiting reverse causation. However, only randomized clinical trials can prove the causal nature of the associations. Such randomized clinical trial testing dietary interventions have proven to be efficient in the prevention of stroke[20] or diabetes[44], for instance, but none are available in the field of AMD.

Selection bias cannot be dismissed completely, because participants included in this analysis were different from nonparticipants in both the RS-I and Alienor Study. Moreover,

participants included from the RS-I were different from those from the Alienor Study regarding some socio-demographic and medical characteristics as well as follow-up duration and frequency. Incidence rates of AMD also were higher in the Alienor Study than in the RS-I. These differences may be explained by the older age at baseline and a closer follow-up (every 2 years instead of every 5 years in the RS-I, with home examinations for participants unable to come to the hospital in the Alienor Study, but not in every RS-I follow-up visit), or by different incidence rates in France and The Netherlands.

The MeDi score uses cutoffs based on each study population and results can be generalizable only to similar populations. To calculate the MeDi score, we used validated FFQs for both studies, adapted to the specific dietary habits of each population (France and The Netherlands). Because the FFQ in the Alienor Study was a 40-item FFQ, we used the 24-hour recall to calculate the MUFAs-to-SFAs ratio and the TEI to increase the exactitude of their ascertainment, as published previously[21]. The distribution of the MeDi score was different between the 2 studies, and participants from RS-I were less adherent. This result was expected in a Northern European population.

Despite these major differences in populations (different countries, different periods, different generations, and different dietary habits) and methods (different follow-up time and frequency and different dietary assessment methods), the association between MeDi and incidence of advanced AMD was similar in both cohorts. This association thus seems to be robust.

To strengthen our analyses, we excluded participants with unusually high or low TEI and adjusted for several factors know to be related to MeDi and AMD. We used a well-known and validated score to assess diet and probable synergistic effects between nutrients and food groups. Our MeDi score was developed by using gender-specific thresholds according to each study to account better for differences between men and women and between studies. Other strengths include a large sample from 2 well-documented and data-harmonized population-based prospective cohorts in the framework of the European EYE-RISK project.

In conclusion, combined results from our 2 observational studies suggest that adopting an energy-unrestricted diet rich in healthful nutrient-rich foods such as fruits, vegetables, legumes and fish and reducing the unhealthful foods such as red and processed meats and savory and salty industrialized products may contribute to the prevention of AMD.

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6

DISCUSSION AND SUMMARY

GENERAL DISCUSSION

Age-related macular degeneration (AMD) is one of the leading causes of blindness in the Western world, and the pathophysiology of this complex disease remains poorly understood. The overall aims of this thesis were to investigate the prevalence and progression of AMD, determine how genetics plays a role in the disease, and determine how lipids and nutrition affect the development of the disease. In this general discussion, we will review the findings of this thesis, discuss our current view regarding AMD, and consider the main findings of this thesis in the context of our current knowledge by discussing the clinical relevance and directions for future research.

The effects of aging

AMD is a disease that affects the elderly. As we age, our physiology changes, leading to loss of function and increasing our vulnerability to a wide range of diseases and conditions[1]. With increasing age, various biological and physiological processes occur, including shortening of our telomeres with each cell division, an accumulation of cellular damage, and cumulative metabolic damage, all of which are regulated by genes involved in repair and conservation[2].

Aging also affects our eyes. At the tissue level, the retina undergoes reorganization, with a loss of rod cells compensated by larger rod inner segments[3,4]. In addition, the so-called on bipolar cells become elongated and extend into the outer nuclear layer at the periphery of the retina, possibly compensating for reduced signal transduction in the aging retina[5].

Importantly, the retinal pigment epithelium (RPE) is also affected by aging; this retinal layer is particularly vulnerable to metabolic waste, as it is composed of terminally differentiated cells and—unlike actively dividing cells—cannot dilute this waste[2]. The fovea contains the highest density of RPE cells and is mainly hexagonal in shape, but changes in later life to other polygonal shapes without changing the number of cells[6]. In addition, as we age the number of desmosomes, which maintain the blood-retina barrier, decreases. Moreover, Bruch's membrane, located below the RPE, becomes thicker with each passing year, doubling or even tripling in thickness at the macula; in contrast, the complex formed between Bruch's membrane and the RPE becomes thinner with each passing year[7]. The increased thickness of Bruch's membrane reduces the bi-directional transport of particles between the RPE and the choroid[8].

Some aging-related changes in the retina are due to changes in cellular metabolism, as the retina has the highest consumption of oxygen per gram tissue, even higher than the brain[9]. Due to this high level of metabolism, molecular damage due to reactive oxygen

species (ROS) is inevitable, and oxidative stress damages both proteins and DNA[10]. High levels of ROS also cause the peroxidation of polyunsaturated fatty acids, which are present in high levels in the cell membranes of photoreceptors and RPE cells, leading to the loss of these cells[11].

Together, these aging-related changes in our physiology create an ideal environment for the development of AMD.

Bruch's membrane

Bruch's membrane forms the basal layer of the RPE and acts as a structural support for the choriocapillaris, consisting of an elastic layer and two collagen layers[12]. Aging effects the structural integrity[13] of Bruch's membrane, resulting in an increase in thickness. To maintain its function, enzymes known as matrix metalloproteinases (MMPs) break down the extracellular matrix; these enzymes are regulated by tissue inhibitors of metalloproteinases (TIMPs), and the activity of TIMP-3 increases in Bruch's membrane with aging[14]. In contrast, MMP-2 and MMP-9 are more frequently found in Bruch's membrane in the elderly compared to younger individuals, but are more often inactive with aging[15]. In addition, genetic variations in the *MMP9* and *TIMP3* genes have been associated with AMD and may contribute to the thickening of Bruch's membrane[11]. Other genetic variants have also been associated with AMD and include genes involved in the extracellular matrix[16], including the *COL8A1* gene, which encodes an alpha chain in type VIII collagen.[17] A common gene variant associated with an increased risk of AMD was previously identified[16]; however, using gene-based analyses we found that rare protein-altering variants are more prevalent in patients with AMD compared to controls. This finding underscores both the importance of the *COL8A1* gene in the pathogenesis of AMD and the key role of Bruch's membrane in this process (**Chapter 4.1**). The structural changes, including thickening of Bruch's membrane, may affect the transport of nutrients and waste products through the membrane, which can lead to the buildup of deposits and waste products in the membrane as we age, as discussed below.

Drusen

Beginning in the second decade of life, debris becomes deposited in the collagen layers of Bruch's membrane[18]. Over time, focal deposits known as drusen are formed[19], containing lipids, minerals, and a wide variety of proteins[20,21]. Drusen are typically the first sign of AMD and are often used to distinguish between AMD and other retinal diseases. A high percentage of the general population has small drusen[22,23]; thus, this finding is considered to be associated with the normal aging process, rather than indicative of AMD. When these small drusen increase in number and/or size, however, the risk of

pathology increases[24]. The lipids and proteins contained in drusen are derived from both the systemic circulation and the retina itself; in the retina, lipid metabolism plays an important role in this process[25-27].

The role of circulating lipids and lipoproteins in AMD has been studied extensively[26]. The RPE expresses receptors that allow it to take up both low-density lipoproteins (LDL) and high-density lipoproteins (HDL) from the systemic circulation into the retina[28,29]. Interestingly, studies regarding lipids and AMD have yielded conflicting results[30]. In **Chapter 5.1,** we report our findings in a large European study showing that patients with AMD have higher levels of circulating HDL-cholesterol and lower levels of triglycerides compared to controls, with the highest increase in the largest subclass of HDL[31]. We also found that high HDL-cholesterol and low triglyceride levels are associated with large drusen (**Chapter 5.1**) and an increased risk of AMD. Moreover, we found that genetic variations are associated with changes in lipid levels (**Chapter 5.1**). A novel finding is, using Mendelian randomization analyses, that these genetic variants act on AMD by changing lipid levels and lipid subclasses[32,33]. Mendelian randomization analysis uses variations in genes in a randomized controlled trial to examine the putative effects of modifiable factors on a disease. This analysis is possible because genes are passed randomly from the parents to their children, and the genotype is not related to possible confounding factors. In addition, the disease does not affect the genetic variation, so reverse causation—which can play a major role in observational studies—is not possible.

The large subclass of HDL in AMD might affect their transport. One of the principal functions of HDL-cholesterol is to carry complement factors such as C2, C3, C4B, C5, and complement factor B, as well as subcomponents of complement 1 and many other proteins[34,35]. Large HDL subclasses are significantly associated with systemic activation of the complement system[31], whereas smaller HDL subclasses reduce complement activation. This finding has led to the hypothesis that the size of HDL particles—a reflection of their composition and function—drives the pathology. For example, large subclasses of HDL molecules are likely to contribute to the formation of drusen by inducing a pro-inflammatory state.

In the eye, the RPE and photoreceptors also produce cholesterol[36] and package cholesterol particles into HDL-cholesterol for bi-directional transport between the RPE and photoreceptors, a process facilitated in part by the enzyme CETP (cholesteryl ester transfer protein)[29,37]. Cholesterol from photoreceptors is re-processed in the RPE by autophagosomes and lysosomes. In AMD, the processing of internalized photoreceptor outer segment disks can be reduced by impaired function of autophagosomes, leading to a disruption in cholesterol metabolism in the RPE[38,39]. To prevent the accumulation of cholesterol in the RPE, cholesterol is secreted into the choriocapillaris in lipoprotein-

like particles by the phospholipid-transporting ATPase ABCA1[refs. 29,40,41]. With aging, this secretion can contribute to the accumulation of debris in Bruch's membrane. Any disruption in cholesterol metabolism in the retina can affect the risk of developing drusen; moreover, oxidation of these lipids can cause the molecules to form crosslinks, thus driving the development of drusen. Finally, the cellular response to the developing drusen and activation of the complement system can cause the disease to progress to the next stage.

Genetics

Genetics has long been known to play a major role in the pathogenesis of AMD, as was shown in twin studies and family aggregation studies[42-44]. Recently, genome-wide association studies (GWAS) and next-generation sequencing techniques have enabled researchers to investigate genetic variations in even more depth. For example, a large GWAS found a set of 52 independent genetic variants associated with AMD[16], to which 12 novel loci and 20 novel genes were subsequently added by the largest, most recent GWAS[45]. Other studies using different sequencing techniques, as well as family studies, identified more than 100 rare AMD-associated variants in at least 13 genes, the majority of which encode complement-related proteins[46]. Although the functional role of the majority of these variants with respect to AMD is currently unknown, they provide new insights into the pathogenesis of AMD. For example, studying the proteins encoded by these genes has revealed several functional pathways, including the complement system, lipid metabolism, and the extracellular matrix (**Chapter 4.2**).

The complement pathway is an important component of the innate immune system, and activation of this pathway initiates a signaling cascade that ultimately leads to cell lysis. Both causal and protective genetic variants in this cascade have been associated with AMD[16], and our population-based studies revealed that the causal variants are more prevalent in late AMD than in newly identified patients (**Chapter 4.2**). It has long been known that the complement system plays an important role in the pathogenesis of AMD[47], and our study described in **Chapter 4.2** revealed significantly higher genetic pathway risk scores in patients with late AMD compared to controls. Importantly, drusen contain complement components[34], and patients with AMD have elevated serum levels of activated complement[48,49], suggesting that the pro-inflammatory component of AMD is not specific to the eye but is likely systemic.

The protein ARMS2 (age-related maculopathy susceptibility 2) plays a major role in the pathogenesis of AMD. Although its function is not fully known, recent studies suggest that it may be part of the complement pathway, serving as a complement regulator expressed at the cell surface. ARMS2 binds to apoptotic and necrotic cells, mobilizing the protein properdin, which activates the complement system[50]. Other, non-complement-

related genes include genes involved in the extracellular matrix, which likely play a role in maintaining Bruch's membrane, and lipid-related genes, which likely play a role in the formation of drusen as described above. We found that for the vast majority of the general population, several genetic pathways contribute to the risk of developing AMD (**Chapter 4.2**). Generally speaking, individuals who have more AMD-associated risk variants are more susceptible to developing AMD. In addition to the common variants, rare variants also contribute to the risk of developing AMD, as discussed previously for the *COL8A1* gene (**Chapter 4.1**)[46].

Many of the genetic variants associated with AMD are common variants. As a consequence, the vast majority of the general population has a genetic risk of AMD, even among individuals who also carry genetic variants that protect against AMD (**Chapter 4.2**). When separating early AMD from late AMD with geographic atrophy (GA) and/or choroidal neovascularization (CNV), we found that the complement pathway is nearly always linked genetically to the development of late AMD (**Chapter 4.2**). Interestingly, risk variants in complement genes are related to a pro-inflammatory state that can protect younger individuals, but can cause degeneration over time when this state is perpetually active[51,52]. In the elderly, the presence of drusen may exacerbate the adverse effects of complement activation, including tissue damage, an influx of immune cells, fibrosis formation, and a burst of ROS. Thus, antioxidants and lifestyle changes can help reduce the formation and effects of ROS, reducing tissue damage.

Lifestyle

In addition to genetic factors, behavior and lifestyle can also affect the risk of developing AMD. Given the high heritability of AMD and the current lack of a cure, research has focused primarily on identifying factors that can be modified in order to affect the development of AMD, including behavior and lifestyle. One such modifiable factor is smoking, which has consistently been associated with a more than twofold increase in the risk of developing AMD[53,54]. Smoking is believed to induce oxidative stress via free radicals, causing lipid peroxidation and inflammation[55,56], thus contributing to the pathophysiology of AMD. A variety of other factors have also been investigated for their possible role in AMD, ranging from iris color[57] and sunlight[58] to cardiovascular-related factors[59], body-mass index[60], and waist-to-hip ratio[61,62]; however, none of these factors has been consistently associated with AMD[61,63-65].

In addition, many studies have investigated the role of nutrition in AMD. Initial studies focused on carotenoids and vitamins[66], but later studies also included vitamin C, vitamin E, lutein, zeaxanthin, and zinc.[67,68] Consuming foods rich in omega-3 fatty acids such as nuts and fatty fish has been associated with a reduced risk of developing late AMD[69]. It is generally believed that these nutrients and compounds protect against AMD by

reducing the pro-inflammatory state and reducing free radicals[70]. In addition, studies have found that consuming food items containing these nutrients, including fruit, fish, and vegetables, can have a protective effect against late AMD[71,72]. Indeed, studies of dietary behavior have found that individuals who adhere to a Mediterranean diet rich in antioxidants and omega-3 fatty acids can reduce their risk of developing late AMD by 41% (**Chapter 5.2**)[73-75]. Thus, diet can play a major role in reducing the risk of developing AMD. Conversely, an unfavorable lifestyle can increase the risk of developing AMD, independent of genetics, for example as shown in a twin study in which differences in lifestyle were associated with different rates of disease progression[76]. These findings are supported by our population-based studies in which a healthy lifestyle that includes not smoking and eating plenty of fruit, fish, and vegetables reduces the risk of developing AMD, regardless of the genetic risk profile (**Chapter 4.2**). Thus, living a healthy lifestyle can reduce the risk of developing AMD, even in individuals who have a low risk based on genetics.

Disease progression

When a patient is initially diagnosed with AMD, a major clinical goal is to determine the patient's prognosis with respect to the course of the disease. For patients with early signs of pathology such as drusen, preventive interventions may be indicated. Even though a relatively large percentage of elderly individuals develop drusen at some point, a small subgroup progresses to early AMD, and an even smaller subgroup will develop late AMD (**Chapter 2.1**)[77]. Specifically, only 0.4% of individuals with small drusen develop late AMD within 5 years[78]. Whether a patient progresses to a more severe or higher pathological stage of AMD depends on a variety of factors, including genetics[79], aging, and lifestyle, which together contribute to structural changes combined with a low-grade inflammatory state (Figure 1).

Compared to the early stages of disease, various phenotypic features in AMD have been associated with disease progression. Both drusen area and the presence of large drusen are risk factors for developing late AMD[59,80,81]. Presence of a specific type of drusen known as calcified drusen appears to be associated with an increased risk of developing GA[82], although calcified drusen can also lead to CNV[82]. However, these results are based on a single study involving 138 patients with one year of follow-up; thus, further research is clearly needed. Another type of drusen, reticular pseudodrusen, present with drusenoid deposits between the RPE and the photoreceptor outer segments and increase the risk of developing late AMD, particularly GA[83,84], as well as more rapid growth of the GA lesion toward the drusen[85]. Pigmentary changes have also been associated with progression to late AMD[78,86]. In addition, we found that both pigmentary changes and drusen area are

associated with the progression of unilateral to bilateral disease and/or the progression from early AMD to late AMD (**Chapter 2.2**); importantly, this association appears to be independent of genetic risk factors and smoking, consistent with previous reports[17,86].

In addition, even late AMD can progress further. For example, the development of CNV has been associated with a genetic variant in the *MMP9* gene[16]. However, a major challenge is quantifying disease progression in this stage. The development of GA is associated with the shape—but not the size—of the baseline lesion[87,88]. For example, we found that estimating the roundness of a lesion can be used to predict the rate of GA growth (**Chapter 3.2**). Furthermore, extrafoveal GA lesions tend to grow more rapidly than foveal GA lesions[89], and lesion growth toward the periphery is more rapid than growth toward the fovea[90]. Moreover, our results confirm[91,92] that GA growth in one eye is correlated with the severity of AMD in the other eye, and bilateral GA growth is closely correlated, with a correlation coefficient of 0.87 (**Chapter 3.1**). However, we also found wide variation with respect to lesion growth; therefore, further research is needed in order to reliably predict lesion growth in individual patients, particularly individuals who are at risk of rapid progression.

Prevalence and prediction

Our population-based studies revealed that the prevalence of late AMD among Europeans ranges from 0.1% among 55-59-year-olds to 9.8% among individuals 85 years of age and older (**Chapter 2.1**), providing the first such overview within the European population. Interestingly, we found that the prevalence decreased over the past decade, and this is likely due to our improved lifestyle and reduced numbers of smokers[93]. Unfortunately, however, even in the context of this downward trend the number of individuals with AMD is expected to increase in the next twenty years due to our aging population (**Chapter 2.1**); thus, ophthalmologists are encouraged to reorganize the healthcare system, and new therapies are urgently needed. Aging provides the foundation for developing AMD, while genetics and environmental factors drive the development and progression of AMD (Figure 1). Nevertheless, changes in lifestyle can reduce the risk and/or severity of AMD.

The risk of developing AMD can be predicted at multiple levels. The first level is to predict individuals who are susceptible to developing AMD. Many studies have focused on distinguishing between high-risk individuals and those with an average or low risk of AMD. Given the disease's high heritability, the large effect of the complement pathway, and the large number of low-risk genes identified to date, one can estimate with high accuracy the AMD risk of an individual and discriminate between risk categories (**Chapter 4.2**). Thus, in principle the risk of developing AMD can be estimated at birth. However, although screening for genetic factors can provide an extremely powerful tool for estimating the life-long cumulative risk of developing AMD, they are a rather poor

predictor of *when* the disease will likely develop. The age at onset is a key factor for high-risk individuals, but is also relevant for optimizing the start of preventive measures. The model can be improved by incorporating environmental and phenotypic factors based on proteomics and/or metabolomics, or by assessing typical AMD features [94].

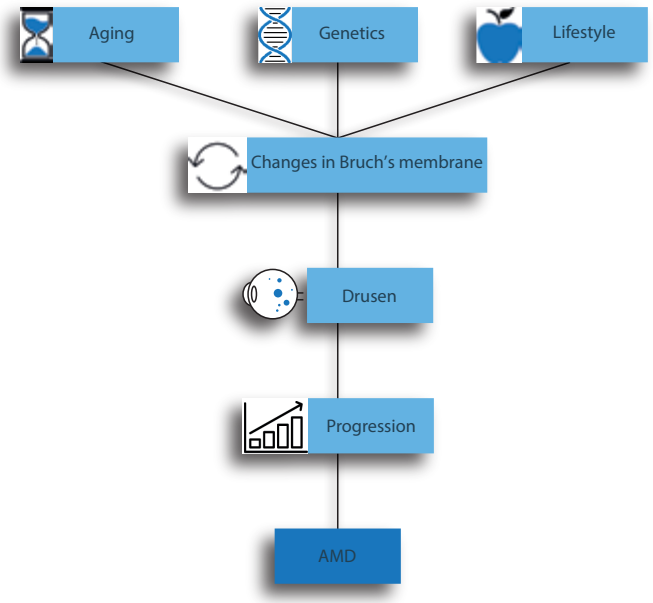

Figure 1. Flow chart summarizing the clinical development of AMD.

Several models have been developed in order to differentiate between patients with late AMD and controls, as well as to predict who will likely develop late AMD over time based on genetics, lifestyle, and/or phenotypic factors. These models have yielded a predictive value ranging from 0.67 in an early study[95] to as high as 0.96[(ref. 96)], with varying levels of validity[97,98]. Achieving such a high prediction level suggests that it might be possible to screen for high-risk individuals. Given that changing one's lifestyle can significantly reduce the risk of developing AMD, these models can be used to identify high-risk individuals as early as possible and begin steering them toward living a healthier lifestyle.

In addition, major progress has been made with respect to predicting the rate of progression from early AMD to late AMD, as well as the rate of lesion growth in GA. This information will be essential for designing robust clinical trials and intervention studies. Risk factors associated with rapid disease progression include bilateral AMD lesions, genetic susceptibility, smoking, and infrequent consumption of fish[99]. With respect to GA,

the shape and location of the lesion can predict the rate of lesion growth (**Chapter 3.2**). Obtaining an accurate prognosis can help researchers investigate the effects of new interventions for GA in clinical trials. Given the high importance of preventing the progression of GA, software designed to analyze multimodal ocular imaging data, as well as clinical tools to identify high-risk individuals, are needed in order to facilitate patient follow-up and provide efficient healthcare options.

Methodological considerations

Each chapter in this thesis addressed the respective methodological considerations; here, we discuss some of the more general considerations. Each chapter used datasets obtained from several studies; in some cases these datasets were combined to increase the sample size. To facilitate the use of several studies and combine datasets, data harmonization is required, which can result in loss of data. In addition, heterogeneity can be introduced due to differences in the study protocols and/or the study location. In our analysis, we addressed heterogeneity either by correcting for it or by repeating the analyses separately for each study site.

The long-term follow-up of population-based studies provides added value, but such studies often involve intervals of five years or longer. The use of follow-up data comes at a price of reduced accuracy with respect to time-estimated outcomes, as the pathology can change in less than five years, and mortality—which can be high in studies involving elderly participants—can limit the ability to examine the participants. Thus, our results provide a rough estimate and will need to be refined in future studies using other designs.

During the follow-up period of the studies included in this thesis, imaging techniques have improved, and the system for grading AMD was refined. This may have affected the results over time, as some diagnoses are now easier to make. Although this may have affected our follow-up data, excluding data obtained using new technologies would likely have compromised our analysis. Advances in medicine and technology are always occurring and are inevitable when conducting longitudinal research, and this must be considered when interpreting the results.

The use of deep learning algorithms is rapidly growing in the fields of image interpretation and disease grading[100,101]. The translation from data to clinical care and prevention is far from clear and differs from classic translation using clinical epidemiological outcomes such as relative risk. Although machine learning–based interpretation is progressing rapidly, the images must still be reviewed manually in order to achieve the highest accuracy.

Clinical implications

Due to changes in our global demographics, the number of individuals who develop AMD is only going to increase in the coming 20 years, placing considerable pressure on efforts to ensure sustainable healthcare. For example, referrals to ophthalmologists will increase, as will the number of anti-VEGF injections administered and the number of OCT scans performed. In addition, the costs associated with treating and educating the visually impaired will increase. Last, but certainly not least, the growing number of patients with late-onset disorders such as AMD will result in a loss of productivity, due in part to the high burden placed on family members and society with respect to caring for these patients.

Adopting a healthier lifestyle can significantly reduce the risk of developing certain diseases, but it may not necessarily prevent the disease altogether, particularly in the case of AMD, which is driven largely by genetic susceptibility. This underscores the importance of not only focusing on prevention, but also investing in developing new interventions that can slow or even stop the disease progression. Importantly, a therapeutic window exists during the development from unilateral AMD to bilateral AMD, as well as during the progression of GA. Ideally, specific interventions should be developed and then administered at the first signs of late AMD; however, the ultimate therapeutic goal is to develop a cure that will halt the pathology.

Preventing blindness due to AMD requires three key steps. First, the general public must be better educated regarding AMD, particularly given the current lack of awareness[102]. This education should include the potential implications of AMD, as well as the possibility of preventing the disease by adopting a healthy lifestyle. In this respect, we can draw lessons from existing awareness campaigns[103] in other parts of the world, including Australia[104], Singapore[105], Ireland, and France[106,107]. These awareness campaigns are designed to inform the public regarding AMD, which symptoms to watch for, when to visit an ophthalmologist, and how to live a healthy lifestyle[104]. In addition, relatives of patients must be made aware of the heritability of AMD and must have easy access to ophthalmic checkups starting at the age of 60. The second step involves a shift in focus from anti-VEGF treatment for neovascularization and checkups for GA toward prevention in high-risk individuals who have a family history of AMD and/or a high-risk genetic profile. This approach could include genetic testing and personalized advice based on algorithms designed to predict one's likelihood of developing AMD, as shown recently[94], and guiding individuals toward living a healthier lifestyle[108]. Lastly, well-designed clinical trials that focus on genetics and/or pathology and have sufficient follow-up duration are needed. These trials can be improved by using deep learning algorithms to predict the development and progression of GA, identify specific patient groups based on the intervention, and follow the disease progression or the effect of treatment more

accurately, as these algorithms can detect relatively small changes in disease status with high accuracy. Importantly, improving patient selection and increasing the ability to detect small changes in disease status will reduce the number of patients needed to effectively measure the effect size of the intervention and will shorten the study duration, thereby reducing study costs and accelerating the discovery process.

Future perspectives

Developing new interventions and therapies for AMD requires additional research that builds upon existing knowledge, as well as new fields of study. With respect to genetic risk, an interesting question is whether combinations of genetic variants occur more often in patient subgroups, for example a risk variant in a complement gene together with a risk variant in an extracellular matrix gene, which when combined increase the risk of developing AMD more than expected based on each separate variant. Thus, linking genetics to the phenotype and disease progression could provide further insight into the pathology of AMD, as shown previously by linking variants in *MMP9* to CNV and variants in *PRMT6* and *LSS* to the progression of GA[16,109].

In addition, the role of lifestyle on the risk of AMD should be studied further by investigating the association between epigenetic changes and AMD. For example, in addition to increased oxidative stress, smokers develop a specific DNA methylation profile that can revert back to the previous profile—at least to a certain extent—when they quit smoking[110]. Moreover, the odds ratio (i.e., relative risk) for AMD is lower among former smokers compared to current smokers[111]; an interesting question is how much of that difference can be attributed to smoking-induced epigenetic changes. Similarly, the benefits of changing to a healthier diet rich in fruit, fish, and vegetables could be investigated.

An emerging field driven by hypothesis-free research focuses on proteomics and metabolomics, giving valuable insights into the pathophysiology of AMD and revealing potential disease biomarkers. Ideally, these biomarkers could be used to detect preclinical disease or indicate whether a specific treatment is likely to be more beneficial than another. Proteomics-based studies have been performed using aqueous humor obtained from patients with GA or CNV, revealing for example proteins related to inflammation and the extracellular matrix[112,113]. Additional studies have also been performed focusing on the RPE and blood samples, showing promising results with respect to putative biomarkers such as proteins and metabolites related to the complement pathway, lipids, and oxidative stress[30]. Imaging biomarkers are also being investigated and could serve as a valuable addition for detecting preclinical disease and/or disease progression.

Recent studies have examined the role of the gut microbiome in AMD, as this microbiome facilitates the uptake of nutrients from food and can affect metabolism and inflammation[114,115]. Thus, altering the gut microbiome in patients with AMD or in high-risk individuals by consuming prebiotics and probiotics can be explored as a potential new therapeutic strategy.

The ability to detect AMD will likely be facilitated by advances in automated grading of retinal imaging data. Although developments in this field are rapidly emerging, the results are currently not sufficiently accurate to become fully independent without the need for human involvement. Further advances in artificial intelligence–based solutions will facilitate the diagnosis of early AMD, thereby helping to prevent or delay the progression to late AMD. Thus, these technological breakthroughs should be embraced by the clinical community and advanced using the growing knowledge base of ophthalmologists.

Ultimately, treating AMD will require an interdisciplinary approach using the latest technologies to detect the disease in its earliest stages. Mathematicians and epidemiologists should work together to develop and optimize algorithms that predict the risk of AMD, and healthcare professionals should work to educate the general public regarding the benefits of living a healthy lifestyle, quitting smoking (or never starting). Finally, scientists and clinicians should work together to develop and administer new therapies for AMD.

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SUMMARY

Age-related macular degeneration (AMD) is a common disease in elderly leading to visual impairment and blindness. It is a multifactorial disease driven by aging, genetics, and environmental factors. On clinical examination, the early stage of AMD is characterized by drusen and pigmentary alterations in the macular area of the retina. The late stages of the disease are choroidal neovascularization (CNV) and geographic atrophy (GA); the first can be treated with anti-VEGF injections, but not cured. The aim of this thesis was to answer questions related to the genetics and epidemiology of AMD. The general introduction and the outline of this thesis are described in **Chapter 1**.

Prevalence

Chapter 2.1 discusses the prevalence of AMD in Europe by using fourteen population based studies from the E3 Consortium. We show that the prevalence of early AMD increases steeply from 3.5% at 55-59 years old to 17.6% in those aged 85 years and older. Late AMD increased up to 9.8% in people aged 85 years and over. Improved visual acuity was observed after the introduction of anti-VEGF therapy in cases with CNV. In addition, we noticed a decreasing age-specific prevalence over time, possibly due to healthier lifestyles. Even with decreasing age-specific prevalences, the number of AMD patients is going to increase in the next twenty years due to the aging population.

Progression

In **Chapter 2.2** we show that a 27 to 68% of the people with unilateral late AMD become bilateral within five years. For any AMD, these numbers are smaller; one fifth to one quarter progresses from any unilateral AMD to bilateral any AMD. We also show that the risk factors for progression are similar to developing the disease; genetic predisposition, smoking and large drusen area with pigmentary changes. The progression of GA is discussed in **Chapter 3.1** in which we show that GA lesions grow on average 0.97mm^2/year, with a very wide variety in the speed of growth, but bilateral growth is highly correlated. 60.4% of the extra foveal GA reaches the fovea within five years. This affects visual acuity and inevitably self-sustainability of these patients such as the ability to drive a car. Half of the people with bilateral GA die visually impaired. In **Chapter 3.2** the growth of GA is predicted using deep learning software. We show that the shape of the lesion is predictive of its growth, factors related to a round shape seem to grow slower. Bilateral growth is not associated with faster growth. We show that computer algorithms are a fast and accurate way to asses and predict growth, a tool that can be used for clinical trials and predictive estimates.

Genetics

The importance of rare protein altering genetic variants not only in families but also in the population is shown in **Chapter 4.1**. Whole exome sequencing data was analyzed in almost 3,000 participants from the Rotterdam Study I and EUGENDA. We observed protein altering variants in *COL8A1* more often in AMD cases (1.0%) than in controls (0.4%), independent from the previously found variant in this gene. We showed that the protein of this gene is located in the Bruchs membrane. As part of the extra-cellular matrix, alterations can affect the integrity of the membrane.

We explored the genetic risk for AMD in the population in **Chapter 4.2**. We showed that the biggest differences in minor allele frequencies and population attributable risks are in *ARMS2* and variants of the complement pathway. When constructing a genetic risk score (GRS) as a measure of total genetic load for AMD, the score increases with the severity of AMD. Constructing GRS per pathway showed that the complement pathway together with *ARMS2* contributed to genetic susceptibility in 90% of the late AMD cases, suggesting that the vast majority of AMD has complement as a molecular driver.

Lipids and lifestyle

In **Chapter 5.1** we investigated the association of lipids with AMD in almost 31,000 Europeans. We showed that a high HDL-cholesterol level and a low triglycerides level were associated with AMD. A high HDL-cholesterol level was associated with large drusen. In a subgroup of the Rotterdam Study I we explored the associations with lipid subfractions and observed that extra-large HDL subfractions were associated with AMD. Genetic variants in the lipid pathway, previously associated with AMD, also showed association with systemic lipids. The *CETP* variant rs17231506 and *APOE* variant rs429358 were both positively associated with HDL-cholesterol, while both *LIPC* variants (rs2043085 and rs2070895) were negatively associated.

Chapter 5.2 focusses on the effect of diet on the development of AMD in two population based cohort studies (Rotterdam Study I and Alienor-3C). A score was constructed to investigate the adherence to a Mediterranean diet. This showed that participants with high adherence to this diet reduced their risk of incident late AMD with 41%. This finding was further underlined in **Chapter 4.2**, where a lifestyle score was constructed based on smoking and a diet rich in fish, fruits, and vegetables. Those with the most favorable lifestyle had almost half the risk of late AMD in all genetic risk groups. This points out the enormous impact of lifestyle on the outcome of AMD genes.

Finally in **Chapter 6** the main findings are placed in the context of my view on the pathophysiology of AMD or on AMD related matters such as prevalence, progression and prediction. This chapter also considers ideas for future research and implications.

SAMENVATTING

Leeftijdsgebonden macula degeneratie (LMD) is een veel voorkomende ziekte onder ouderen die zorgt voor slechtziendheid en blindheid. Het is een multi-factoriele ziekte die veroorzaakt wordt door veroudering, genetica en omgevingsfactoren. Bij funduscopie wordt het vroege stadium van LMD gekarakteriseerd door drusen en pigmentveranderingen in de macula. De late stadia van de ziekte zijn choroidale neovascularisaties (CNV) en geografische atrofie (GA); de eerste kan behandeld worden met anti-VEGF injecties maar niet worden genezen. Het doel van dit proefschrift was om vragen te beantwoorden die gerelateerd zijn aan de genetica en epidemiologie van LMD. De algemene introductie en de opzet van het proefschrift zijn beschreven in **Hoofdstuk 1**.

Prevalentie

Hoofdstuk 2.1 beschrijft de prevalentie van LMD in Europa op basis van veertien populatie studies uit het E3 Consortium. Wij laten hierin zien dat de prevalentie van vroege LMD sterk toeneemt met de leeftijd; van 3.5% op 55-59 jarige leeftijd tot 17.6% in mensen van 85 jaar en ouder. De prevalentie van late LMD stijgt tot 9.8% in mensen van 85 jaar en ouder. In de mensen met CNV zagen wij een verbeterde visus na de introductie van anti-VEGF. Daarnaast zagen we dat, over de tijd, de leeftijdsgebonden prevalentie afnam. Mogelijk door een toename in gezondere levensgewoontes. Zelfs met de afnemende leeftijdsspecifieke prevalenties gaat het absolute aantal mensen met LMD toenemen in de komende twintig jaar door de vergrijzende populatie.

Progressie

In **Hoofdstuk 2.2** laten we zien dat 27 tot 68% van de mensen met unilaterale late LMD binnen 5 jaar ook het tweede oog late LMD krijgt. Voor alle vormen van LMD samen is dit aantal wat kleiner; een vijfde tot een kwart krijgt een bilaterale vorm van LMD binnen 5 jaar. We laten ook zien dat de risico factoren voor progressie gelijk zijn aan de risicofactoren voor het krijgen van de ziekte; namelijk genetische predispositie, roken, grote drusen oppervlakte, en pigment veranderingen. De progressie van GA wordt beschreven in **Hoofdstuk 3.1**, waarin we laten zien dat GA laesies gemiddeld met 0.97mm^2/jaar toenemen. Er is een grote spreiding in de groeisnelheid, maar de groei van GA in bilateraal aangedane patiënten is sterk gecorreleerd. Een groot deel (60.4%) van de laesies die buiten de fovea zit, groeien binnen 5 jaar in de fovea. Dit heeft effect op het zicht en op de zelfredzaamheid van deze patiënten, bijvoorbeeld of ze nog kunnen autorijden. De helft van de mensen met bilaterale GA is slechtziend als ze overlijden. In **Hoofdstuk 3.2** wordt de groei van GA voorspeld middels deep learning software. We tonen aan dat de vorm van de laesie de groeisnelheid voorspelt. Factoren gerelateerd

aan hoe rond de laesie is, zijn gerelateerd aan een langzamere groei. Bilaterale GA zorgt niet voor snellere groei. We laten zien dat computer algoritmes snel en precies de groei van een laesie kunnen inschatten, dit kan gebruikt worden voor klinische studies en om een prognose te geven voor de patiënt.

Genetica

Zeldzame eiwit veranderende genetische varianten zijn niet alleen belangrijk in families met LMD maar ook in de populatie, dat laten we zien in **Hoofdstuk 4.1**. Hiervoor analyseerden wij de sequenties van alle exonen uit het genoom van bijna 3000 deelnemers uit de Rotterdam Studie I en EUGENDA. Wij vonden dat eiwit coderende varianten in het *COL8A1* gen vaker voorkomen in mensen met LMD (1.0%) dan in controles (0.4%). Deze bevinding is onafhankelijk van de eerder gevonden variant in dit gen dat geassocieerd is met LMD. Wij toonden aan dat het COL8A1 eiwit in het Bruchs membraan zit. Omdat het een onderdeel is van de extra-cellulaire matrix kunnen veranderingen in het eiwit de integriteit van het membraan aantasten.

Ook onderzochten wij het genetische risico voor LMD in de populatie, **Hoofdstuk 4.2**. We lieten zien dat *ARMS2* en varianten gerelateerd aan complement activiteit het meest voorkomen in patiënten met LMD. Ook het populatie risico op LMD kan voornamelijk toegeschreven worden aan deze varianten. Wij maakten een genetische risico score op basis van alle varianten uit het tot dan toe grootste genome wide association study. Deze score nam toe met elk stadium van LMD. Daarnaast hebben wij ook genetische scores gemaakt per "pathway", dit liet zien dat in 90% van de late LDM patiënten de complement "pathway" samen met *ARMS2* bijdraagt aan de genetische aanleg om LMD te krijgen. Dit suggereert dat de meerderheid van de patiënten met late LMD complement heeft als een moleculaire stimulans.

Lipiden en leefstijl

In **Hoofdstuk 5.1** hebben wij de associatie van lipiden met LMD onderzocht in bijna 31.000 Europeanen. We lieten zien dat een hoog HDL-cholesterol in het bloed en een laag triglyceriden niveau geassocieerd waren met LMD. Een hoog HDL-cholesterol was ook geassocieerd met grote drusen. In een subgroep van de Rotterdam Study I onderzochten wij de associaties met lipiden subfracties en zagen wij dat de extra grote HDL-subfracties geassocieerd waren met LMD. Genetische varianten in de lipiden "pathway" die eerder al geassocieerd waren met LMD, lieten ook een associatie met de lipiden in het bloed zien. De *CETP* variant rs17231506 en *APOE* variant rs429358 waren beiden positief geassocieerd met HDL-cholesterol, terwijl beide *LIPC* varianten (rs2043085 en rs2070895) negatief waren geassocieerd.

Hoofdstuk 5.2 focust op het effect van dieet op de ontwikkeling van LMD in twee populatie cohorten (Rotterdam Study I en Alienor-3C). Een dieet score werd gemaakt om vast te kunnen stellen in hoeverre iemand een Mediterraans dieet volgt. Dit toonde aan dat deelnemers die het Mediterraanse dieet goed volgden het risico op het ontwikkelen van late LMD verkleinden met 41%. Deze bevinding werd verder ondersteund door **Hoofdstuk 4.2** waar een score voor levensstijl werd gemaakt op basis van roken en het eten van groente, fruit en vis. De personen die de meest gunstige levensstijl hadden reduceerden het risico op late LMD met ongeveer de helft, ongeacht de genetische belasting voor LMD. Dit laat zien wat de enorme impact van de levensstijl is op de uitkomst van LMD geassocieerde genen.

Tenslotte, in **Hoofdstuk 6** worden de bevindingen uit dit proefschrift besproken in de context van mijn kijk op de pathofysiologie van LMD of LMD gerelateerde zaken zoals de prevalentie, progressie van de ziekte en predictie. Ook worden de implicaties en de overwegingen voor toekomstig onderzoek besproken.

7

EPILOGUE

PHD PORTFOLIO

Summary of PhD training

Name PhD student:	Johanna Maria Colijn
Erasmus MC Departments:	Ophthalmology and Epidemiology
Research school:	NIHES
PhD-period:	2014-2021
Supervisors:	prof.dr. C.C.W. Klaver and prof.dr.ing C.M. van Duijn, copromotor Dr M.A. Meester-Smoor

PhD training	Year	Workload (ECTS)
Courses		
Master of Health Sciences, Genetic Epidemiology (NIHES)	2014-2017	70
Biomedical English Writing and Communication	2016	4.0
Scientific integrity	2015	0.3
Seminars, symposia and workshops		
Ingenuity Pathway Analysis (IPA)	2016	0.3
5th European Eye Epidemiology Workshop, London, UK (oral presentation)	2015	1.0
6th European Eye Epidemiology Workshop, Lisbon, Portugal (oral presentation)	2016	1.0
7th European Eye Epidemiology Workshop, Mainz, Germany (oral presentation)	2017	1.0
8th European Eye Epidemiology Workshop, Rotterdam, Netherlands (oral presentation)	2018	1.0
9th European Eye Epidemiology Workshop, Belfast, UK (oral presentation)	2019	1.0
10th European Eye Epidemiology Workshop, online, (oral presentation)	2021	0.3
Weekly seminars, department of Ophthalmology, Erasmus MC	2014-today	
Research seminars, department of Epidemiology, Erasmus MC	2014-2018	
Macula Degeneratie symposium, Rotterdam	2014	0.1
Eye Nutrition Meeting, Barcelona, Spain	2015	0.4
EYE-RISK Big Data symposium (oral presentation)	2016	1.0
Myopie Controle Symposium	2016	0.1
EYE-RISK Lipids meeting (oral presentation)	2017	1.0
EYE-RISK risk scores, Applied Mathematics Kings College London (oral presentation)	2017	0.6
EYE-RISK Bordeaux Website development	2018	0.4

PhD training	Year	Workload (ECTS)
EYE-RISK kick-off meeting, London, UK	2015	0.6
EYE-RISK meeting, Mallorca, Spain (oral presentation)	2015	1.0
EYE-RISK meeting, Seattle, USA (oral presentation)	2016	1.0
EYE-RISK meeting, Barcelona, Spain (oral presentation)	2016	1.0
EYE-RISK meeting, Tubingen, Germany (oral presentation)	2017	1.0
EYE-RISK meeting Rotterdam, Netherlands (oral presentation)	2017	1.0
EYE-RISK meeting Rotterdam, Netherlands (oral presentation)	2019	1.0
EYE-RISK meeting Rotterdam, Belfast (oral presentation)	2019	1.0
National Conferences		
Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering, Groningen	2015	0.6
NOG jaarvergadering, Maastricht (oral presentation)	2016	1.0
NOG jaarvergadering, Maastricht (oral presentation)	2017	1.0
Dutch Ophthalmology PhD Students (DOPS) conference, Nijmegen (poster presentation)	2015	1.0
DOPS conference, Nijmegen (poster presentation)	2016	1.0
DOPS conference, Nijmegen (poster presentation)	2017	1.0
International conferences		
6th International DOG-Symposium on AMD (oral presentation)	2015	1.0
EURETINA winter meeting (oral presentation)	2016	1.0
ARVO Annual Meeting, Seattle, USA (poster presentation)	2016	1.0
ARVO Annual Meeting, Baltimore, USA (oral presentation)	2017	1.0
Young researchers vision camp, Beuron, Germany (invited oral presentation)	2017	1.0
ARVO Annual Meeting, Hawaii, USA (oral presentation)	2018	1.0
Other		
Chair of genetics session, DOPS conference, Nijmegen	2017	
Reviewer for several international journals - Scientific Reports - Ophthalmic Epidemiology - IOVS - European Journal of Ophthalmology	2014-today	
Supervising students	2014-2017	

LIST OF PUBLICATIONS

Publications on which this thesis is based:

1. **Colijn JM***, Buitendijk GHS*, Prokofyeva E, Alves D, Cachulo ML, Khawaja AP, Cougnard-Grégoire A, Merle BMJ, Korb C, Erke MG, Bron A, Anastasopoulos E, Meester-Smoor MA, Segato T, Piermarocchi S, de Jong PTVM, Vingerling JR, Topouzis F, Creuzot-Garcher C, Bertelsen G, Pfeiffer N, Fletcher AE, Foster PJ, Silva R, Korobelnik JF, Delcourt C, Klaver CCW; EYE-RISK consortium; European Eye Epidemiology (E3) consortium. Prevalence of Age-Related Macular Degeneration in Europe: The Past and the Future. Ophthalmology. 2017 Dec.

2. Joachim N, **Colijn JM**, Kifley A, Lee KE, Buitendijk GHS, Klein BEK, Myers CE, Meuer SM, Tan AG, Holliday EG, Attia J, Liew G, Iyengar SK, de Jong PTVM, Hofman A, Vingerling JR, Mitchell P, Klaver CCW, Klein R, Wang JJ. Five-year progression of unilateral age-related macular degeneration to bilateral involvement: the Three Continent AMD Consortium report. Br J Ophthalmol. 2017 Sep.

3. **Colijn JM**, Liefers B, Joachim N, Verzijden T, Meester-Smoor MA, Biarnés M, Monés J, de Jong PTVM, Vingerling JR, Mitchell P, Sánchez CI, Wang JJ, Klaver CCW, EyeNED Reading Center, EYE-RISK Consortium. Progression of Geographic Atrophy from first diagnosis to life's ending: results from population studies *Accepted in JAMA Ophthalmology*

4. Liefers B, **Colijn JM**, González-Gonzalo C, Verzijden T, Wang JJ, Joachim N, Mitchell P, Hoyng CB, van Ginneken B, Klaver CCW, Sánchez CI. A Deep Learning Model for Segmentation of Geographic Atrophy to Study Its Long-Term Natural History. Ophthalmology. 2020 Feb 15.

5. Corominas J*, **Colijn JM***, Geerlings MJ, Pauper M, Bakker B, Amin N, Lores Motta L, Kersten E, Garanto A, Verlouw JAM, van Rooij JGJ, Kraaij R, de Jong PTVM, Hofman A, Vingerling JR, Schick T, Fauser S, de Jong EK, van Duijn CM, Hoyng CB, Klaver CCW, den Hollander AI. Whole-Exome Sequencing in Age-Related Macular Degeneration Identifies Rare Variants in COL8A1, a Component of Bruch's Membrane. Ophthalmology. 2018 Apr 26.

6. **Colijn JM**, Meester-Smoor MA, Verzijden T, de Breuk A, Silva R, Merle BMJ, Cougnard-Grégoire A, Hoyng CB, Fauser S, Coolen A, Creuzot-Garcher C, Hense HW, Ueffing M, Delcourt C, den Hollander AI, Klaver CCW, EYE-RISK Consortium Genetic risk, lifestyle, and AMD in Europe. The EYE-RISK consortium Ophthalmology. 2020 Nov 27.

7. **Colijn JM**, Hollander AID, Demirkan A, Cougnard-Grégoire A, Verzijden T, Kersten E, Meester MA, Merle BMJ, Papageorgiou G, Ahmad S, Mulder MT, Costa MA, Benlian P, Bertelsen G, Bron A, Claes B, Creuzot-Garcher C, Erke MG, Fauser S, Foster PJ, Hammond CJ, Hense HW, Hoyng CB, Khawaja AP, Korobelnik J, Piermarocchi S, Segato T, Silva R, Souied EH, Williams KM, van Duijn CM, Delcourt C, Klaver CCW; E3 Consortium and EYE-RISK Consortium. Increased High Density Lipoprotein-levels associated with Age-related Macular degeneration. Evidence from the EYE-RISK and E3 Consortia. Ophthalmology. 2018 Oct 10.

8. Merle BMJ, **Colijn JM**, Cougnard-Grégoire A, de Koning-Backus APM, Delyfer MN, Kiefte-de Jong JC, Meester-Smoor M, Féart C, Verzijden T, Samieri C, Franco OH, Korobelnik JF, Klaver CCW, Delcourt C; EYE-RISK Consortium. Mediterranean Diet and Incidence of Advanced Age-Related Macular Degeneration: The EYE-RISK Consortium. Ophthalmology. 2018 Aug 13.

Other publications:

9. Thee E, **Colijn JM**, Meester-Smoor MA, Verzijden T, Ueffing M, den Hollander AI, Klaver CCW on behalf of the E3 consortium and the EYE-RISK project The phenotype of the ARMS2 risk variant in age-related macular degeneration. The E3&EYE-RISK consortium. *In preparation*

10. Zuber V, **Colijn JM**, Klaver C, Burgess S. Selecting likely causal risk factors from high-throughput experiments using multivariable Mendelian randomization. Nat Commun. 2020 Jan 7.

11. Biarnés M, **Colijn JM**, Sousa J, Ferraro LL, Garcia MOD, Verzijden T, Meester-Smoor MA, Delcourt C, Klaver CCW, den Hollander AI, Lengyel I, Peto T, Monés J, on behalf of the EYE-RISK Consortium Genotype- and phenotype-based subgroups in geographic atrophy secondary to age-related macular degeneration. The EYE-RISK Consortium Ophthalmology Retina 2020 May 1.

12. Mutlu U, **Colijn JM**, Ikram MA, Bonnemaijer PWM, Licher S, Wolters FJ, Tiemeier H, Koudstaal PJ, Klaver CCW, Ikram MK. Association of Retinal Neurodegeneration on Optical Coherence Tomography with Dementia: A Population-Based Study. JAMA Neurol. 2018 Jun 25.

13. Joachim N*, Kifley A*, **Colijn JM**, Lee KE, Buitendijk GHS, Klein BEK, Myers C, Meuer SM, Tan AG, Flood V, Schoufour JD, Franco OH, Holliday EG, Attia J, Liew G, Iyengar SK, de Jong PTVM, Hofman A, Vingerling JR, Mitchell P, Klein R, Klaver CCW, Wang JJ, Joint Contribution of Genetic Susceptibility and Modifiable Factors to the Progression of Age-Related Macular Degeneration over 10 Years: The Three Continent AMD Consortium Report Ophthalmol Retina. 2018 July.

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