AGE-RELATED MACULAR DEGENERATION
FROM RISK PROFILES TOWARD PREDICTION MODELS

GABRIËLLE H.S. BUITENDIJK

Age-related Macular Degeneration:
from risk profiles towards prediction models

Gabriëlle H.S. Buitendijk

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Chapter 1.1

General introduction

GENERAL INTRODUCTION

This thesis comprises studies on the common eye disorder age-related macular degeneration (AMD). In the introduction, I will first focus on the structures of the eye most important to the disease, then explain the state of knowledge prior to my studies, and subsequently discuss the outline of my thesis.

Anatomy and physiology of the eye

To create vision, light needs to travels through many structures of the eye: cornea, anterior chamber, through the pupil, lens, vitreous body, and retina (Figure 1). Photoreceptor cells in the retina absorbs light photons by the visual pigment and translate these first in a biochemical message and then in an electrical signal that can stimulate the succeeding neurons of the retina. This signal is subsequently transmitted through the optic nerve to the occipital cortex of the brain via the visual pathway.

The retina consists of two primary layers: the neurosensory retina and the retinal pigment epithelium (RPE). Directly underneath the RPE lies Bruch's membrane, which separates the RPE from the choriocapillaris and the choroid, which are vascular structures that nourishes the retina. The deepest outer fibrous layer of the eye is the sclera, which functions as the external shell of the eye.

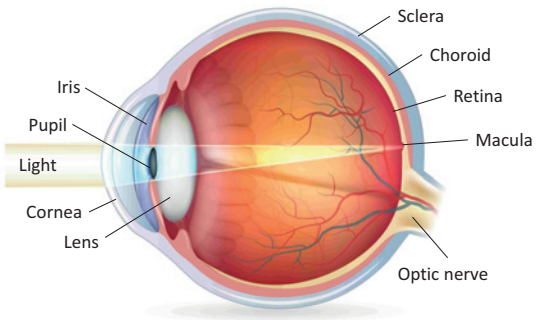

FIGURE 1 - Anatomy of the human eye (Figure adapted from www.biology-questions-and-answers.com/images/Human-Eyes.jpg).

The neurosensory retina consists of eight layers, including the photoreceptor cell layer (Figure 2). There are two types of photoreceptor cells, rods and cones. Cones have optimal function in bright light and are responsible for fine resolution, spatial resolution, and color vision, while rods function optimal in dim-light and sense contrast, brightness, and motion. A yellow colored pigment is highly concentrated in the ganglion cells, cone axons, and Muller cells of the optical center of the posterior pole, called macula lutea. This macular pigment consists of lutein, zeaxanthin and meso-zeaxanthin, and these pigments offer protection to the retina by absorbing hazardous ionizing blue and ultraviolet light. Fine detailed and color vision is mainly acquired in the fovea, which is located in the center of the macula. Underneath the photoreceptor layer lies the RPE, a monolayer of epithelial cells that are in close contact with the photoreceptors. This cell layer has many functions that are critical to the visual process, such as phagocytosis of photoreceptor outer segments, synthesis of interphotoreceptor matrix, absorption of light, vitamin A metabolism, and transport of other molecules. If the RPE becomes dysfunctional, like in AMD, the neurosensory retina will not function properly and vision is disturbed.

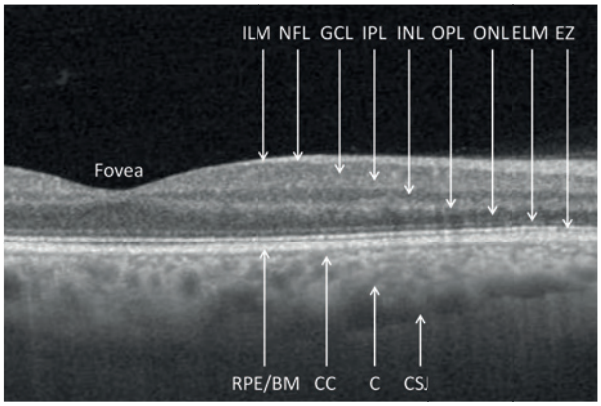

FIGURE 2 - Layers of the human retina. The following layers are disclosed in the optical coherence tomography image of a human macula, from top to bottom: internal limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), ellipsoid zone (EZ), retinal pigment epithelium/Bruch's membrane (RPE/BM), choriocapillaris (CC), choroid (C), choroid sclera junction (CSJ).

Age-related macular degeneration

AMD is a chronic disease of the macula and is the leading cause of blindness in elderly, particularly in those of European descent. About 30-50 million persons are affected in the world and this number is expected to increase dramatically with the exponentially aging population.[1] AMD can be stratified in two severity stages: early and late AMD (Figure 3). Early AMD is mostly asymptomatic and characterized by drusen (sub-RPE deposits), reticular pseudodrusen (deposits above the RPE), and pigmentary changes. Late AMD is the visual threatening end-stage of AMD which can be subdivided into geographic atrophy (dry AMD) and choroidal neovascularization (wet AMD). Geographic atrophy is characterized by atrophy of the RPE and neurosensory retina. In choroidal neovascularization abnormal new blood vessels from the choroid grow into the retina, which can easily bleed, leak fluids and cause fibrovascular scarring.[2] Having signs of early AMD will increase the risk of developing late AMD. The larger the area, size and the type of drusen and pigmentary changes, the higher the risk of developing late AMD.[3,4]

Epidemiology

Disease frequency

The prevalence of early and late AMD has been established in several parts of the world. For Caucasians in the United States of America, over 40 years of age, the overall prevalence of early AMD (indicated by large drusen) showed much more variation than that of late AMD; overall prevalence of early AMD was estimated at 6.12%; prevalence of late AMD at 1.47%.[5] Age-specific prevalence for both early and late AMD increased with advancing age in all ethnicities. As for incidence figures, the 10-year risk of late AMD was estimated to be virtually nil for those aged 55 years, but increased to 11% for those aged 80 years and older.[6] Studies in Europe show comparable estimates for both early and late AMD.[4,6,7]

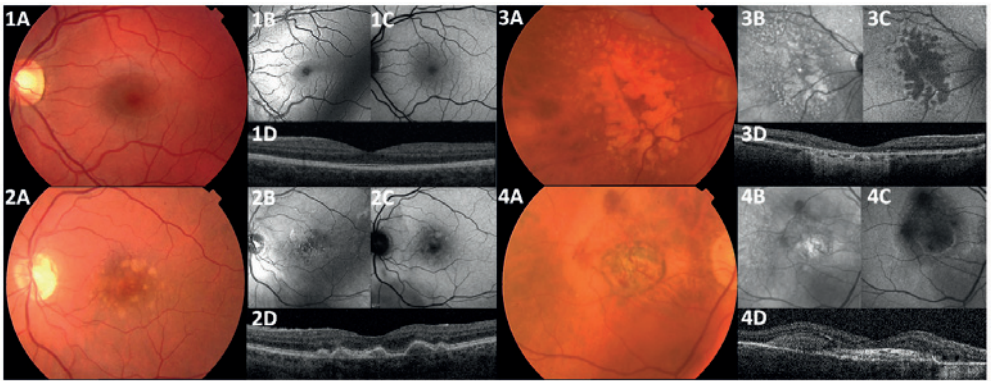

FIGURE 3 - Stages of age-related macular degeneration (AMD) disclosed using different imaging methods. The different images represent 1) normal retina without signs of AMD, 2) early AMD, 3) late AMD – subtype geographic atrophy, and 4) late AMD – subtype choroidal neovascularization, and were obtained using A) fundus color photographs, B) near-infrared imaging, C) fundus autofluorescence, D) optical coherence tomography.

Risk factors

Many risk factors for AMD have been identified; including age, smoking, higher body mass index and increased serum complement activation. Cardiovascular risk factors such as hypertension and lipid levels may be associated with an increased risk of AMD.[6,8-10] Intake of antioxidants, in particular lutein and zeaxanthin, from diet and supplements have been associated with a protective effect.[11-13] Positive family history has been known for many years to be an important risk factor[14], indicating that genetic predisposition plays an significant role. Genetic studies have confirmed this hypothesis and found several genes associated with AMD. These genes include commonly occurring risk variants. The most important and frequently replicated variants are located in the *CFH, ARMS2, C2/CB* and *C3* genes. Smaller effects were found in several other genes, including *APOE*, *LIPC, LPL, CETP, ABCA1* and *TIMP3* genes.[6] The known variants in these associated genes do not fully explain the heritability of AMD, which has been determined to be between 65-70%.[15,16] Unknown variants, gene-gene interactions, and gene-environment interactions could explain the missing heritability in AMD.

Treatment

Preventive measures like cessation of smoking, healthy diet and supplementation of anti-oxidants are at this time the only option for those with early and dry AMD. For wet AMD, anti-vascular endothelial growth factor treatment aimed at cessation of blood vessel growth and impermeability of endothelium is currently the only available treatment.[17,18] However, this is not a definite cure and will improve and maintain visual acuity only for a limited period of time.[19] Many trials are currently testing newly developed medication focusing on other disease mechanisms in early and dry AMD.

Imaging techniques

AMD features can be disclosed using various imaging techniques (Figure 3). Ophthalmoscopy can be used to identify the majority of disease features, and the obtained image can be captured using color fundus photography (CFP). Other techniques can help identify lesions with an increased sensitivity. Confocal scanning laser ophthalmoscopy can be used for non-invasive imaging like fundus autofluorescence (FAF) and near-infrared imaging (NIR). FAF uses short wavelengths (blue

light) to excite lipofuscin in the retina, while NIR uses wavelengths in the infra-red spectrum and reveals structures based on light reflectivity. CFP, FAF and NIR imaging are two dimensional, while optical coherence tomography (OCT) provides a cross-sectional image of the retina and thereby reveals the Z-axis. OCT distinguishes the retinal layers based on differences in light reflectivity. In particular anatomical changes and their relation to location can easily be identified using this non-invasive technique.

Gaps of Knowledge

AMD is a chronic complex disease of a predominantly unknown etiology for which limited treatment options are available. In general, the late stages of the disease ultimately cause severe visual impairment provided that the patient lives long enough. Identification of new risk factors and in depth comprehension of interactions between risk factors may help elucidate the intricate pathogenesis of this disease. Current epidemiologic studies often do not allow valid extrapolation of findings, because they are too small or lack appropriate study designs to obtain conclusive results. They barely go beyond the study of single relationships. In order to expand current genetic and epidemiologic knowledge, large, well designed longitudinal studies, international collaborations using harmonized methodology and grading protocols, and applications of new imaging techniques are needed. Implementation of these strategies will help gain more homogeneous phenotypes and a plethora of risk factors for analysis. Improved risk profiling is likely to lead to better identification of high-risk groups, and may offer new leads for therapy. Validated prediction models can be used in the clinic for patient management. These gaps of knowledge and the inference that arises by filling them was the driving force behind this thesis.

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Chapter 1.2

Aims of this thesis

AIMS OF THIS THESIS

This thesis describes epidemiologic and genetic studies on AMD. The major goals of our studies were:

Chapter 2: to assess the frequency and impact of AMD in Europeans
Chapter 3: to evaluate the merit of new imaging techniques for diagnosis and risk assessment
Chapter 4: to identify new environmental risk factors for AMD
Chapter 5: to investigate genetic associations and gene-environment interactions in large study populations
Chapter 6: to assess the predictive value of risk factors associated with AMD

STUDY POPULATIONS ON WHICH THIS THESIS IS BASED

We have addressed these aims in various study populations. We joined efforts with study populations outside the Netherlands to enlarge the study population in order to improve statistical power for analysis, and enable risk calculations for relatively rare exposures in a relatively infrequent disease outcome (Late AMD occurs in ~1-2% of the elderly population). A short description of these studies and consortia is listed below.

The Rotterdam Study[1] – a population-based study which started in 1990. The current study consists of three cohorts and includes almost 15,000 participants living in the suburb Ommoord, a district of Rotterdam, the Netherlands. The aim of this study is to identify risk factors in cardiovascular, endocrine, hepatic, neurological, ophthalmic, psychiatric and respiratory diseases in elderly people. (Chapter 2, 3 & 4)

BRAMD Study[2] –a double blind randomized-controlled multicenter trial comparing the efficacy of intravitreal bevacizumab versus ranibizumab in persons diagnosed with exudative AMD. (Chapter 4)

Three Continent AMD Consortium[3] – a consortium of three population-based studies representing three continents. The entire study population consists of almost 24,500 participants which are derived from the Beaver Dam Eye study, from Beaver Dam, Wisconsin, United States of America; the Blue Mountains Eye study from Sydney, Australia, and our own Rotterdam Study. Epidemiology of AMD is their main focus. (Chapter 5 & 6)

European Eye Epidemiology (E3) Consortium[4] –a collaborative network of 41 studies across Europe, including the Rotterdam Study, providing ophthalmologic data on 170,000 European participants. The aim of this consortium is to increase understanding of eye diseases and vision loss in Europe. (Chapter 2)

AMD Gene Consortium[5] – a worldwide collaborative study analyzing the genetics of AMD, involving 18 studies (including the Rotterdam Study) with over 17,000 cases of late AMD and 60,000 controls. (Chapter 5)

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Chapter 2.1

Prevalence of age-related macular degeneration in Europe: the past and the future

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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 1990-2013 using the European Eye Epidemiology (E3) consortium, and made projections for the future.

Design Meta-analysis of prevalence data.

Participants A total of 42080 individuals aged 40 years of age and older participating in fourteen population-based cohorts from ten countries in Europe.

Methods AMD was diagnosed 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 early and 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 14.9-21.5 million, and for late AMD between 3.9-4.8 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 two 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 two subtypes; geographic atrophy (GA) and choroidal neovascularization (CNV).[2]

Worldwide estimates approximated that 30 to 50 million people are affected by AMD[3,4], and these numbers are expected to increase over time due to the aging population.[1,5-9] Although multiple small studies have assessed the prevalence of AMD and its relation with 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 due to improved diagnostic procedures and hence earlier diagnosis, and the introduction of anti-vascular endothelial growth Factor (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 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 of the E3 consortium 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 older provided summary data. Participants were recruited between 1990 and 2013 from the following countries: Estonia, France, Germany, Greece, Italy, Northern Ireland, Norway, Netherlands, Spain and Portugal[19,20], United Kingdom (Table 1).[16] 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 worse eye was used for classification of the person. To harmonize classification of AMD, studies were graded or re-classified 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 in 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, Thessaloniki Eye Study and 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 three studies only participated in the Late AMD analysis.

Visual impairment

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

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Projection of AMD

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 onwards and for late AMD 65 years and onwards. The projected prevalences were 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, for ten-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 (StataCorp. 2013. Stata Statistical Software: Release 13, version 13.1. College Station, TX: StataCorp LP.) using metaprop. Graphical outputs were constructed with GraphPad Prism 7 (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

TABLE 1 - Description of the European Eye Epidemiology Consortium studies included in the meta-analysis

Region		Study	Data collection period	Total participants	Age Range yrs	Median age group	Gender, % Male	European ethnicity %	Crude prevalence % Early AMD	Crude prevalence % 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
	Thessaloniki	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; EPIC = European Prospective Investigation into Cancer; EUREYE = European Eye Study; GHS= Gutenberg Health Study; POLA= Pathologies Oculaires Liées à l'Age Study; PAMDI = Prevalence of Age-Related Macular Degeneration in Italy; RS= Rotterdam Study.

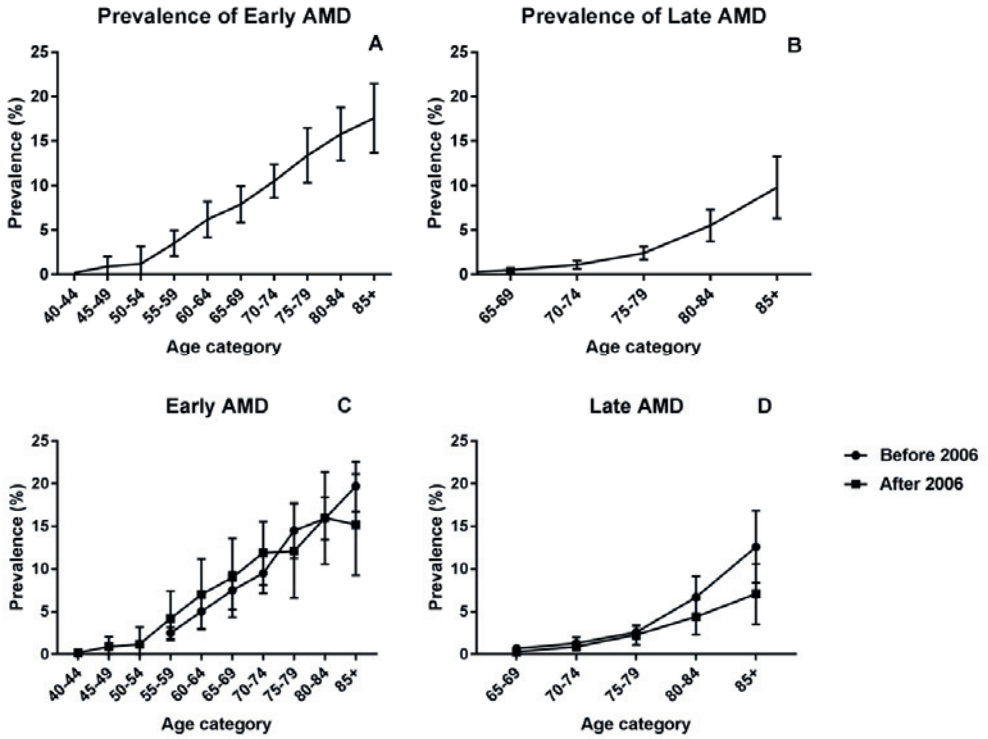

FIGURE 3 - Meta-analysis of Early (A) and Late (B) AMD in Europe per age category for the participating studies**.** Meta-analysis of the prevalence of Early (C) and Late (D) AMD before and after 2006

RESULTS

The total study population included in this analysis comprised of 42,080 individuals from 14 studies with a median age group 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) (Figure 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 III age-category 85+). 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 the 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+ years (Figure 3A, and Table 2, available at www.aaojournal.org). The prevalence of late AMD rose from virtually naught in the youngest age group to 9.8% (95% CI 6.3-13.3) for those in the highest age group (Figure 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 splitting the E3 consortium into studies conducted before and after 2006. The prevalence of early AMD before and after 2006 seemed to rise similarly. For late AMD, a trend of decreasing prevalence was observed for the higher age categories after 2006 (Figure 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). 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 (Figure 4A and B).

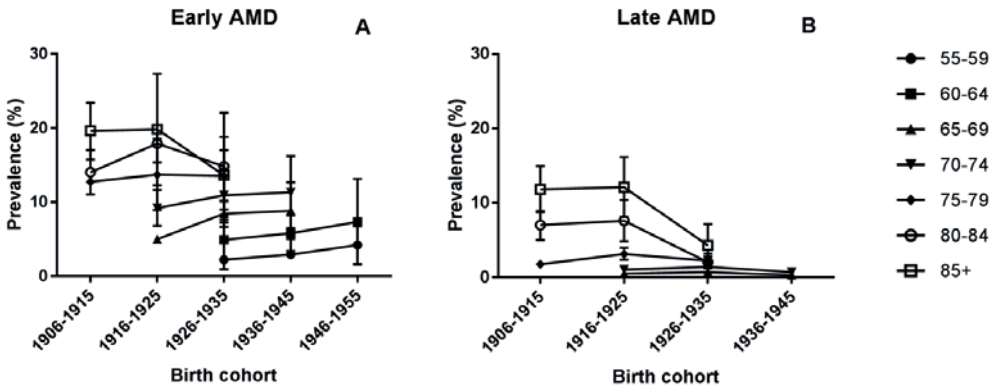

FIGURE 4 - Meta-analysis of Early (A) and Late (B) AMD in Europe by ten year birth cohort.

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 (Figure 5A and B, available at www.aaojournal.org). This category shows a trend for a higher prevalence in women compared to men, although confidence intervals overlap.

To address differential distribution of AMD in Europe, we stratified studies according to three regions defined by the United Nations[24]. In older individuals, we observed a trend towards 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]) (Figure 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]) (Figure 6B, available at www.aaojournal.org). More detailed analyses showed that a frequency difference was only present for CNV (Figure 6C and D, available at www.aaojournal.org), however, confidence intervals of the regional differences overlapped.

Visual consequences

As most countries implemented anti-VEGF therapy for CNV from 2006 onwards, 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 onwards, 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 (Figure 7A). This improvement was also observed for the number of bilaterally visually impaired persons; 120 out of 345 (34.8%) before 2006 to 75 out of 259 (28.9%, $P = 0.13$) after 2006. The largest drop was seen for people aged 80 years and older; 85 out of 175 (48.6%) before 2006 to 46 out of 132 (34.8%, $P = 0.016$) after 2006 (Figure 7B).

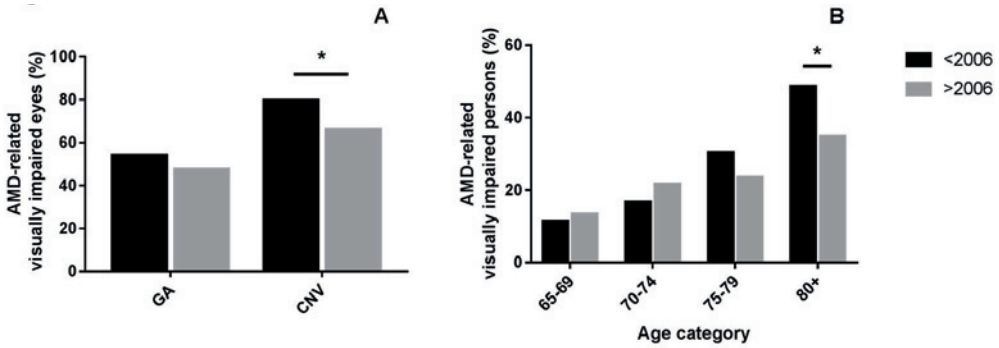

FIGURE 7 - A) Proportion of visually impaired eyes within each subgroup of Late AMD. The proportion of visually impaired eyes remained the same for GA (47.6%, $P = 0.40$), but dropped to 66.2% ($P = 0.026$) for CNV after 2006. B) Proportion of persons with Late AMD with bilateral visual impairment before and after 2006 ($P = 0.016$).

* Corresponds with $P < 0.05$.

Projections of AMD 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.

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 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 by 2040 (Figure 8).

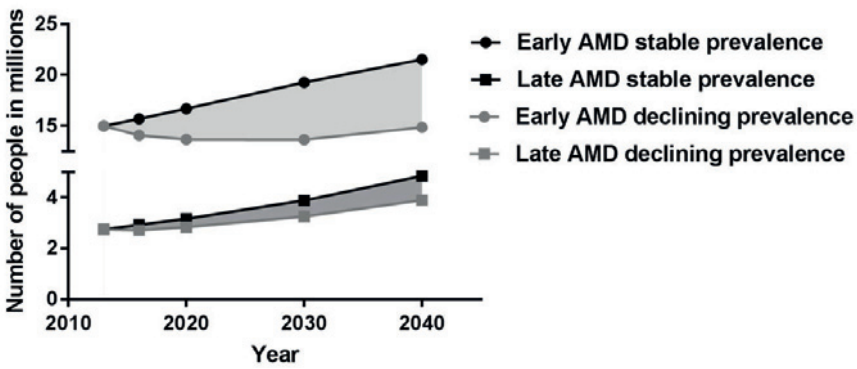

FIGURE 8 - Predicted number of persons with AMD in years 2013-2040 as a function of two prevalence scenarios

DISCUSSION

AMD prevalence and its time trends

Our study provides insight in the prevalence of both early and late AMD in Europe. Based on meta-analyzed data from fourteen 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 from European descent living in other continents.[3,25]

Our data showed a trend towards 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 [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. (Figure 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 AMD in the North. Our findings are in concordance with the results earlier published by the Tromsø Eye Study[40], but 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 to the other studies, these findings might be more legitimate. No consistent differences were observed for West and South 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 onwards.[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 which were initiated after the introduction of anti-VEGF have undoubtedly contributed to the reduction of visual loss, as they made elderly more aware of the symptoms and stimulated prompt therapy.[48,49]

Projections of AMD in Europe

It is unclear whether the prevalence rates of AMD will decrease even more in the coming years, but an increase is not likely to be expected. Therefore, we performed projections of the estimated number of AMD affected persons until the year 2040 based on two different scenarios: one based on a stable prevalence and one 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 USA 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 2.9 million in 2016 to 4.0 million by 2040. Considering the declining rates of smoking and implementation of healthier diets in elderly, 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 golden 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, auto-fluorescence 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 showed a slight decrease. Nevertheless, we observed a significant reduction in the proportion of visually impaired eyes due 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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Chapter 2.2

Visual consequences of refractive errors in the general population

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ABSTRACT

Objective To study the frequency and causes of visual impairment in relation to refractive error.

Design Population-based cohort study

Participants: A total of 6,597 participants from Rotterdam Study I (baseline and 4 follow-up examinations) and of 2,579 participants from Rotterdam Study II (baseline and 2 follow-up examinations), all aged 55+ years, were included.

Methods Participants underwent an extensive ophthalmic examination including best-corrected visual acuity and objective refraction, fundus photography, visual field perimetry, and OCT imaging of macula and optic disc. We calculated cumulative risks and odds ratios of visual impairment for various refractive error categories, determined causes by using all screening information as well as medical records.

Main Outcome Measures Unilateral and bilateral low vision (WHO criteria: VA <0.3 and VA ≥0.05; US criteria: VA <0.5 and VA ≥0.1) and blindness (WHO criteria: VA <0.05; US criteria: VA<0.1).

Results Cumulative risks of visual impairment ranged from virtually 0 in all refractive error categories at age 55 to 9.5% (standard error (se) 0.01) for emmetropia, 15.3% (se 0.06) for high hyperopia to 33.7% (se 0.08) for high myopia, at age 85. The major causes of visual impairment in highly hyperopic persons were age-related macular degeneration (AMD), cataract, and combined causes (each 25%); in highly myopic persons the major cause was myopic macular degeneration (38.9%). The major causes of visual impairment for the other refractive error categories were AMD and cataract. Compared to emmetropes, high myopes had a significantly increased risk of visual impairment; those with ≤-6 D & ≥-10 D had a risk of OR 3.4 (95% CI 1.4-8.2) of visual impairment; those with <-10 D had OR 22.0 (95% CI 9.2-52.6).

Conclusion Of all refractive errors, high myopia has the most severe visual consequences. Irreversible macular pathology is the most common cause of visual impairment in this group.

INTRODUCTION

Refractive errors - both myopia and hyperopia - are very common human eye disorders and leading causes of visual impairment worldwide.[1-3] Myopia is characterized by an elongation of the eye, and is accompanied by structural changes of the retina and choroid.[4] These changes can lead to potentially blinding complications such as myopic macular degeneration, open-angle glaucoma and retinal detachment.[5,6] Although all myopic eyes are at risk for complications,[4,7,8] highly myopic eyes, i.e., -6 diopters (D) or worse, are particularly at risk to develop functional blindness at a relatively young age. Hyperopia (farsightedness), by contrast, is a condition in which the eye is shortened. For this refractive error category, the risks of visual impairment are less well studied, but it is known that persons with hyperopia have a higher risk of amblyopia, strabismus and closed-angle glaucoma.[9] An association with age-related macular degeneration (AMD) has also been described.[10]

Although numerous studies have addressed population frequencies of low vision and blindness none have focused on visual loss as a function of the full spectrum of refractive errors. In addition, frequency of causes of blindness and low vision specified per refractive error category have not been described until now. Given the current rise in prevalence of this trait[11-13], this information can be useful for clinicians, patients, and researchers, and will increase awareness of the visual consequences of refractive errors.

In this study, we investigated the frequency and causes of blindness and low vision stratified for various refractive error categories in 2 independent cohorts of the population-based prospective Rotterdam Study.

MATERIAL AND METHODS

Study population

The rationale and design of the Rotterdam Study have been described in detail elsewhere.[14] In brief, this prospective population-based follow-up study focuses on chronic ophthalmologic, neurologic, cardiovascular, and locomotor diseases in middle aged and elderly participants living in Ommoord, a city district of Rotterdam, the Netherlands. Baseline data for the ophthalmic part were collected between 1991 and 2002 and follow-up examinations were performed at 2-4 years (Figure 1). A total of 99% of study participants were from European descent. For this analysis, we included 9,176 participants from two independent cohorts of the Rotterdam Study. The first is Rotterdam Study I (RS-I): 6,597 participants aged 55 years and older. Baseline examinations took place between 1990 and 1993, and four follow-up examinations were performed in 1993-1995, 1997-1999, 2002-2004, and 2009-2011 (Figure 1). The second cohort is Rotterdam Study II (RS-II), which included 2,579 participants aged 55 years and older. Baseline examinations took place in between 2000 and 2002, and two follow-up examinations were performed in 2004-2005 and 2011-2012 (Figure 1). Persons with bilateral pseudophakia or aphakia at baseline with no knowledge of prior refractive error were excluded (n = 278). From these two cohorts, 9,176 participants with data on refractive error and visual acuity at baseline were eligible for the current analysis. The Medical Ethics Committee of the Erasmus University had approved the study protocols, and participants had given a written informed consent in accordance with the Declaration of Helsinki.

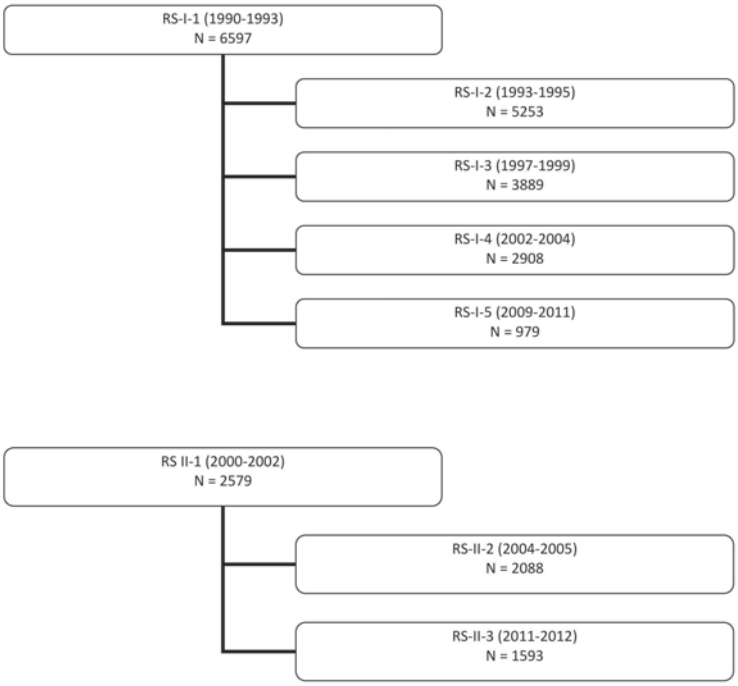


FIGURE 1 - Participation and ophthalmological measurement from each examination interval of the Rotterdam Study

Abbreviations: RS = Rotterdam Study

Ophthalmic data collection

All patients underwent an extensive ophthalmological examination. Visual acuity was measured using the Lighthouse Distance Visual Acuity Test, a modified Early Treatment Diabetic Retinopathy Study chart.[15] To evaluate the best-corrected visual acuity (BCVA), refraction was initially obtained after objective autorefraction (Topcon RM-A2000, Topcon Optical Company, Tokyo, Japan), and then subjectively adjusted. Screening of visual fields was performed using a modified 76-point supra-threshold perimetry test (Humprey Visual Field Analyzer, Zeiss, Oberkochen, Germany); visual field defects were confirmed by Goldmann perimetry. After pupil dilation, optic nerve head and macular area imaging was performed using simultaneous stereoscopic photography (Topcon TRC-SS2, Topcon optical Company, Tokyo, Japan), followed by a 35° film fundus camera (Topcon TRV-50VT, Topcon Optical Company, Tokyo, Japan). During the last examination rounds, RSI-4, RSI-5 and RSII-2 respectively, a Topcon digital 35° colour fundus camera (Topcon TRC 50EX with a Sony DXC-950P digital camera; 0.44 megapixel) was used.

Low vision and blindness were classified according to the WHO criteria[16] and US criteria:

Low Vision: WHO: VA < 0.3 and ≥ 0.05; US: VA < 0.5 and ≥ 0.1 Blindness: WHO: VA < 0.05; US: VA < 0.1

For participants with bilateral blindness and low vision, three clinical investigators (C.C.W.K, V.J.M.V., and K.T.W.) reached consensus on the final determination of the cause of visual impairment after reviewing all screening information, fundus transparencies, and medical information provided by ophthalmologists.

Statistical analysis

Mean spherical equivalent (SE) was calculated according to the standard formula (SE=spherical value + ½*cylinder). When data from only one eye were available, the SE of this eye was used. Mean SE was categorized into high myopia (≤ -6 diopters (D)), moderate myopia (>-6D & ≤-3D), low myopia (<-3D & ≤-0.75D), emmetropia (>-0.75D & <0.75D), low hyperopia (≥0.75D & <3D), medium hyperopia (≥ 3D & <6D), and high hyperopia (≥ 6D), using previously defined criteria.[17] High myopia and high hyperopia were further classified as high myopia <-10 D and ≤-6 D & ≥-10 D and high hyperopia >10 D and ≥6 & ≤10 D. Visual acuity at last visit was categorized into normal vision, low vision, and blindness according to WHO and US criteria as defined above. For bilateral visual impairment, BCVA was used. Unilateral visual impairment was defined as visual impairment in only one eye.

We calculated the number of cases with bilateral and unilateral blindness and low vision as a percentage of the total number of all cases with blindness and low vision at the endpoint of the study per refractive error category.

Cumulative risks of bilateral visual impairment were estimated per refractive error category using Kaplan Meier product limit analysis. We assigned the age at diagnosis of blindness or low vision as the mean between the examination at which this endpoint was first observed and the previous examination. For participants who did not develop the endpoint, we used age at last examination for censoring. Participants who died or were lost to follow-up were counted at the time of the last examination. All participants aged 85+ years were censored at age 85 years to maintain unbiased estimates. Cumulative risks per refractive error category were compared with the log-rank test of equality (Mantel-Cox) using emmetropia as the reference group.

Causes of bilateral blindness and low vision (according to the WHO criteria) were categorized, and frequencies of causes were calculated per refractive error category. We calculated mean age at diagnosis of bilateral visual impairment per refractive error category, and calculated mean spherical equivalent per refractive error category, stratified by normal vision, low vision and blindness. Statistical differences at nominal *P*-value <0.05 between refractive error categories for age at diagnosis and between visual acuity categories for mean SE were calculated using Student's T test. The risk of blindness and low vision (reference normal vision) for persons with various refractive error categories (reference emmetropia) was assessed using logistic regression analysis with blindness and low vision as a combined outcome, correcting for age and sex. We used SPSS version 20.0.0 (SPSS Inc.) for all analyses.

RESULTS

General characteristics of the 9,176 study participants are presented in Table 1. At baseline, we identified 98 prevalent cases (1.1%) with bilateral low vision and 29 cases (0.3%) with bilateral blindness (WHO criteria). After a mean follow-up time of 9.6±6.1 years, respectively 62 and 26 persons developed incident bilateral low vision and blindness. Subjects in RS-I were generally younger (mean age at inclusion 69.0 versus 64.1 years) and were less myopic (mean SE 0.84 vs. 0.47 D) than those in

RS-II, due to a cohort effect described in our previous work.[17] The characteristics of all cases who had received a diagnosis of bilateral low vision or blindness by the end of the study can be found in Table 2 (WHO-criteria) and Table 3 (US-criteria; available at http://aaojournal.org).

The distribution of bilateral and unilateral blindness and low vision (WHO criteria) per refractive error category is shown in Figure 2. The high myopia group showed the highest percentage of bilateral blindness (9.6%) and low vision (25.0%). Persons from the high hyperopia group had the highest proportion of unilateral blind eyes (39.1%).

TABLE 1 - Characteristics of the study population

	Rotterdam Study I	Rotterdam Study II	Total
N at baseline	6597	2579	9176
Follow-up time, mean ± sd (yrs)	9.8 ± 6.0	8.9 ± 2.9	9.6 ± 6.1
Baseline age, mean ± sd (yrs)	69.0 ± 9.0	64.1 ± 7.4	67.6 ± 8.8
Sex, % men	41.0	45.0	42.0
Visual acuity at last measurement - WHO criteria			
Bilaterally visually impaired subjects	2.2	0.5	1.7
Bilaterally blind subjects	0.8	0.1	0.6
Unilaterally visually impaired subjects	6.1	3.8	5.5
Unilaterally blind subjects	3.4	2.1	3.0
Visual acuity at last measurement - US criteria			
Bilaterally visually impaired subjects	6.6	1.8	5.2
Bilaterally blind subjects	1.1	0.1	0.8
Unilaterally visually impaired subjects	12.5	4.8	10.3
Unilaterally blind subjects	3.4	2.2	3.1
Refractive error			
Spherical equivalent, mean ± sd (D)	0.84 ± 2.54	0.47 ± 2.49	0.74 ± 2.53
High myopia ≤-6D	1.8	1.8	1.8
Medium myopia >-6D & ≤-3D	5.2	7.3	5.8
Low myopia -3D & ≤-0.75D	9.5	12.8	10.4
Emmetropia >-0.75D & <0.75D	25.4	26.9	25.8
Low hyperopia ≥0.75D & <3D	44.4	41.1	43.4
Medium hyperopia ≥3D & <6D	12.3	9.2	11.4
High hyperopia ≥6D	1.5	1.0	1.3

Numbers displayed are percentages, unless stated otherwise.
Abbreviations:; D = diopters, sd = standard deviation, WHO = World Health Organization

TABLE 2 - Characteristics of subjects with bilateral blindness, low vision and normal vision (WHO criteria)

	Bilaterally blind subjects	Bilaterally visually impaired subjects	Subjects with bilateral visual acuity ≥ 0.3
	N = 55	N = 160	N = 8961
Age of onset, mean ± sd (yrs)	78.1 ± 11.3	79.7 ± 10.1	-
Range age of onset	55.4-96.3	56.4-106.2	-
Sex, % men	31.0	53.0	51.0
Spherical equivalent, mean ± sd (D)	-0.05 ± 5.78	0.09 ± 4.03	0.75 ± 2.45
Range spherical equivalent	-19.13; 12.25	-15.31; 8.50	-19.13; 15.13
High myopia ≤-6D, %	9.1	8.1	1.7
Moderate myopia >-6D & ≤-3D, %	5.5	7.5	5.7
Low myopia -3D & ≤-0.75D, %	10.9	10.6	10.4
Emmetropia >-0.75D & <0.75D, %	16.4	19.4	26.0
Low hyperopia ≥0.75D & <3D, %	38.2	38.1	43.6
Moderate hyperopia ≥3D & <6D,%	12.7	13.8	11.4
High hyperopia ≥6D, %	7.3	2.5	1.3

Abbreviations: D = diopters, sd = standard deviation;

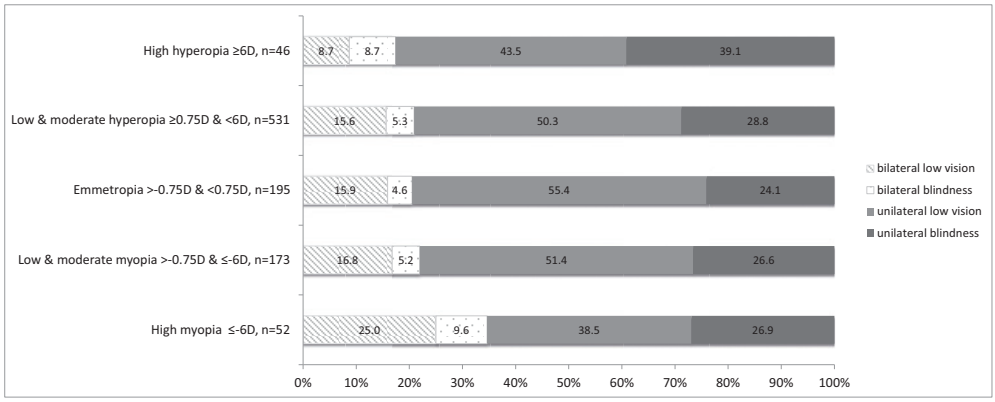


FIGURE 2 - Bar graph showing the distribution of bilateral and unilateral blindness and low vision (World Health Organization criteria) per refractive error category. The number of cases with bilateral and unilateral blindness and low vision is shown as a percentage of the total number of prevalent and incident cases with blindness and low vision per refractive error category. For data of visual impairment as a percentage of the entire population, see Table 1.

Abbreviations: D = diopters.

Kaplan Meier curves showing cumulative risk of visual impairment for high myopia, emmetropia and high hyperopia appear in Figure 3. Cumulative risks ranged from virtually 0 in all refractive error categories at age 55 to 9.5% (standard error (se) 0.01) for emmetropia, 15.3% (se 0.06) for high hyperopia to 33.7% (se 0.08) for high myopia, at age 85. Risks for high myopia started to increase gradually before age 60; for high hyperopia between 60 and 70 years of age, whereas emmetropia

showed a more steady increase in risk from the age of 70. Cumulative risks for persons with low to moderate myopia and hyperopia were not significantly different from persons with emmetropia (*P* 0.09; *P* 0.78). Kaplan Meier curves for US criteria can be found in Figure 4 (available at http://aaojournal.org). Cumulative risks ranged from virtually 0 in all refractive error categories at age 55 to 28.9% (standard error (se) 0.03) for emmetropia, 41.5% (se 0.08) for high hyperopia to 59.2% (se 0.08) for high myopia, at age 85.

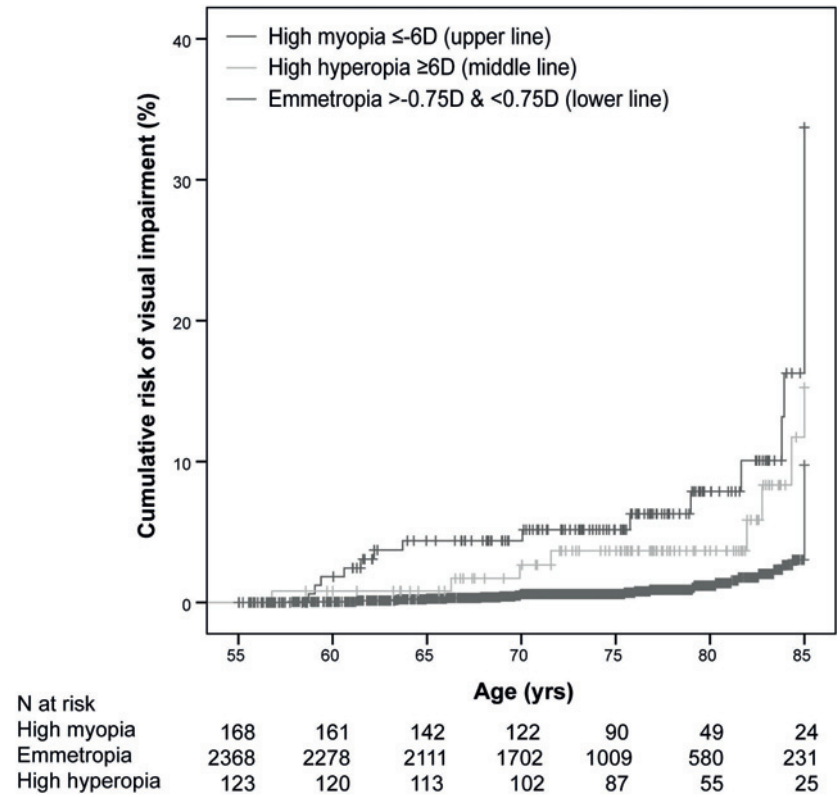
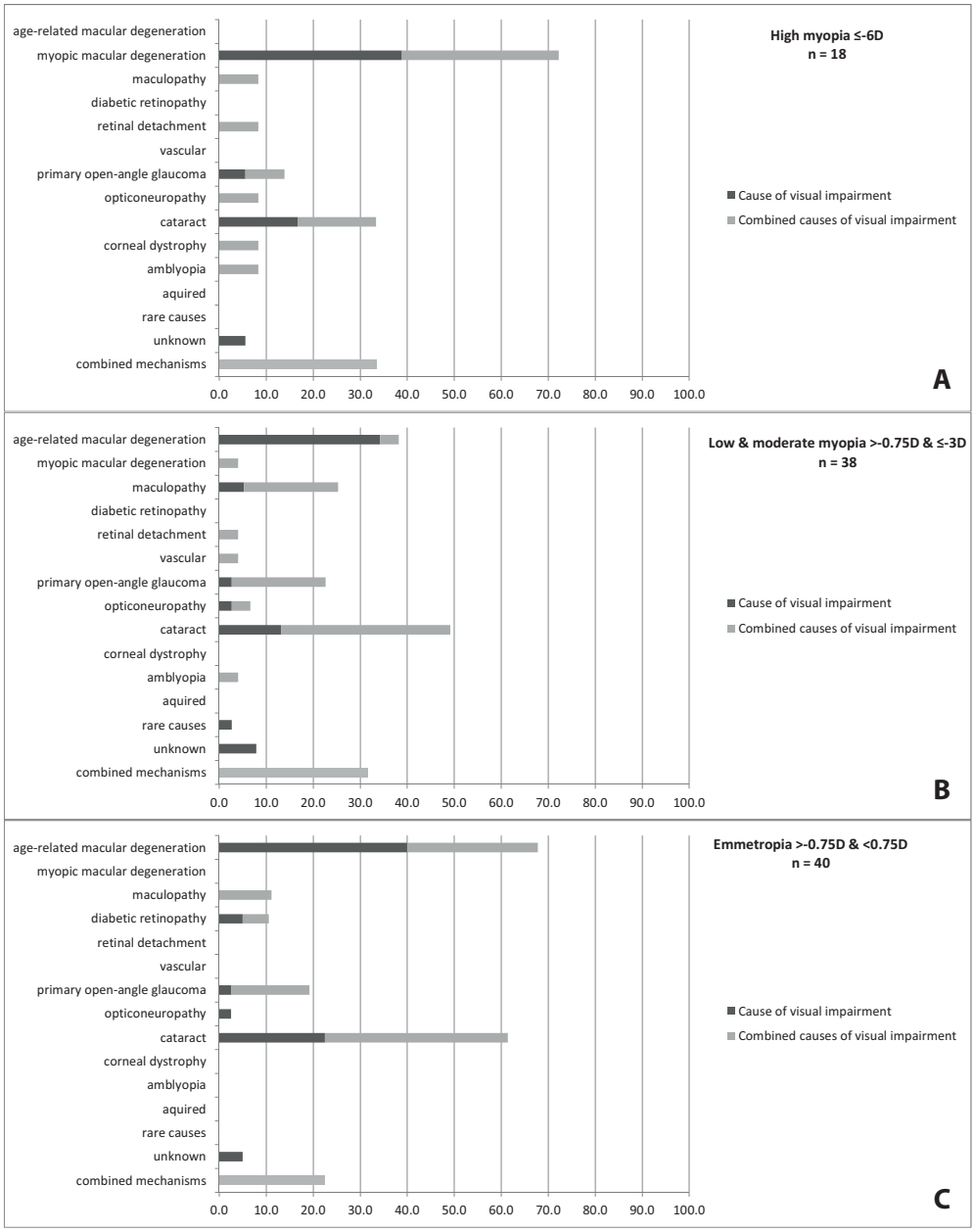


FIGURE 3 - Cumulative risk of bilateral visual impairment (WHO criteria) stratified for high myopia, emmetropia and high hyperopia. The X-axis represents the age at diagnosis for all cases with blindness or low vision at the end point of the study and age at last examination for non-cases; the Y-axis represents the cumulative risk for persons with visual impairment. The number of persons at risk at each decade per refractive error category is presented below.

Abbreviations: D = Diopters.

The causes of bilateral visual impairment according to WHO criteria are provided in Figure 5. For persons with emmetropia, low to moderate myopia, and low to moderate hyperopia, AMD was the major cause of visual impairment. The most important cause of visual impairment in high myopic persons was myopic macular degeneration (38.9%), followed by combined mechanisms (33.3, including myopic macular degeneration, cataract, and maculopathy) and cataract (16.7%). In highly

hyperopic persons, the major causes of visual impairment were AMD (25%), cataract (25.0%), and combined causes (25%, including amblyopia, corneal dystrophy, cataract, maculopathy, age-related macular degeneration).



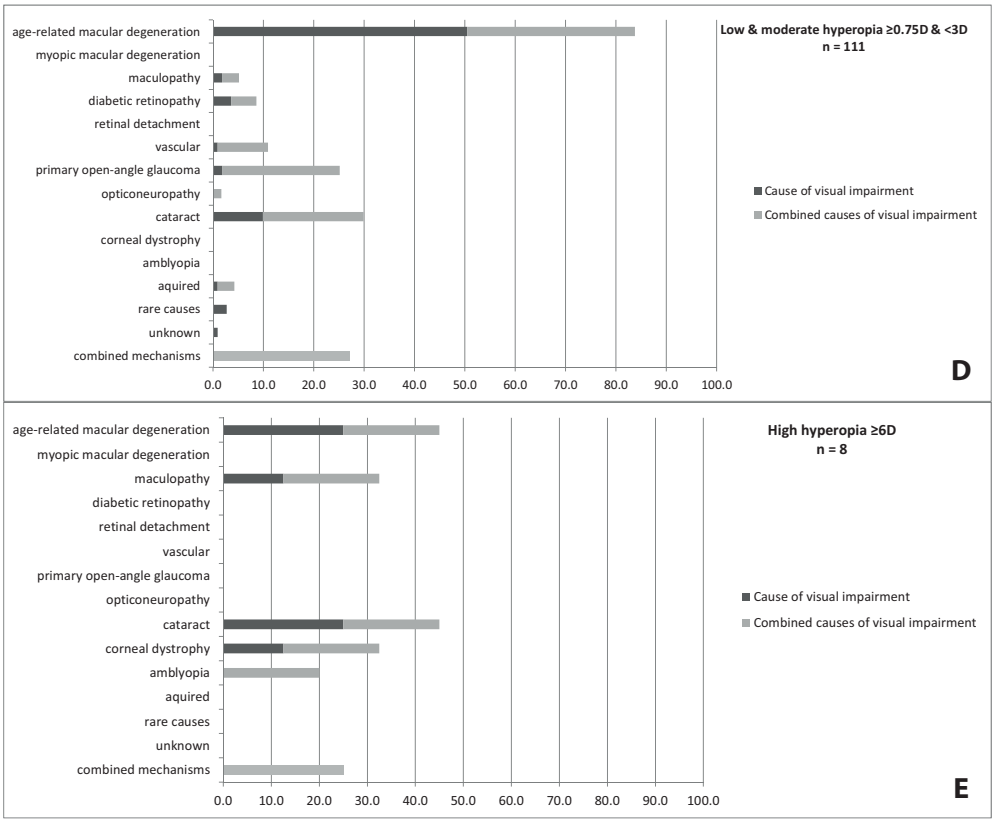


FIGURE 5 - Causes of bilateral low vision and blindness (WHO criteria) stratified by refractive error category. The X-axis represents the percentage of visual impairment explained by the different causes mentioned on the Y-axis stratified by subjects with high myopia (A), low & moderate myopia (B), emmetropia (C), low & moderate hyperopia (D) and high hyperopia (E).

Abbreviations: D = diopters, NA = not applicable, WHO = World health organization.

The age at diagnosis of visual impairment for persons with high myopia (75.4±13.7 yrs) and high hyperopia (75.4±10.0 yrs) was slightly, albeit non significantly, lower than for persons with emmetropia (80.3±11.0 yrs; *P* =0.152; and *P* = 0.250, respectively).

Boxplots of the SE distribution among visually impaired participants with high myopia and high hyperopia are provided in Figure 6. Among the high myopes, persons with bilateral blindness (SE=-15.25 ± 5.23 D; *P* = 0.034) and low vision (SE=-10.91 ± 2.57 D, *P* = 0.0036) had a significantly lower SE (i.e. more myopia)than persons with normal vision (SE=-8.25 ± 2.59 D). In the other refractive error groups, no statistical SE differences were found between the visual acuity categories (data not shown). The risk of blindness or low vision for high myopes versus emmetropes was OR 3.4 (95% CI 1.4-8.2, *P*<0.001) for those with SE ≤-6 D & ≥-10 D, and OR 22.0 (95% CI 9.2-52.6, *P* 0.01) for those with SE <-10 D (Figure 7).

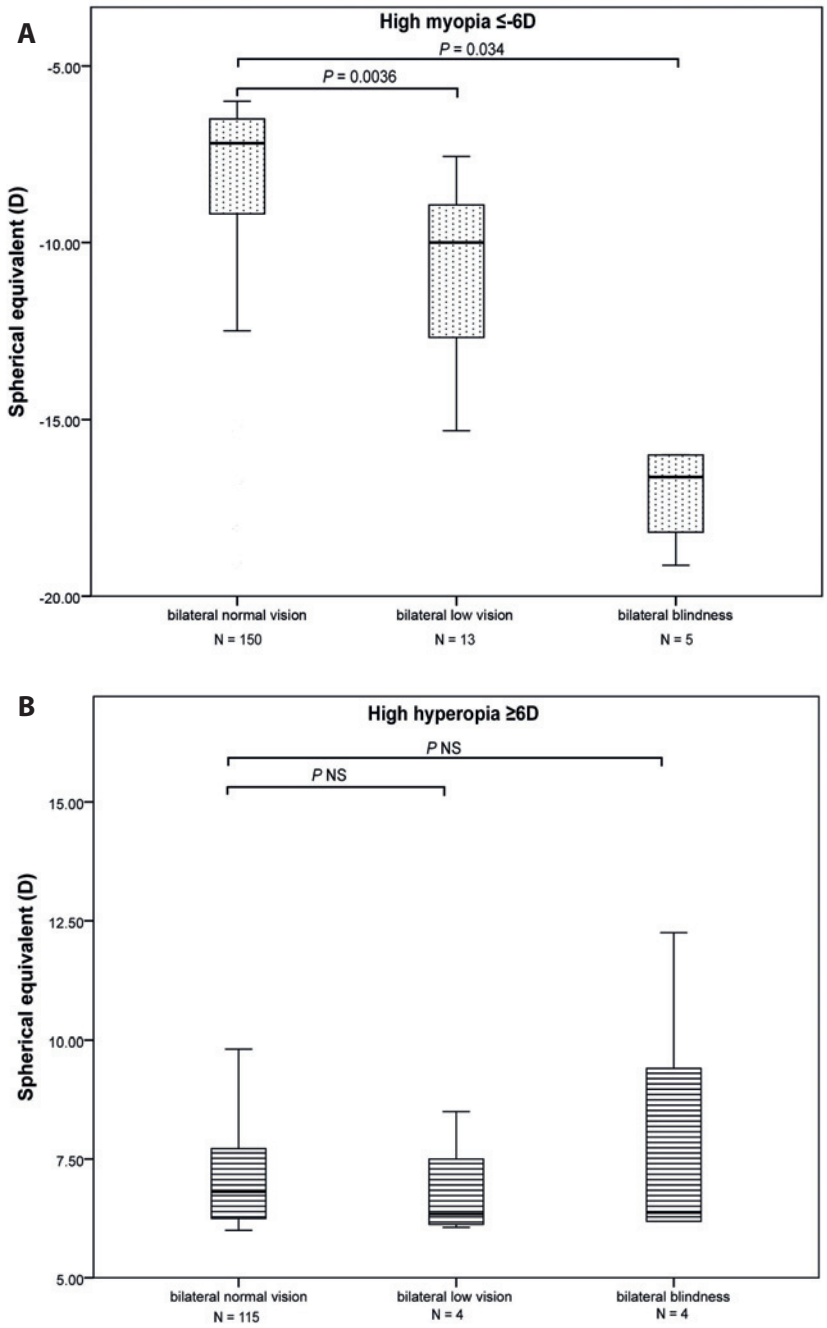


FIGURE 6 - Distribution of spherical equivalent in relation to bilateral visual impairment (WHO criteria) in participants with high myopia (A) and high hyperopia (B). Boxplots for the distribution of spherical equivalent stratified by bilateral blindness, bilateral low vision and normal vision (based on WHO criteria) for all subjects with high myopia SE ≤-6D (A) and high hyperopia ≥ 6 D (B).

Abbreviations: D= diopters, NS = not significant, WHO= World Health Organisation

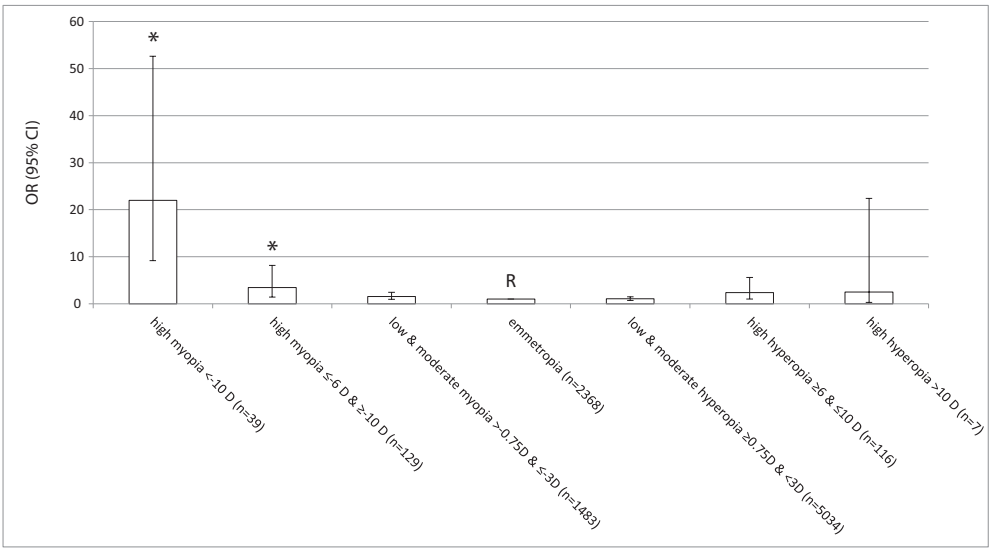


FIGURE 7 - Risk of bilateral blindness and low vision (WHO criteria) for high myopes. Odds ratios with 95% confidence intervals for blindness and low vision (reference normal vision) for persons with high myopia with SE ≤-6 D & ≥-10 D or SE <-10 D (reference emmetropia) are shown.

Abbreviations: CI = Confidence Interval, D = Diopters, OR = Odds Ratio, R = reference, WHO = World Health Organisation; *, statistically significant OR (P < 0.05) compared to the reference group

DISCUSSION

In this population-based longitudinal study, we found that persons with high myopia (SE≤-6D) and high hyperopia (SE≥6D) are at a considerable risk of visual impairment. Blindness or low vision occurred in one third of high myopes, mainly caused by myopic macular degeneration. The blind and visually impaired persons within this group had a higher degree of myopia than the ones with normal vision; the risk of visual impairment was 22x increased for those with refractive errors of -10 D or more when compared to emmetropes, but also 6x higher than those with refractive errors between -6 and -10 D. The onset of visual impairment appeared to occur at a younger age; cumulative risks of visual impairment rose at least 10 years earlier for high myopia (before the age of 60) than for emmetropia (from the age of 70). For high hyperopia, we found that 15% of the persons were visually impaired. Causes of visual impairment for this refractive error showed more variation, and included cataract, AMD, and combined mechanisms.

This is the first report on refractive error specific risks and causes of blindness and low vision. Strengths of this study are the investigation of the full spectrum of refractive errors, the large sample size, and the lengthy follow-up time. In addition, our ophthalmic examination was extensive, which enabled an accurate determination of the cause of visual impairment. Our study also had limitations. Despite the large sample size, subgroup numbers were relatively small, jeopardizing precision of the risk estimates. Also, we focused on causes of visual impairment in persons with bilateral low vision, and did not study those with unilateral visual impairment. Therefore, we may have missed refractive error specific causes of visual impairment that are more likely to occur unilaterally, such

as rhegmatogenous retinal detachment[18], and closed-angle glaucoma[19] in (high) myopes and amblyopia in (high) hyperopes. Lastly, selective non-participation of disabled persons may have caused an underestimation of the frequencies of blindness and visual impairment.

Our findings are in line with results from previous studies that showed a highly increased risk for high myopes (SE≤-6D).[8,20] Except for one person with moderate myopia, all persons with myopic macular degeneration were highly myopic. Those with extreme refractive error values of ≥ -10 D had the highest risk of visual impairment. We could not confirm the previously described mildly increased risk of visual impairment for persons with low to moderate myopia.[8,20]

Myopia is a growing public health problem since the prevalence is rapidly increasing, particularly in East Asia.[11-13] With time, this trend is predicted to occur in other regions as well, and the increase in myopia and high myopia prevalence will result in a higher frequency of complications. Atropine eyedrops can currently be used as a therapy in children to slow the progression of myopia and decrease the final adult value of myopic refractive error.[21] Our data underscore the objective of this therapy, because realisation of a lower refractive error will lower the risk of visual impairment later in life.[22]

It was previously shown that clinically significant pathological changes can be noted in highly myopic patients who are middle-aged or even younger.[23,24] Our mean age at diagnosis of visual impairment is likely to be overestimated, since we included persons over age 55 years with visual impairment at baseline; baseline age was 69 years for RS-I and 64.1 years for RS-II. We did not have information on the actual age of onset of visual impairment occurring before this age.

The frequency of visual impairment in the high hyperopia group was relatively high. The number of cases with blindness or low vision in this group was very small (n = 8). Also, the proportion of high hyperopes in our older study sample was quite large, so these data are not necessarily applicable to the general population. Previous research has mainly focused on high myopia rather than on high hyperopia, but our results at least show that high hyperopia should be subject to further studies as well. Cataract was an important cause of visual impairment in all refractive error categories. This may be an overestimation of the current situation, since the majority of the data had been collected in the 1990's, and since then cataract surgery has become a more easily accessible and safer procedure. Several studies showed an increased incidence of nuclear cataract and subcapsular posterior cataract in high myopes.[25] We considered whether the exclusion of pseudophakic and aphakic persons might have introduced a selection bias and an underestimation of the risks of visual impairment in high myopic persons in our study. This does not seem to be the case, since only 2 out of 287 excluded participants (0.7%) with pseudophakia or aphakia were blind or visually impaired due to myopic macular degeneration diagnosed on the fundus photograph.

In summary, our data indicate that risks and causes of visual impairment vary with refractive error. The risks for high myopes are by far the highest with more than 1 in 3 persons with high myopia developing bilateral blindness or low vision. This large health risk requires public awareness and a focus to initiate strategies to reduce this burden in those at risk of myopia.

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Chapter 3.1

Epidemiology of reticular pseudodrusen in age-related macular degeneration: The Rotterdam Study

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ABSTRACT

Purpose Reticular pseudodrusen (RPD) are considered to be a distinct feature in age-related macular degeneration (AMD). Population studies have studied the epidemiology of RPD using standard color fundus photographs (CFP). However, recent studies have shown that RPD are better imaged using near-infrared (NIR) imaging. We studied the epidemiology of RPD in a large population-based study using NIR and CFP.

Methods Participants aged 65+ years from the Rotterdam Study underwent ophthalmological examination including NIR and CFP. Both images were graded for the presence of RPD and soft indistinct drusen (SID). Associations with demographic and environmental factors, 26 genetic variants, and total genetic risk score were analyzed using logistic regression analysis.

Results RPD were detected in 137 (4.9%) of 2,774 study participants; of these, 92.7% were detected with NIR imaging and 38% on CFP. The majority of eyes with RPD showed presence of SID, while other drusen types coincided less frequently. RPD were significantly associated with age (Odds Ratio (OR) 1.21 (95% Confidence Interval (CI) 1.17-1.24)) and female sex (OR 2.10 (95% CI 1.41-3.13)). Environmental factors did not show a significant association with RPD. Major AMD risk variants were significantly associated with RPD and SID, however, *ARMS2*, *C3* and *VEGFA* were more associated with RPD (RPD vs SID $P<0.05$). Total genetic risk score did not differ significantly ($P=0.88$).

Conclusion Detection of RPD was better with NIR imaging than on CFP in a population-based setting. Presence of RPD often coincided with presence of SID, however, they showed quantitative differences in genetic risk profile.

INTRODUCTION

Reticular pseudodrusen (RPD), also known as subretinal drusenoid deposits (SDD), are depositions located in the subretinal space between the outer segments of the photoreceptors and the retinal pigment epithelium (RPE).[1-3] RPD carry a much higher risk of developing end-stage AMD than other AMD lesions, such as soft indistinct drusen (SID).[4-7] RPD have first been described in 1990 and it was already then pointed out that RPD are better visualized using blue reflectance photography than regular color fundus photographs (CFP).[8] RPD can also be detected using other imaging modalities. A comparative study between various image modalities showed that near-infrared imaging is one of the image modalities that has the highest sensitivity for RPD detection[9], and that sensitivity of RPD detected only on CFP can be as low as 36%.[10]

Several risk factors have been identified for RPD, which include age, smoking, higher body mass index, female predominance, and genetic risk factors including *CFH*(Y402H) and *ARMS2*(A69S).[4,6,7,11-13] Previous studies have shown that RPD were present in 60-90% of patients with late AMD.[14-16] Boddu et al. studied risk profiles of RPD versus large soft drusen in a small clinic-based study.[13] These researchers could not find many significant differences between these patient groups, however, individuals with RPD were older and more often female.

Since the population-based studies on RPD have based their grading solely on CFP, and the clinic-based studies were carried out only in small groups, risk estimates are likely to be imprecise.[11] Improved detection of RPD in a large population-based study may provide more accurate prevalence figures, and could enhance risk profiling for RPD.

In this study, we aimed to investigate the epidemiology of RPD using CFP and NIR images and compare this with SID in a large, unselected population. We have chosen to use the term RPD instead of SDD, since this is more commonly used in clinical-based papers. However, where RPD is written SDD can be read.

METHODS

Study population

The Rotterdam Study is a prospective population-based cohort study that focuses on chronic ophthalmologic neurologic, cardiovascular, and locomotor diseases in middle aged and elderly subjects living in Ommoord, a suburb of Rotterdam. The aims and design of the Rotterdam study have been described elaborately elsewhere.[17] In brief, the study started in 1989 and since then every 2-4 years follow-up examinations were performed. During these follow-up examinations new techniques and devices were implied, such as the Heidelberg Retina Angiograph 2, a scanning laser ophthalmoscope (SLO). The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC and by the Ministry of Health, Welfare and Sport of the Netherlands, implementing the "Wet Bevolkingsonderzoek: ERGO (Population Studies Act: Rotterdam Study)". All participants provided written informed consent to participate in the study and to obtain information from their treating physicians.

For the current cross-sectional analysis, we included 3,108 participants from the last examination round from two independent cohorts of the Rotterdam Study; 1,542 participants from the Rotterdam Study I (RS-I), aged 70 years and older and 1,566 participants from the Rotterdam Study II (RS-II), aged 65 years and older. Participants were excluded from the analyses if they had ungradable CFP (N=17)

or ungradable NIR images (N=68). Since the Heidelberg Retina Angiograph 2 was operational a few weeks after the start of the examination round an additional 266 participants were excluded from the study due to absence of NIR images. From these two cohorts, 2,774 participants with gradable CFP and gradable NIR imaging were eligible for analysis.

Grading of AMD, SID and RPD

All eligible participants underwent 35° digital CFP of the macula (Topcon TRC-50EX, Topcon Optical Company, Tokyo, Japan with a Sony DXC-950P digital camera; 0.44 megapixel, Sony Corporation, Tokyo, Japan) after pharmacologic mydriasis. Next, NIR images, (λ=820 nm) 30°x30° of the macula, were taken with a Heidelberg Retina Angiograph 2 (Heidelberg engineering, Heidelberg, Germany).

CFP were graded for presence of all AMD-related features according to the Wisconsin Age-Related Maculopathy Grading[18] and Rotterdam classification (for definition see Table 1), a modified International Classification System, using the standard grading grid for AMD (central circle 1000 µm, inner circle 3000µm and outer circle 6000µm in diameter).[19,20] In short, the Rotterdam classification consists of 5 grades with grade 0 defined as no AMD, grade 1 as preliminary early AMD, grade 2 and 3 together as Early AMD and grade 4 as Late AMD. SID were defined as yellow lesions with indistinct borders and ≥ 125µm in size. These type of soft drusen are associated with a higher risk of developing advanced AMD compared to soft distinct drusen.[20] RPD were defined as indistinct yellowish lesions interlacing in networks125-250µm in width.[18,19]

NIR images of the macula were graded based on the presence of RPD, detectable as groups of hypo-reflectant lesions against a mildly hyper-reflectant background in regular patterns.[9,10,21,22] Since SID and other drusen types are less visible on NIR imaging, these lesions were not graded on NIR, only on CFP.

On CFP, RPD and/or SID were graded in and outside the ETDRS grading grid . On NIR, RPD was graded if present on the image, this equals grading in and outside the ETDRS grid on CFP.

All images were graded by trained graders, while being masked for the grading of the other image modality, under the supervision of senior retinal specialists (P.T.V.M.d.J., J.R.V., C.C.W.K.). Between grader comparisons were assessed. For drusen grading on CFP, the weighted κ values ranged from 0.60 for hard drusen to 0.82 for soft distinct drusen. For RPD grading on CFP, the κ value was 0.72. For NIR imaging, this κ value was 0.84. The eyes of each participant were graded and classified separately, and the eye with the more severe grade was used to classify the person.

Genotyping and selection of genetic variables

Genomic DNA was extracted from peripheral blood leukocytes. All study participants in the RS-I were genotyped with the Illumina Infinium II HumanHap550 array or Taqman assays (Applied Biosystems, Foster City, CA). Study participants from the RS-II were genotyped with the Illumina Human610-Quad array. HapMap CEU data (release #22) was used for imputation. Genetic variables associated with AMD were selected based on previous publications.[23,24]

Risk score Three Continent AMD Consortium prediction model

The Three Continent AMD Consortium (3CC) developed a validated prediction model including a total risk score based on 31 variables; 26 genetic variants associated with AMD, age, sex, smoking, BMI, and AMD phenotype.[23] The total risk score was based on the sum of the beta coefficients from a

Cox proportional hazard analysis, which included all the selected variables. In this analysis we used the total genetic risk score, which is a variant of the total risk score and has been calculated using only the beta coefficients from the genetic variables of the 3CC prediction model.

Assessment of nongenetic variables

Information on the history of diabetes mellitus, education level and cigarette smoking were derived from computerized questionnaires administered during home interviews. Smoking was categorized in never, past and current smokers. Blood pressure, systolic and diastolic, was calculated as the average of two consecutive measurements, using a random-zero mercury sphygmomanometer. Hypertension was defined as having a systolic blood pressure ≥ 140 mmHg or a diastolic blood pressure ≥ 90mmHg or using anti-hypertensive medication at baseline. Cholesterol, high density lipoprotein (HDL) and triglycerides were measured at baseline by the Central Clinical Chemical Laboratory of the Erasmus University Medical Center. A subgroup of measurements was carried out in the laboratory of the Department of Epidemiology & Biostatistics, Erasmus University Medical Center. Body-mass index (BMI) was calculated as weight kilograms divided by height squared in meters. Waist circumference and hip circumference were measured in centimeters.

Statistical analysis

Prevalence of RPD as a function of age was calculated per image modality. The prevalence of Early AMD based on CFP diagnosis of AMD lesions was compared with the prevalence of Early AMD based on both NIR and CFP grading.

We investigated the association with demographic, genetic and environmental variables using various outcomes: soft indistinct drusen versus no drusen; RPD versus no drusen, and RPD versus soft indistinct drusen. Outcomes were binary and the association was assessed with logistic regression analysis. Total genetic risk scores were calculated for each individual using only the genetic variables from the 3CC prediction model. Risk scores were grouped and stratified for RPD, SID and no RPD/SID; strata were compared using Chi-Square statistic test. Analyses investigating concurrence of drusen types were eye-based, all other analyses were person-based, which were eye based. All statistical analyses were performed using SPSS version 21 (SPSS IBM, New York, U.S.A).

RESULTS

RPD were detected in 137 (4.9%) of 2,774 study participants, of which 52 (38.0%) were identified on CFP and 127 (92.7%) on NIR imaging (numbers do not add up to 100% due to overlap). RPD were mostly present from age 70 years onwards (Figure 1) and were bilaterally present in 69.3%. Only one person had been diagnosed with RPD at the age of 64.0 years, and RPD were visible on CFP as well as on NIR imaging. Frequency of RPD increased per age category, however, the steep rise in those aged 90+ years was most pronounced when RPD were diagnosed on NIR imaging.

In the Rotterdam classification system, RPD are part of the criteria for staging. To investigate how much the improved detection of RPD by NIR imaging influenced AMD prevalence, we classified each person using the two gradings, one solely based on CFP and the other based on CFP and NIR imaging. Inclusion of the NIR imaging increased the prevalence of Early AMD (stage 2 & 3) from 19% to 20.5% (Table 1).

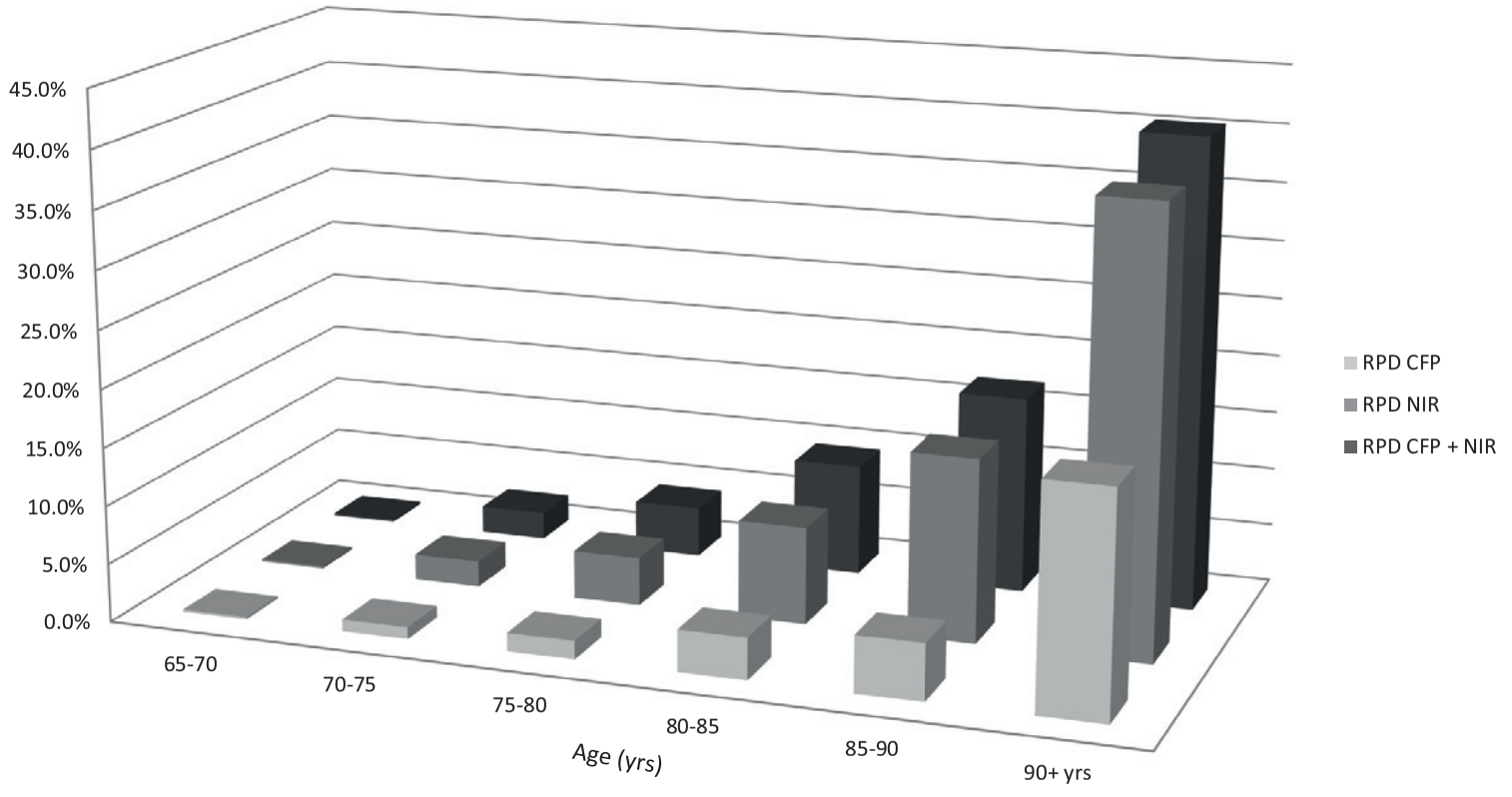

FIGURE 1 - Reticular pseudodrusen detection with color fundus photographs and near-infrared imaging. Frequency of reticular pseudodrusen was plotted per age category for the different imaging types: color fundus photographs, near-infrared imaging and both imaging types combined. X-axis: age category in years, y-axis: percentages Abbreviations: CFP = Color fundus photographs, NIR = near-infrared imaging, yrs = years

TABLE 1 - Prevalence per AMD severity grade according to the Rotterdam Classification based on color fundus photographs only versus color fundus photographs and near-infrared imaging

Grade	Definition	Grading CFP N=2774	Grading CFP + NIR N=2774
0	No signs of AMD at all **OR** hard drusen (< 63 µm) only	42.70%	42.30%
1	Soft distinct drusen (≥ 63 µm) only **OR** pigmentary abnormalities only	36.10%	35.00%
2	Soft indictinct drusen (≥ 125 µm) / reticular drusen only **OR** soft distinct drusen (≥ 63 µm) **AND** pigmentary abnormalities	13.60%	14.50%
3	Soft indictinct (≥ 125 µm) / reticular drusen **AND** pigmentary abnormalities	5.40%	6.00%
4	Atrophic, neovascular or mixed AMD	2.20%	2.20%

Abbreviations: AMD = age-related macular degeneration, CFP = color fundus photographs, NIR = near-infrared imaging

We then investigated the number and type of other drusen present in eyes with RPD (N=232) (Figure 2 and Supplementary Figure 1). We performed this analysis for drusen within and outside the grid. The majority of eyes with RPD had SID, both within and outside the grid. These eyes also presented with other types of drusen, but they were less frequent compared to SID. Of eyes with RPD, only 14 eyes (6.0% of eyes with RPD) did not have any type of soft drusen, and 9 eyes (3.9% of eyes with RPD) had no other type of drusen at all. Of these, the contralateral eye of 2 persons had drusen. Only 5 persons with RPD had no drusen at all, not even in the contralateral eye.

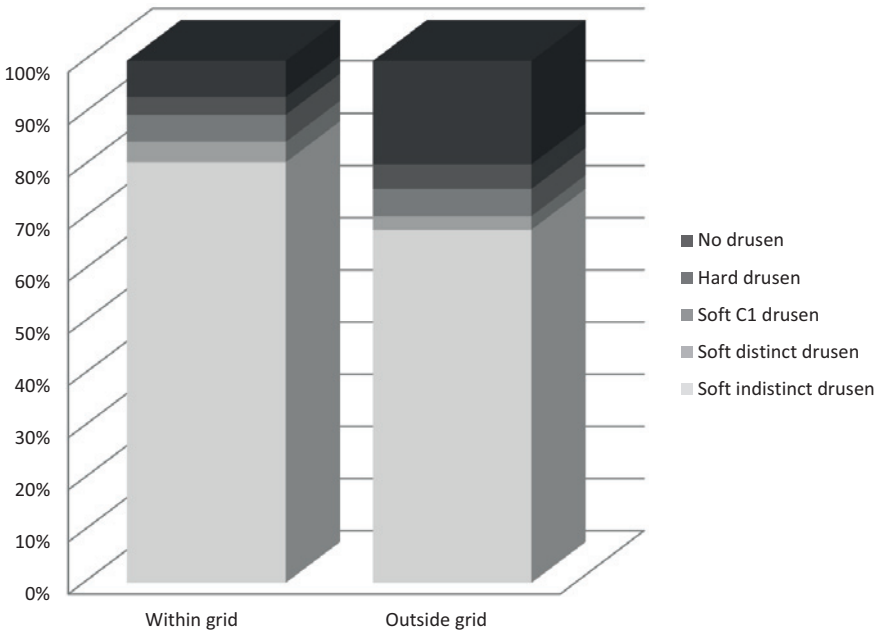

FIGURE 2 - Frequency of other drusen types in eyes with reticular pseudodrusen. Other drusen types coinciding with reticular pseudodrusen. Grading based on most severe drusen type, beside reticular pseudodrusen, on color fundus photographs, according to the Wisconscin Age-Related Maculopathy Grading. Frequencies of other drusen types were stratified for drusen presence within and outside the grading grid.

Table 2 shows the frequency of characteristics between persons with SID, RPD, and those without SID or RPD. Table 3 shows the associations of these characteristics with SID only, with RPD, and with RPD versus SID. The odds ratio represents the risk for a certain parameter on a drusen type (RPD or SID) in reference to the other drusen type or to absence of RPD/SID. Both SID and RPD were associated with age (OR 1.09 (95% CI 1.06-1.11) and OR 1.21 (95% CI 1.17-1.24)), respectively. For SID there was no association with sex (OR 1.02 (95% CI 0.77-1.34)) while occurrence of RPD was strongly associated with female sex (OR 2.10 (95% CI 1.41-3.13)). HDL cholesterol (OR 1.83 (95% CI 1.30-2.59)) and triglycerides (OR 0.66 (95% CI 0.51-0.86)) in serum were both significantly associated with SID, but not with RPD. No other demographic or environmental risk factors were significantly associated with SID or RPD. Table 4 shows the association of RPD/SID with genetic factors. Genetic variants in the *CFH* gene (rs1061170, rs12144939, rs800292), the *ARMS2* gene (rs10490924) and the *C2/CFB* gene (rs641153) were significantly associated with both drusen types. A variant in the *IER3/DDR1* gene (OR 2.54 [95% CI 1.32-4.86] for rs3130783, GG vs AA) was only associated with SID, while a variant in the *C3* gene (OR 1.61 (95% CI 1.08-2.40)] for rs22130199, CG vs CC) was only associated with RPD. Two variants were significantly more associated with RPD than SID. These included the variant in the *ARMS2* gene (OR 2.48 (95% CI 1.001-6.17) for rs10490924, TT vs GG) and a variant in the *VEGFA* gene (OR 2.00 (95% CI 1.05-3.8) for rs943080, CT vs CC). Genetic risk score frequencies did not differ significantly between SID and RPD ($P = 0.88$, Chi-Square test) (Supplementary Table 1).

TABLE 2 - Frequency of clinical parameters stratified for drusen type

Variables	No RPD/SID N=2416	SID N=221	RPD N=137
Age, yrs (sd)	75.0 (5.6)	77.8 (6.2)	82.1 (6.2)
Sex, % females	54.6	54.8	71.5
Education, %			
< 12 years	26.8	23.3	29.1
≥ 12 years	73.2	76.7	70.9
Smoking, %			
never	31.2	32.1	37
past	57	57	48.9
current	11.9	10.9	14.1
Diabetes Mellitus, %	13	12.4	13
Hypertension, %	86	86.8	89.7
Systolic bloodpressure, mmHg (sd)	152.3 (21.4)	154.0 (21.4)	154.0 (23.7)
Diastolic bloodpressure, mmHg (sd)	85.2 (11.0)	85.9 (11.1)	85.3 (13.3)
Cholesterol, mmol/l (sd)	5.4 (1.1)	5.4 (0.9)	5.3 (1.1)
HDL cholesterol, mmol/l (sd)	1.5 (0.4)	1.6 (0.4)	1.5 (0.4)
Triglycerides, mmol/l (sd)	1.4 (0.7)	1.3 (0.6)	1.4 (0.8)
BMI, kg/m^2	27.5 (4.1)	27.1 (4.1)	27.2 (3.9)
Waist circumference, cm (sd)	93.7 (12.0)	92.0 (11.7)	91.5 (11.0)
Hip circumference, cm (sd)	102.9 (8.2)	102.6 (9.3)	102.6 (8.7)

Abbreviations: BMI= body mass index, HDL = high density lipoprotein, LDL= low density lipoprotein, RPD = reticular pseudodrusen, sd = standard deviation, SID = soft indistinct drusen

TABLE 3 - Comparison of clinical parameters among drusen types

Variables	SID vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs SID OR (95% CI) age and sex adjusted
Age*	1.09 (1.06-1.11)	1.21 (1.17-1.24)	1.12 (1.07-1.16)
Sex **			
male	1	1	1
female	1.02 (0.77-1.34)	2.10 (1.41-3.13)	2.16 (1.34-3.49)
Education			
< 12 years	1	1	1
≥ 12 years	1.31 (0.93-1.83)	1.24 (0.82-1.89)	0.90 (0.53-1.52)
Smoking			
never	1	1	1
past	0.98 (0.70-1.35)	1.06 (0.69-1.62)	0.97 (0.57-1.65)
current	0.98 (0.60-1.62)	1.77 (0.97-3.23)	1.54 (0.71-3.32)
Diabetes Mellitus			
No	1	1	1
Yes	0.96 (0.59-1.55)	1.05 (0.57-1.94)	1.41 (0.64-3.09)
Hypertension			
No	1	1	1
Yes	0.88 (0.58-1.33)	0.89 (0.49-1.61)	0.91 (0.44-1.89)
Cholesterol, mmol/l	1.06 (0.93-1.21)	0.89 (0.75-1.06)	0.78 (0.61-1.00)
HDL cholesterol, mmol/l	1.83 (1.30-2.59)	1.28 (0.80-2.05)	0.68 (0.39-1.20)
Triglycerides, mmol/l	0.66 (0.51-0.86)	0.95 (0.70-1.28)	1.39 (0.97-1.98)
BMI			
≤ 25 kg/m^2	1	1	1
> 25 kg/m^2	0.83 (0.61-1.11)	1.13 (0.75-1.70)	1.50 (0.90-2.48)
Waist circumference			
≤ 90 cm	1	1	1
> 90 cm	0.82 (0.61-1.11)	1.21 (0.82-1.80)	1.51 (0.93-2.47)
Hip circumference			
≤ 100 cm	1	1	1
> 100 cm	0.87 (0.65-1.15)	1.18 (0.81-1.72)	1.29 (0.81-2.06)

Abbreviations: BMI = body mass index, CI = confidence interval, HDL = high density lipoprotein, LDL = low density lipoprotein OR = Odds' Ratio, RPD = reticular pseudodrusen, SID = soft indistinct drusen
* Adjusted for sex
** Adjusted for age

TABLE 4 - Genetic risks among drusen types

Genes	SID vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs SID OR (95% CI) age and sex adjusted
CFH (Y402H) rs1061170			
TT	1	1	1
CT	1.65 (1.17-2.34)	1.45 (0.95-2.23)	0.91 (0.52-1.58)
CC	3.84 (2.50-5.90)	2.78 (1.57-4.93)	0.83 (0.42-1.66)
CFH rs12144939			
GG	1	1	1
GT/TT	0.34 (0.23-0.51)	0.28 (0.17-0.47)	0.89 (0.47-1.71)
CFH rs800292			
GG	1	1	1
GA	0.54 (0.39-0.76)	0.55 (0.36-0.84)	0.88 (0.51-1.50)
AA	0.38 (0.17-0.83)	0.30 (0.11-0.87)	0.96 (0.24-3.74)
ARMS2 (A69S) rs10490924			
GG	1	1	1
GT	1.81 (1.33-2.47)	2.41 (1.60-3.62)	1.24 (0.75-2.05)
TT	3.25 (1.70-6.20)	7.85 (3.75-16.45)	2.48 (1.001-6.17)
C2/CFB (L9H) rs4151667			
TT	1	1	1
TA/AA	0.65 (0.35-1.19)	0.43 (0.17-1.10)	0.78 (0.25-2.44)
C2/CFB (R32Q) rs641153			
GG	1	1	1
GA/AA	0.56 (0.34-0.90)	0.38 (0.19-0.75)	0.66 (0.29-1.54)
C3 (R102G) rs2230199			
CC	1	1	1
CG	1.19 (0.86-1.64)	1.61 (1.08-2.40)	1.32 (0.80-2.19)
GG	1.28 (0.64-2.56)	1.81 (0.80-4.13)	1.18 (0.41-3.40)
C3 rs433594			
GG	1	1	1
GA	0.80 (0.58-1.12)	0.84 (0.55-1.28)	1.16 (0.68-1.96)
AA	0.83 (0.52-1.32)	0.94 (0.51-1.71)	1.01 (0.47-2.16)
CFI rs10033900			
CC	1	1	1
CT	1.27 (0.87-1.85)	0.82 (0.52-1.30)	0.68 (0.38-1.24)
TT	1.33 (0.86-2.06)	0.90 (0.52-1.54)	0.62 (0.31-1.24)
LPL rs256			
CC	1	1	1
CT/TT	0.88 (0.62-1.25)	0.75 (0.48-1.19)	0.83 (0.46-1.47)
LIPC rs12912415			
AA	1	1	1
AG/GG	0.90 (0.64-1.26)	0.75 (0.48-1.17)	0.98 (0.56-1.71)
MYRIP rs2679798			
AA	1	1	1
AG	1.04 (0.73-1.49)	1.42 (0.88-2.27)	1.34 (0.75-2.42)
GG	1.23 (0.81-1.87)	1.36 (0.77-2.39)	1.22 (0.61-2.47)
SKIV2L rs429608			
GG	1	1	1
GA/AA	0.57 (0.38-0.84)	0.55 (0.33-0.91)	1.01 (0.53-1.92)
ABAC1 rs1883025			
CC	1	1	1
CT	0.78 (0.56-1.09)	1.00 (0.67-1.50)	1.03 (0.61-1.74)

TABLE 4 - (continued)

Genes	SID vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs no RPD/SID OR (95% CI) age and sex adjusted	RPD vs SID OR (95% CI) age and sex adjusted
TT	1.09 (0.61-1.94)	0.53 (0.19-1.53)	0.38 (0.11-1.28)
CETP rs3764261			
CC	1	1	1
CA	1.27 (0.92-1.76)	1.15 (0.76-1.73)	1.06 (0.63-1.79)
AA	1.40 (0.84-2.32)	1.02 (0.50-2.07)	0.68 (0.29-1.63)
TIMP3 rs5749482			
GG	1	1	1
CG/CC	0.87 (0.60-1.27)	0.62 (0.37-1.04)	1.17 (0.40-3.40)
VEGFA rs943080			
CC	1	1	1
TC	0.81 (0.56-1.17)	1.34 (0.79-2.24)	2.00 (1.05-3.81)
TT	1.09 (0.72-1.65)	1.51 (0.84-2.73)	1.50 (0.73-3.08)
COL8A1 rs13081855			
GG	1	1	1
GT/TT	1.15 (0.78-1.68)	0.90 (0.53-1.52)	0.85 (0.45-1.59)
TNFRSF10A rs13278062			
TT	1	1	1
GT	0.92 (0.65-1.30)	0.73 (0.46-1.14)	0.89 (0.51-1.57)
GG	0.72 (0.46-1.12)	0.77 (0.45-1.31)	1.11 (0.57-2.18)
FRK/COL10A1 rs3812111			
TT	1	1	1
AT	0.93 (0.68-1.29)	0.92 (0.61-1.39)	0.96 (0.57-1.60)
AA	0.86 (0.52-1.44)	0.53 (0.24-1.15)	0.71 (0.28-1.79)
SLC16A8 rs8135665			
CC	1	1	1
CT	1.17 (0.85-1.61)	0.83 (0.54-1.28)	0.73 (0.43-1.24)
TT	1.31 (0.61-2.82)	0.74 (0.21-2.63)	0.74 (0.17-3.18)
ADAMTS9 rs6795735			
CC	1	1	1
TC	0.96 (0.68-1.36)	1.03 (0.66-1.61)	1.10 (0.63-1.92)
TT	1.34 (0.88-2.06)	1.35 (0.77-2.37)	0.90 (0.45-1.80)
TGFBR1 rs334353			
TT	1	1	1
GT	0.92 (0.66-1.27)	1.09 (0.72-1.64)	1.29 (0.76-2.18)
GG	1.03 (0.55-1.94)	0.62 (0.24-1.63)	0.61 (0.20-1.87)
RAD51B rs8017304			
AA	1	1	1
AG	0.82 (0.59-1.13)	0.85 (0.56-1.29)	1.26 (0.74-2.14)
GG	0.63 (0.38-1.03)	0.85 (0.46-1.56)	1.46 (0.67-3.19)
IER3/DDR1 rs3130783			
AA	1	1	1
AG	0.85 (0.61-1.19)	0.83 (0.54-1.28)	0.95 (0.55-1.64)
GG	2.54 (1.32-4.86)	1.48 (0.49-4.51)	0.34 (0.10-1.20)
B3GALTL rs9542236			
TT	1	1	1
CT	0.77 (0.55-1.09)	1.11 (0.70-1.74)	1.57 (0.89-2.76)
CC	0.92 (0.60-1.39)	1.02 (0.58-1.83)	1.49 (0.73-3.04)

Abbreviations: CI = Confidence Interval, OR = Odds' Ratio, RPD = reticular pseudodrusen, SID = soft indistinct drusen

DISCUSSION

In this cross-sectional analysis of a population-based study, we showed that NIR imaging is superior to traditional CFP in the detection of RPD. We also found that RPD generally coincide with soft drusen, and were seldom seen as the only AMD feature. However, the AMD risk profile of RPD showed quantitative differences with that of SID. Those with RPD were more likely to be women, have older age, and carry risk variants in the *C3*, *ARMS2* and *VEGFA* genes.

Our study confirms that NIR imaging is preferred over the use of CFP for detection of RPD.[11,13,21,25] Of all persons diagnosed with RPD, 62.7% were not visible on CFP, only on NIR. By contrast, 7% of those with RPD were diagnosed on CFP and failed to be detected on NIR. This implies that multimodal imaging for complete AMD grading is better than using single imaging devices. Suzuki et al. described three subtypes of RPD: two were better visualized by NIR-imaging, and one, the ribbon like subtype, was more easily spotted on CFP.[26] Indeed, we acknowledge that diagnosis of RPD on CFP is mostly based on recognition of the pattern of this feature. Aside from NIR and CFP, other image modalities can also reveal RPD, such as fundus auto fluorescence, optical coherent tomography (OCT), confocal blue reflectance, and indocyanine green angiography. Whether incorporation of all these methods further improves detection of RPD is questionable. A recent study assessed the accuracy of RPD detection using all these modalities, and found that NIR and OCT have the highest sensitivity, both 94.6%.[9]

Using NIR imaging increased our estimate of the overall prevalence of RPD to 4.9%. All previously reported population estimates of RPD were based on CFP only, and were therefore lower; the Beaver Dam Eye Study reported an overall prevalence of 0.7% in a population aged 45+ years,[6] the Blue Mountain Eye Study reported an overall prevalence of 1.95% in a population aged 50+ years,[12] and the Melbourne Collaborative Cohort Study recently reported an overall prevalence of 0.41% in a population 48-86 years of age.[4] In our study, RPD were bilateral in 69% of cases, while other population-based cohorts reported a slightly lower frequency of bilaterality, 51-63% respectively.[4,6,12] The higher rates in our study could be due to the improved detection of RPD using NIR.

We identified several risk factors for RPD and SID. For RPD, demographic risk factors were older age and female sex, factors which have been reported by several studies.[6,7,11-13,27] We did not find any statistically significant environmental risk factors, although the relationship with current smoking was suggestive. Other studies reported risks of RPD for hypertension, lower income, lower education, higher body mass index, angina pectoris, HDL cholesterol, and triglycerides, but we and many others could not confirm these findings. [6,7,11-13,21,27] Associations which showed a preference for SID were increased HDL-cholesterol, and decreased triglycerides. These trends have been observed previously for Late AMD, and have not been explained thus far.[20] With respect to AMD risk variants, those in the *CFH, C2/FB* and *ARMS2* genes were significantly associated with both RPD and SID in this study. *C3,* however was only associated with RPD. The lack of association with other risk variants may be due to our limited statistical power to find minor associations. Remarkably, *ARMS2* appeared to have a strong predilection for RPD. Homozygous carriers of the *ARMS2* risk variant were twice as likely to have RPD than SID. We did not find this differential distribution for the *CFH* nor *C2/FB* risk variants. Prior reports found stronger associations with *ARMS2* than *CFH*.[27,28] Another gene showing a tendency for RPD was *VEGFA*. Although this gene was not significantly associated with either phenotype, it showed a significant risk difference between RPD and SID.

Eyes featuring RPD without any type of drusen were rare in our study. We evaluated the risk profiles of these participants (N=5), and these were not remarkably different from the entire group of RPD (Table 2 and 3). Noteworthy, none of the RPD in these participants were observed on CFP, only on NIR imaging. RPD without any type of drusen may represent other phenotypes than AMD; in literature, RPD have been reported to accompany Sorsby fundus dystrophy, pseudoxanthoma elasticum, acquired vitelliform lesions, and systemic disorders such as vitamin A deficiency, cardiovascular disease and complement mediated IgA nephropathy.[28-33] In our study, these 5 patients showed no other retinal pathology aside from RPD. A focus on unravelling the etiology of isolated RPD may help shed light on overarching molecular mechanisms in retinal disease.

Current insights into RPD pathogenesis are still highly limited, but a vascular etiology has been suggested.[27,28] The genetic predilections in our study may point toward this hypothesis. *ARMS2* was found to be expressed in the ellipsoid layer of the photoreceptors and in the intercapillary pillars of the choroid.[34,35] The first is close to the location of RPD,[34] and the latter may help explain the vascular hypothesis.[35] Another hint in this direction is the higher susceptibility of *VEGFA* and *ARMS2* for neovascular AMD[24], although RPD do not preferentially accompany this AMD subtype.[4,6,7,36] Furthermore, the genes *CFH, C3,* and, *VEGFA* have been associated with cardiovascular and coronary artery disease.[27,28] Morphologic changes in eyes with RPD, suggest that these lesions follow the pattern of the watershed zones of the choroid, and correspond with local thinning of this layer.[37] Spaide found that choroidal thinning is even more pronounced when RPD regress and photoreceptors shorten[38]. A recent study of retinal imaging with adaptive optics showed that photoreceptor changes precede RPD regression.[39] Several histopathologic studies have studied the molecular content of RPD and SID and found significant overlap: both contain unesterified cholesterol, complement factor H, apolipoprotein E, and vitronectin.[16,40] There are also remarkable differences: RPD lack immunoreactivity for photoreceptors, Müller cells, and RPE marker proteins, and have only low concentrations of esterified cholesterol and other neutral lipids.[16,40] Why the deposit in RPD is located above rather than below the RPE cell is unclear. Several hypotheses have been made. Rudolf et al. suggested that loss of RPE cell polarity may lead to deposition of unphagocytized photoreceptor outer segments above rather than below the RPE cell.[41] Curcio et al. suggest that perturbation of cholesterol homestasis and the lipid transfer between the RPE cell and the photoreceptor cell in the context of an outer retinal lipid-recycling program, could explain the formation of these deposits.[16] Another hypothesis may be that shortening of the RPE villi[42] jeopardize close contact with photoreceptor outer segments, and may hamper uptake of shedded discs from the photoreceptor by the RPE cell. In AMD, the formation of RPD appears to follow that of soft drusen, since the concurrence of these lesions carry a higher risk of progression to Late AMD.[4,6,20] How the associated genes, the choroidal anatomic changes, and formation of drusenoid material above the RPE are related remains an intriguing question for future studies.

To our knowledge, this is the first population-based study, studying RPD using another imaging device aside from CFP. Other strengths include the large sample size of unselected persons, the study of RPD within the context of other Early AMD features, and the comparison of risk profiles of RPD versus soft drusen. Other reports did not specify the frequency of concurrent AMD lesions in eyes with RPD. Furthermore, we analyzed a much more comprehensive set of demographic, environmental and genetic risk factors for RPD than previous population-based and clinical studies, which led to new insights into the genetic background of RPD. A limitation of our study is the lack of follow up data with NIR imaging, hampering the study of RPD incidence and progression. This needs to be addressed in prospective studies.

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Chapter 3.2

Automatic identification of reticular pseudodrusen using multimodal retinal image analysis

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ABSTRACT

Purpose To examine human performance and agreement on reticular pseudodrusen (RPD) detection and quantification using single- and multi-modality grading protocols and to describe and evaluate a machine learning system for the automatic detection and quantification of reticular drusen using single- and multi-modality information.

Methods Color fundus, fundus autofluoresence and near infra-red images of 278 eyes from 230 patients with or without presence of RPD were used in this study. All eyes were scored for presence of RPD during single- and multi-modality setups by two experienced observers and a developed machine learning system. Furthermore, automatic quantification of RD area was performed by the proposed system and compared with human delineations.

Results Observers obtained a higher performance and better inter-observer agreement for RPD detection using multi-modality grading, achieving areas under the Receiver operating characteristics (ROC) of 0.940 and 0.958, and a kappa agreement of 0.911. The proposed automatic system achieved an area under the ROC of 0.941 using a multi-modality setup. Automatic RPD quantification resulted in an intra-class correlation (ICC) value of 0.704, which was comparable with ICC values obtained between single modality manual delineations.

Conclusion Observer performance and agreement for RPD identification improved significantly using a multi-modality grading approach. The developed automatic system showed similar performance as observers and automatic RPD area quantification was in concordance with manual delineations. The proposed automatic system allows for a fast and accurate identification and quantification of RD, opening the way for efficient quantitative imaging biomarkers in large dataset analysis.

INTRODUCTION

Age-related macular degeneration (AMD) is a progressive eye disease affecting mainly the elderly and causing vision loss at advanced stages.[1] The early stages of AMD are characterized by the presence of pigmentary changes and drusen, which are deposits accumulating between the retinal pigment epithelium (RPE) and the Bruch's membrane. A sub-type of drusen, commonly addressed as subretinal drusenoid deposits or reticular pseudodrusen (RPD), present different characteristics and distribution than normal drusen and have been proven to be a strong risk factor for progression to advanced AMD.[2-7] Therefore, their identification and quantification is of paramount importance for a better understanding of disease progression.

RPD are visible on color fundus (CF) photography, fundus autofluorescence (FAF) imaging and near infra-red (NIR) imaging amongst other retinal imaging modalities such as confocal blue reflectance, indocyanine green (ICG) angiography, spectral-domain optical coherence tomography (SD-OCT) and fluorescein angiography.[8-13] On CF images, RPD are described as indistinct, yellowish interlacing networks of 125 µm to 250 µm wide.[14] On FAF images, RPD are characterized as hypofluorescent lesions, while on NIR images, RPD are characterized as groups of hyporeflectant lesions against a mild hyper-reflectant background.[15-17] Previous studies have reported a difference in sensitivities for RPD detection among image techniques.[12,17] However, RPD identification using a single image modality is challenging as the characteristic changes associated to RPD are often subtle and might not always be detected using only one imaging technique. Therefore, for an accurate diagnosis, RPD detection should be performed using two or more image modalities.[11] Although other studies have investigated and compared the performance of individual image techniques for RPD detection,[12,17] a study of the performance obtained using multiple image modalities simultaneously has not been performed as far as we are concerned.

Despite its expected higher accuracy, grading of multi-modality images represents a considerable workload for a human grader. Machine learning algorithms have huge potential for dealing with complex information extracted from different image modalities. Furthermore, automatic systems are not influenced by fatigue and mindset and, therefore, less prone to variability than humans. Previously developed systems for the automatic detection of drusen showed good performance on CF images.[18-21] Whether they also perform well fusing information from different image modalities is currently unknown. As far as we are concerned, there is no method for the automatic identification of RPD fusing information from different image modalities.

The aim of the present study is two-fold. Firstly, we evaluate the performance and the agreement between human observers using single- as well as multi-modality grading approaches for RPD detection. In the single-modality approach, RPD detection is performed using only one image technique (namely, CF, FAF or NIR). In contrast, during the multi-modality grading session, the observers evaluate the three available image modalities simultaneously. Secondly, we aim to investigate the effectiveness of a novel machine learning algorithm for the automatic identification and quantification of RPD using combined information from different image modalities by comparing its performance to human observers.

METHODS

Study dataset

A set of subjects with and without RPD were selected from the Rotterdam Study, a prospective cohort study aimed to investigate risk factors for chronic diseases in the elderly.[22] The study adhered to the tenets set forth in the Declaration of Helsinki and Investigational Review Board approval was obtained. Only patients with CF, FAF and NIR images available were included in this study. CF images were taken using a 35° field of view Topcon TRC 50EX fundus camera (Topcon Optical Company, Tokyo, Japan) with a Sony DXC-950P digital camera with a resolution of 768 x 576 pixels. FAF and NIR images were taken with a Heidelberg Retina Angiograph 2 (Heidelberg engineering, Heidelberg, Germany) with a field of view of 30° and a resolution of 768 x 768 pixels. In total 278 eyes of 230 patients aged 65 years and older were selected from the last examination round of the Rotterdam Study. All CF images were graded according to the Wisconsin Age-related Maculopathy Grading[23] and the International Classification and Grading System for age-related maculopathy and age-related macular degeneration[24] by local graders of the Rotterdam Study using visual assessment. These annotations constitute the reference standard for our study. We selected all the eyes where RPD based on CF images were identified in this round (N=72). Status of RPD was also confirmed on FAF and NIR. For positive and negative controls we selected eyes that were graded by the local Rotterdam Study graders as having soft distinct or soft indistinct drusen but not with RPD (N=108) and eyes that did not contain any type of drusen (N=98). The positive and negative controls did not have any signs of RPD in the other modalities (FAF and NIR). As the database did not contain any information about the extent of RPD area, two human observers (G.B. and C.B.) made in consensus RPD area delineations using the three modalities simultaneously for the eyes containing RD. These delineations were used as reference standard for the quantification of RPD area.

Observer study: single- versus multi-modality grading

All images were evaluated independently by two human observers (G.B. and C.B.) for evidence of RD. RPD were defined as indistinct, yellowish interlacing networks of 125 µm to 250 µm wide on CF image;[14] groups of hyporeflectant lesions in regular patterns on FAF[15-17] and groups of hyporeflectant lesions against a mildly hyper-reflectant background in regular patterns on NIR images images.[17] Observer 1 has 4 years of reading experience for all three imaging modalities, whereas Observer 2 has 19 years of reading experience on CF imaging and 5 years on FAF and NIR imaging. The observers were asked to give a score ranging from 0 to 1, indicating the likeliness of presence of RD. Two different grading approaches were performed: single- and multi-modality grading. During single-modality grading, the observers graded each image modality separately in a randomized order. CF, FAF and NIR images were pooled and shown randomly to the observers. Observers were also asked to indicate whether the image was of sufficient quality for grading. Bad quality was assigned if the observer was not confident in assessing the image for RPD as a result of low image quality. During multi-modality grading, observers were asked to diagnose RPD after observing CF, FAF and NIR images from the same eye simultaneously. The eyes were shown in a randomized order in this grading session as well.

In a separate grading session, the observers manually delineated in consensus the area covered by RPD based on one single modality, i.e. single-modality RPD delineation on CF, FAF or NIR images. Only the 72 eyes containing RPD as indicated by the reference were taken into account for the quantification of RPD area.

Automatic reticular drusen identification

The proposed machine learning algorithm analyzed simultaneously the available modalities from an eye examination to automatically identify reticular drusen areas. The algorithm assigned the complete eye examination a probability between 0 and 1 indicating the probability of presence of RPD and provided a quantification of the area covered by them. To accomplish this, the algorithm performed three steps: preprocessing, feature extraction, and classification and quantification.

Preprocessing

In the preprocessing step, two different methods were applied to the images: image registration and vessel removal.

1. Registration will provide a geometrical alignment across modalities to identify corresponding pixels that represent the same scene. This multi-modal image registration was performed using a semiautomatic affine method, where the images are deformed to accurately match user specified points or landmarks.[25] In this study, three corresponding landmarks on prominent image locations, such as vessel bifurcations, were manually selected on each modality and used to perform the registration.
2. To reduce intensity variations due to presence of vessels, the retinal vasculature was removed from the images. The vasculature was automatically extracted using a previously developed algorithm[26] and used as input in an inpainting algorithm,[27] which removes the vessels by interpolating intensities at the supplied image locations. Figure 1 shows an example eye after vessel removal.

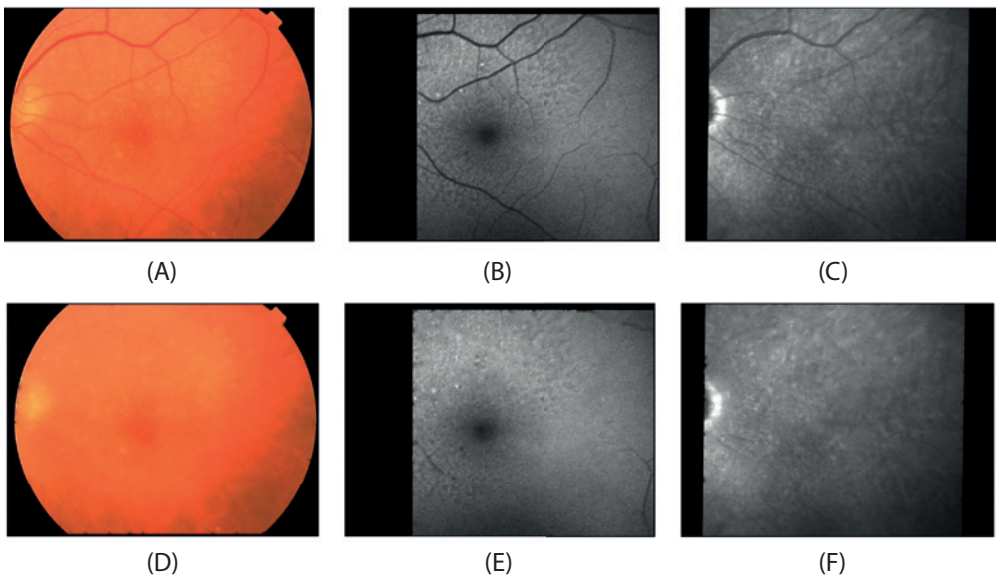

FIGURE 1 - Co-registered (A) color fundus photograph, (B) fundus autofluorescence and (C) near infra-red images and their corresponding results after vessel removal (D-F).

Feature extraction

To perform an automatic analysis of the images, the machine learning algorithm uses information which is extracted from the images and encoded in numerical values or so called features. To do so, each color channel of the CF image as well as the FAF and NIR image were separately convolved with a set of Gaussian filters. These filters are based on Gaussian derivatives up to second order at different scales and are invariant to rotation and translation.[28] For each resulting filtered image, the mean, standard deviation, skewness and kurtosis values in a circular neighborhood around each pixel were calculated. The corresponding features for each pixel were then obtained by concatenating these extracted values in a single feature vector.

Classification and quantification

To determine whether a pixel is part of a RPD area, a random forest classifier was used to obtain an automatic classification based on the calculated features. This classifier operates by constructing a multitude of decision boundaries (trees) to make a separation between multiple classes.[29] After training, the random forest classifier provided a probability between 0 and 1 indicating the probability that the pixel belongs to a RPD area based on labeled training examples and the input pixel feature vector. Figure 2 shows an example eye exam with the output of the classifier. Finally, an image score indicating the likelihood of the eye exam to contain RD, was assigned by taking the 99th percentile of the obtained probability map.

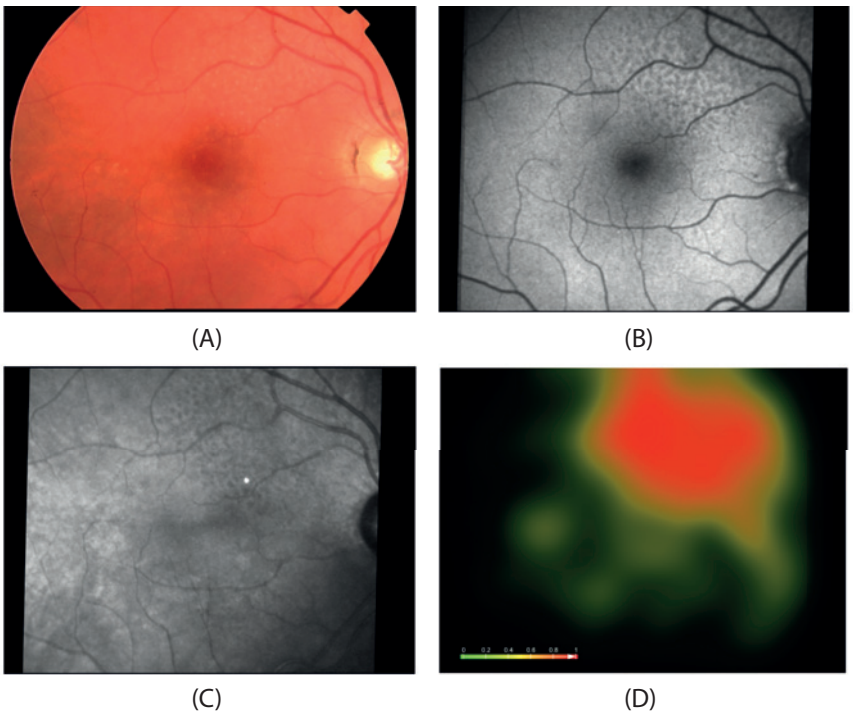

FIGURE 2 - Example of the classification result obtained by the proposed machine learning algorithm. Given an eye exam consisted of (A) a color fundus photograph, (B) a fundus autofluorescence image and (C) a near infrared image; the algorithm outputs (D) a probability map indicating the likelihood for each pixel to be part of a reticular drusen area. Red values indicate higher probability to be reticular drusen.

To quantify the area covered by RD, a threshold was set on the probability map. This threshold was image based and experimentally determined as the 55th percentage of the maximum value of the probability map. Only the area inside the Early Treatment Diabetic Retinopathy Study (ETDRS) grading grid was taken into account for the quantification.

Statistical analysis

The performance of the observers and the proposed machine learning algorithm for the single- and multi-modality approaches was evaluated by measuring the area (Az) under the Receiver Operating Characteristic (ROC) curve.[30] Statistical comparisons were made using bootstrap analysis with 5000 bootstraps.[31] Bootstrap analysis is an nonparametric test that is commonly used to estimate the variance of ROC analysis. Results with a p-value lower than 0.05 were seen as statistically significant. Bonferroni correction was applied to counteract the problem of multiple comparisons.[32] For observers, kappa statistics were also reported to assess inter observer variability.[33] As the proposed machine learning algorithm requires labeled example data for training, the evaluation was performed using a patient-based leave-one-out strategy.[34]

Automatic quantification of RPD area was evaluated by calculating the percentage of detected RPD area inside the ETDRS grading grid and was compared with the observer delineations. RPD area agreement with observers was measured using intra-class correlation (ICC) statistics.

RESULTS

Image quality assessment

Table 1 shows the image quality analysis of the observers for the different image modalities. Of the 278 eyes, only 172 (61.9%) were graded by both observers to have all image modalities with good quality and were established as the "good quality" set for the subsequent data analysis. Bad quality of the FAF image was the main reason for a bad quality indication for the multi modal exam (CF+FAF+NIR).

TABLE 1 - Number and percentage of good quality images as indicated by observers for the different image modalities independently. Last column shows the number of images where both observers agree that the image is of good quality.

	Observer 1	Observer 2	*Consensus*
CF	272 (97.8%)	268 (96.4%)	264 (95.0%)
FAF	211 (75.9%)	195 (70.1%)	185 (66.5%)
NIR	269 (96.8%)	265 (95.3%)	264 (95.0%)

Abbreviations: CF = color fundus photographs, FAF = fundus autofluorescence images, NIR = near infra-red images.

Comparison of single- and multi-modality grading

Figure 3 shows the ROC curves for the single- and multi-modality grading approaches. The point on the curve closest to the upper left corner in the ROC curve is used to compute sensitivity/specificity pairs.

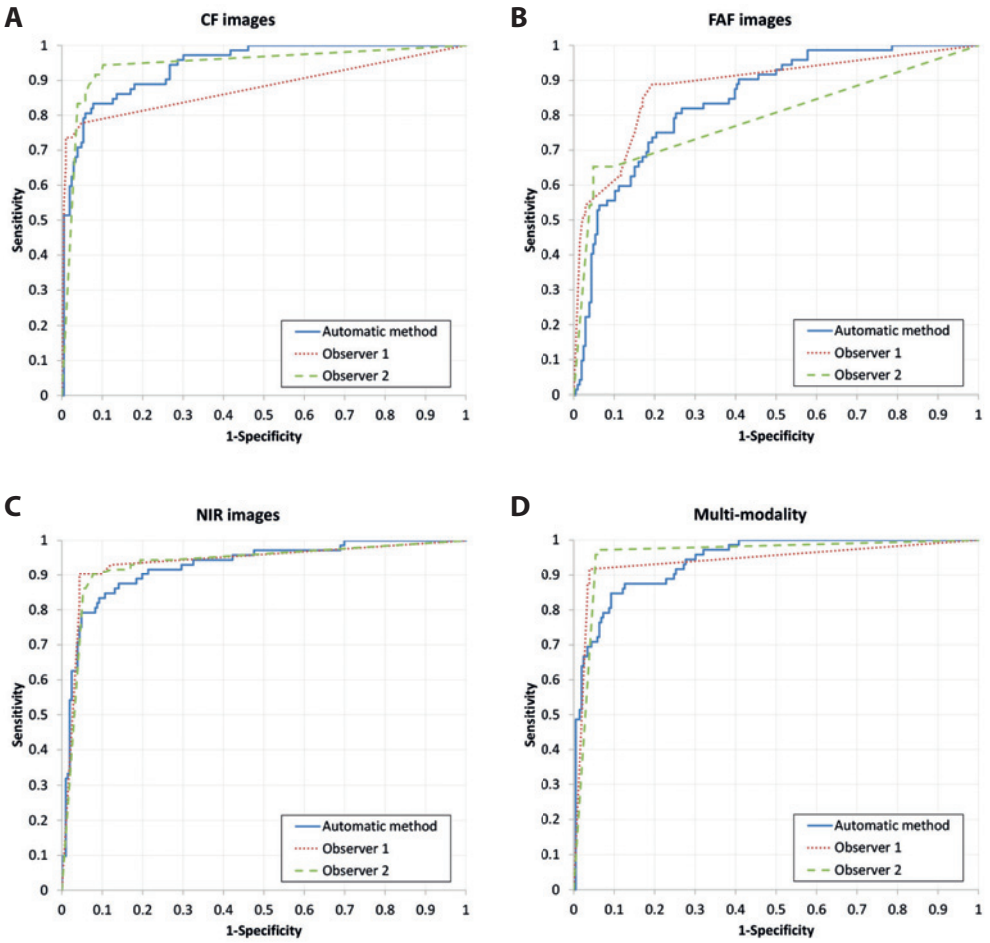

FIGURE 3 - ROC curves for the identification of eyes with reticular drusen using (A) color fundus photographs, (B) fundus autofluorescence images, (C) near infra-red images and (D) a multi-modality setup.

Table 2 shows the Az values and sensitivity/specificity pairs for the single- and multi-modality grading of Observer 1 and Observer 2, respectively, calculated on the full data set and on the subset of good quality images as indicated by both observers. The performance of both observers for RPD detection considerably increased when performing a multi-modality grading.

Kappa statistics were calculated to measure inter-observer variability during single-modality and multi-modality grading sessions. Table 3 shows the kappa values between the observers for the different grading sessions. Observers achieved a higher agreement using multi-modality grading. When considering only good quality images, observers also achieved high agreement using FAF images.

TABLE 2 - Performance of Observer 1 and 2 for RPD detection using single- and multi-modality grading.

			All			Good quality		
			Az	Se	Sp	Az	Se	Sp
Observer 1	Single-modality	**CF**	0.879*	0.778	0.951	0.888*	0.789	0.974
		FAF	0.881*	0.889	0.806	0.959	0.946	0.966
		NIR	0.936	0.903	0.956	0.936	0.929	0.918
	Multi-modality		0.940	0.917	0.961	0.956	0.944	0.963
Observer 2	Single-modality	**CF**	0.944	0.944	0.989	0.944	0.930	0.917
		FAF	0.793*	0.653	0.951	0.961	0.973	0.946
		NIR	0.932	0.903	0.922	0.929*	0.900	0.918
	Multi-modality		0.958	0.972	0.942	0.974	1.000	0.949

Area (Az) under the ROC values and optimal sensitivity (Se) and specificity (Sp) values are reported.
*Indicates a statistical significant difference of the Az value with respect to the multi-modality approach.
Abbreviations: CF = color fundus photographs, FAF = fundus autofluorescence images, NIR = near infra-red images, RPD = reticular pseudodrusen, ROC = receiver operating curve.

TABLE 3 - Kappa agreement and 95% confidence intervals between observers for single-modality and multi-modality reading sessions.

		All		Good quality	
		κ	95% CI	κ	95% CI
Single-modality	**CF**	0.654	(0.556-0.752)	0.724	(0.632-0.817)
	FAF	0.468	(0.363-0.572)	0.938	(0.879-0.998)
	NIR	0.884	(0.822-0.945)	0.839	(0.767-0.910)
Multi-modality		0.911	(0.857-0.965)	0.936	(0.874-0.998)

Abbreviations: CF = color fundus photographs, CI = confiodence interval, FAF = fundus auotofluorescence images, NIR - near infra-red images.

Performance of the automatic method

The ROC curves for the proposed machine learning algorithm are shown in Figure 3. The corresponding Az values and the sensitivity/specificity pairs for the single- and multi-modality approaches are summarized in Table 4.

Quantification of the area covered by reticular drusen

The boxplots in Figure 4 show the RPD area percentage inside the ETDRS grading grid as delineated by the observers and as identified by the automatic system. Only eyes which were of good quality as indicated by both observers were taken into account. The multi-modality area delineations made during consensus grading of the two observers was used as the reference for the RPD area quantification.

The agreement between single-modality RPD area delineations made by the observers and the reference delineations set using multi-modal information reached ICC values of 0.580 (-0.034;0.830), 0.790 (0.409;0.920) and 0.930 (0.763;0.976) for the CF, FAF and NIR delineation, respectively. For the automatic quantification of the RPD area, ICC values of 0.637 (0.395;0.796), 0.389 (0.082;0.631) and 0.557 (0.280;0.747) were obtained for the single-modality analysis of CF, FAF and NIR with respect to the reference delineations. Comparing the automatic multi-modality approach with the reference standard, an ICC value of 0.704 (0.495;0.837) was obtained.

TABLE 4 - Performance of the automatic system for RPD detection using single- and multi-modality grading.

		All			Good quality		
		Az	**Se**	**Sp**	**Az**	**Se**	**Sp**
Single-modality	**CF**	0.942	0.833	0.922	0.939	0.887	0.860
	FAF	0.844*	0.806	0.747	0.935	0.919	0.891
	NIR	0.927	0.847	0.893	0.919	0.814	0.902
Multi-modality		0.941	0.875	0.873	0.949	0.861	0.882

Area (Az) under the ROC values and optimal sensitivity (Se) and specificity (Sp) values are reported.
*Indicates a statistical significant difference of the Az value with respect to the multi-modality approach.
Abbreviations: CF = color fundus photographs, FAF = fundus autofluorescence images, NIR = near infra-red images, RPD = reticular pseudodrusen, ROC = receiver operating curve.

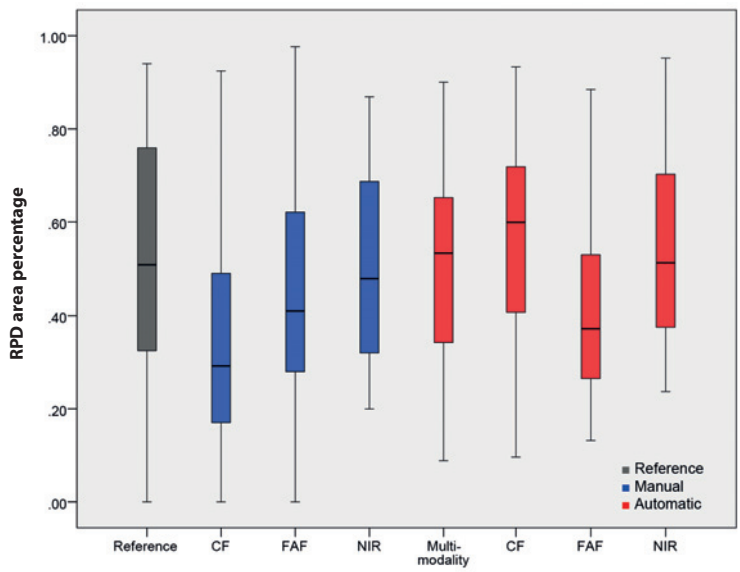

FIGURE 4 - Box-plots showing the percentage of reticular drusen area inside the ETDRS grading grid. Manual multi-modality was seen as the reference and is shown in grey. Single modality manual area percentages are shown in blue. The area percentage obtained by the automatic method for single- and multi-modality analysis are shown in red.

Abbreviations: CF = color fundus photographs, ETDRS = Early Treatment Diabetic Retinopathy Study, FAF = fundus autofluorescence images, NIR = near infra-red images, RPD = reticular pseudodrusen.

DISCUSSION

In this study, we assessed the performance achieved for RPD detection using multimodal information and compared it to the one obtained using several single image techniques. In our larger dataset,[11-13,17] we demonstrated that a significantly higher performance, as well as a better inter-rater agreement, was achieved when the reticular pattern was assessed in a multi-modality grading approach. Moreover, our automatic machine learning algorithm for RPD detection and quantification using multimodal information proved to perform within the same range as the human graders.

Two independent human observers identified RPD areas using two different grading protocols. During the single-modality grading session, only information from a single image techniques was available; while, during the multi-modality approach, the observers evaluated evidence of RPD using all the modalities simultaneously. Both observers achieved higher performance using the multi-modality approach reaching Az values of 0.940 and 0.958, respectively (see Figure 3 and Table 2). Although previous studies only evaluated the accuracy for detecting RPD of single image modalities,[8,11,12] our results confirm their conclusions that a more accurate diagnosis of RPD is obtained using multiple image modalities.

In contrast to observer 1, observer 2 achieved high performance on RPD assessment using CF images. Possible reasons for this are the larger experience of this observer on this modality and the lower sensitivity that this image technique has.[12] The disparity between observers' performance was substantially reduced when the assessment was performed using multiple image modalities (see Table 2). When the observers scored FAF images, the performance was significantly lower than when they used multi-modality reading. This may be due to the poor quality level of the FAF images. Only 66.5% of the images were considered of good quality by both observers, as it is shown in Table 1. During the FAF acquisition, a mean intensity image is constructed to reduce noise in the image. However, eye movements may cause displacement errors resulting in a lower contrast and thus lower quality of the FAF image. Another reason is the presence of cataracts in the study population. The wavelength used for FAF imaging is more affected by cataract than the one used in NIR imaging, resulting in lower image quality. As it is shown in Table 2, the adoption of a multi-modality grading approach can overcome image quality issues, maintaining a high detection performance independently of the quality level of a particular image techniques. When considering only the subset of good quality images, the performance of both observers increases for both single- and multi-modality gradings.

Inter-observer agreement was also investigated using the two grading protocols. Table 3 shows that the agreement between observers substantially increased when multiple imaging techniques were used to evaluate the evidence of RD. When taking only the subset of good quality images into account, the agreement between observers improved using CF, FAF and the multi-modal approach. However, the agreement using CF images is still substantially lower than using the other modalities. Other studies included multiple graders but no information about inter-observer agreement was reported.[12]

In this study, we also developed and evaluated a machine learning algorithm for the automatic identification and quantification of RPD using multimodal information. The results showed that the proposed system achieved similar performance as the observers, see Figure 3 and Table 4. Similar to the observers' gradings, the incorporation of multimodal information improved the performance of the algorithm. Using multimodal information, the proposed algorithm achieved an Az value of 0.941 and a sensitivity/specificity pair of 0.875/0.873. Compared to the observers, that reached a

kappa agreement of 0.87 with the reference, the automatic system has a kappa agreement of 0.70. However, 20% of the misclassified cases corresponds to cases where the observers also disagree. Of the false positive cases, 9 cases contained low quality images, 3 cases presented geographic atrophy, 1 case showed a neovascular macular detachment and 12 cases contained soft indistinct drusen. As described in other publications[11,35,36], RPD and drusen have very similar characteristics and might therefore more easily be misinterpreted by the automatic system. Better discriminant features, such as image context information or local intensity changes, might improve the performance of the automatic system, but this has to be further investigated.

Quantification of RPD area is a more difficult task due to the undefined boundaries of RD.[15-17] When comparing the manual delineations performed on CF images with the reference delineations based on multi-modal information, an ICC value of 0.580 was achieved. When comparing the FAF or NIR delineations with the reference delineations, the agreement was better, reaching ICC values of 0.790 and 0.930, respectively. As presented in Figure 4, the RPD area was underestimated using CF images when compared to the other image techniques. As reported in previous publications,[12,17] the visibility of RPD differs over modalities, causing these differences. As RPD are more pronounced on FAF and NIR, the delineations on these modalities were more similar to reference delineations. The quantified RPD area, which was automatically obtained by the proposed algorithm, was in agreement with the area delineated by the observers, reaching an ICC value of 0.704. It has to be noted that only images of good quality were used for RPD area quantification as images with insufficient quality were not suitable as it was nearly impossible for observers to delineate RPD area on these images. Another limitation of this study was that the multi-modal approach only included fundus images, excluding information obtained with spectral domain optical coherence tomography (SD-OCT). Including this modality in the multimodal protocol, a better understanding of the reticular pattern might be obtained and, consequently, an increased accuracy in their identification.[9,10,35] SD-OCT can provide 3D information about RPD formation and is essential for RPD volume measurements. This enhancement will be of great importance for clinical trials studying the development and progression of RPD. We will investigate this improvement in further studies.

In conclusion, we were able to show that a multimodal approach significantly increased observer performance and inter-observer agreement for detection of reticular drusen in fundus images when the information of different imaging modalities was evaluated simultaneously. Furthermore, an automatic machine learning algorithm for detection and quantification of RPD using multimodal information was developed and evaluated, showing comparable results with those obtained by observers. The area covered by RPD was also automatically quantified by the algorithm, tallying the values manually provided by the observers. The absence of SD-OCT is regarded as a limitation of this study and will be investigated in future work. This automatic algorithm yields a quick and reliable diagnosis and quantification of reticular drusen, allowing for large dataset analysis within population studies and to gain insights into risk factors involved in AMD and disease progression.

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Chapter 4.1

Trace elements, vitamins, and lipids and age-related macular degeneration. An overview of the current concepts on nutrients and AMD

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SUMMARY

In this chapter we provide an overview of the current concepts on trace elements, vitamins, lipids in their relationship to age-related macular degeneration (AMD). Intake zinc, and omega-3 fatty acids can help lower the risk of AMD, in particular in those at high genetic risk. Vitamin D and nutrients influencing epigenetics appear to have a beneficial effect, but more research is needed before recommendation of these nutrients is warranted.

Dietary nutrients have been implicated in the development of age-related macular degeneration (AMD) for years. The first paper reporting a beneficial effect of a nutrient on AMD was published in 1988, in which the National Health and Nutritional Examination Survey 1 (NHANES-1) study investigated the dietary intake of vitamin A (*P* trend =0.058) in AMD.[1] Since then, many more papers on this topic have been published. This chapter will provide an overview of nutrients investigated in relation with AMD.

CAROTENOIDS

There are many different carotenoids; over 600 are known to date. These can be split in two groups: carotenes, which refer to hydrocarbon carotenoids, and xanthophyll, a carotenoid with one or more oxygen groups. Carotenoids are pigments and can be found in chloroplasts and chromoplasts in predominantly plants and algae. Their function is to absorb blue light to protect the plants and algae from photo-damage, and absorb the light energy for use in photosynthesis. In the eye, lutein and zeaxanthin are xanthophylls which protect the macula from blue and ultraviolet (UV)-light damage. All dietary carotenoids have antioxidant function; α carotene, β carotene, γ carotene and β cryptoxanthin also have vitamin A activity. These four carotenoids are converted to retinal in herbivores and omnivores.[2]

Astaxanthin

Astaxanthin is a red pigmented xanthophyll, and can be found in salmon and other red colored sea foods (Table 1). Two studies added this carotenoid to a cocktail of anti-oxidants, including lutein and zeaxanthin. Both studies reported a positive effect of this cocktail on visual functions. Nonetheless, a specific beneficial effect for astaxanthin cannot be concluded from these results.[3,4]

α Carotene

This carotene is an antioxidant and has vitamin A activity (Retinal Activity Equivalents; RAE); 1 RAE = 1 µg retinol or 24µg α carotene. A meta-analysis based on four prospective cohort studies reported no significant association for α carotene and the risk of developing early AMD (Odds' ratio (OR) 1.05; 95% CI 0.87-1.26).[5] However, one of these studies did find a trend for intake of α carotene ten years prior to baseline examination and incident large drusen (*P* trend = 0.02).[6] Another study found a trend with pigmentary changes (*P* trend = 0.03), but not with drusen.[7]

Recently, a study investigated serum levels of carotenoids and the risk of AMD in a Chinese population sample.[8] Serum levels of α carotene were significantly lower in subjects with exudative AMD versus controls ($P < 0.001$), while subjects with early AMD had higher serum levels versus controls ($P < 0.001$). The relative risk was reduced for exudative AMD (Relative risk (RR) 0.24; 95% CI 0.12-0.51), and increased for early AMD (RR 2.22; 95% CI 1.37-3.58). Why α carotene would increase the risk for early AMD and not for late is not clear.

β Carotene

This antioxidant has vitamin A activity; 1 RAE = 12µg β carotene and has been studied intensively in many diseases. A meta-analysis on this nutrient reported no association with incident early AMD (RR 1.04; 95% CI 0.86-1.25).[5] Contradictory results were found for late AMD. Two studies from the US on exudative AMD reported a lower risk for those with higher intakes; [9,10] in contrast, a study from Australia found a higher risk (*P* trend = 0.027).[11] β carotene supplements have also been tested. A meta-analysis based on two randomized controlled trials showed neither a significant association

with any AMD (RR 1.03; 95% CI 0.89-1.19) nor with advanced AMD (RR 0.97; 95% CI 0.69-1.36).[12] Serum levels of β carotene were investigated in a Chinese population. The investigators found higher levels to be associated with an increased risk of exudative AMD (RR 2.36; 95% CI 1.30-4.29).[8] This would favor the diet findings from the earlier mentioned Australian study. Differences in population structure, definition of the outcome, and measurement error of the exposure may explain the difference of study results.

TABLE 1 - Nutrients in Foods

Nutrient	Foods
Astaxanthin	Salmon, throut, shrimp, crayfish
Betaine	Grain products, fish, spinach, sugar beets
α Carotene	dark-leafy vegetables (spinach, kale) yellow/orange vegetables (carrots, bell peppers)
β Carotene	dark-leafy vegetables (spinach, kale) yellow/orange vegetables (carrots, bell peppers)
β Cryptoxanthin	Orange rind, egg yolk, papaya, apples
Lutein	dark-leafy vegetables (spinach, kale) yellow/orange vegetables (carrots, bell peppers)
Lycopene	Red fruits and vegetables (tomatoes, bell peppers, watermelon)
Meso-zeaxanthin	Sea foods
Methionine	Poultry, fish, dairy products
Omega-3 fatty acids - ALA	vegetable oils (flaxseed, canola oil)
Omega-3 fatty acids - DHA/EPA	Oily fish (herring, salmon, sardines, trout)
Omega-6 fatty acids - LA	vegetable oils (canola oil, safflower oil, corn oil)
Omega-6 fatty acids - AA	poultry, meat
Resveratrol	Skin red grapes, other fruits, red wine
Selenium	Shellfish and crustacea, egg yolk
Vitamin A	liver, butter, cheddar cheese, milk
Vitamin C	Fruits and vegetables (kiwi, peppers, parsley, rose hips)
Vitamin D*	Oily fish, dairy products, beef and fish liver
Vitamin E#	Corn oil, soybean oil, margarine, dressings
Zinc	Fortified cereals, meats, dairy products, nuts, seeds
Zeaxanthin	dark-leafy vegetables (spinach, kale) yellow/orange vegetables (carrots, bell peppers)

Abbreviations: AA, arachidonic acid; ALA, α-liolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid

* Levels of vitamin D depends on country. Some foods (mostly dairy products) are fortified with vitamin D

There are many different forms of vitamin E, the most common is α-tocopherol

β carotene may have interaction with genetic factors. Dietary intake was analyzed for AMD genotypes and the risk of early AMD in the Rotterdam Study.[13,14] Carriers of the risk variant *CFH* (Y402H) had a higher risk of developing AMD in particular in those that had a low intake of β carotene. Carriers could reduce their AMD risk by increasing their intake (*P* trend = 0.05) (Figure 1). There are several explanations. β Carotene is inversely related to CRP levels, and CRP is related to the *CFH* (Y402H) genotype and AMD.[15] These results imply that the genetic risk can be 'eaten' away. Whether supplementation would further decrease the risk is not clear and requires a note of caution. The

results of two randomized controlled trials indicated that β carotene supplementation in smokers increased risk of developing lung cancer, and many commercial supplements do not include this nutrient anymore.[16,17]

β Cryptoxanthin

This carotenoid is very similar to β carotene, except for one oxygen group. The retinal activity of β cryptoxanthin is two times lower than β carotene; 1 RAE = 24 µg β cryptoxanthin. In the literature, an inverse correlation of β cryptoxanthin with lung cancer has been reported in in vitro studies. This nutrient could potentially act as a chemoprotective agent in cancer, [18] but this beneficial effect is questionable since it appears to cause a lower survival in patients with malignant glioma.[19]

No consistent associations have been found for β cryptoxanthin and AMD.[5,7-11,20,21]

Lutein, zeaxanthin and meso-zeaxanthin

The pigment of the macula consists of lutein, zeaxanthin and meso-zeaxanthin. These carotenoids absorb blue light and UV, and protect the retina from damage by free radicals. Dietary intake (Table 1) is very important, because the human body cannot synthesize lutein/zeaxanthin. However, there are some reports that meso-zeaxanthin might be synthesized from lutein in the retina.[22] Because of the macular location, these nutrients have been of high interest in ophthalmic research, especially in AMD.

Serum levels lutein/zeaxanthin

Many studies have investigated serum levels of lutein/zeaxanthin in patients and controls. In the majority of the studies an inverse association was found for lutein/zeaxanthin levels in serum with early, late and any AMD. In stratified analysis, zeaxanthin was more significantly associated with AMD than lutein.[23]

Dietary lutein/zeaxanthin

A meta-analysis of observational studies on diet has been performed with data from six studies. Overall, the risk of early AMD was not significantly associated with intake of lutein/zeaxanthin (RR 0.96; 95% CI 0.78-1.17).[24] The meta-analysis for advanced AMD did show an association with intake of lutein/zeaxanthin (RR 0.74; 95% CI 0.57-0.97), most prominent for exudative AMD (RR 0.68; 95% CI 0.51-0.92).

Case control studies also reported a significant association of lutein/zeaxanthin intake with a lower risk for advanced AMD.[9,10] Seddon et al (1994) also showed an inverse trend with intake of lutein/zeaxanthin, a trend which was particularly present in smokers.

As β carotene, lutein/zeaxanthin also interacted with genetic factors. High intake of these nutrients was associated with a reduced genetic risk for carriers of CFH risk variants (*P* trend = 0.05) (Figure 1).[15]

Supplementation of lutein/zeaxanthin

A number of small studies have investigated this topic. Some studied the retina of healthy subjects who received supplementation of lutein and/or zeaxanthin, with or without meso-zeaxanthin. After duration of 8-52 weeks, the macular pigment optical density (MPOD) was significantly higher than at baseline.[23] Consumption of lutein/zeaxanthin rich foods like egg yolks or spinach improved serum lutein and zeaxanthin levels significantly. MPOD did improve in all groups, but only significantly in those with low MPOD at baseline and consumption of 4 egg/yolks per day.[25,26]

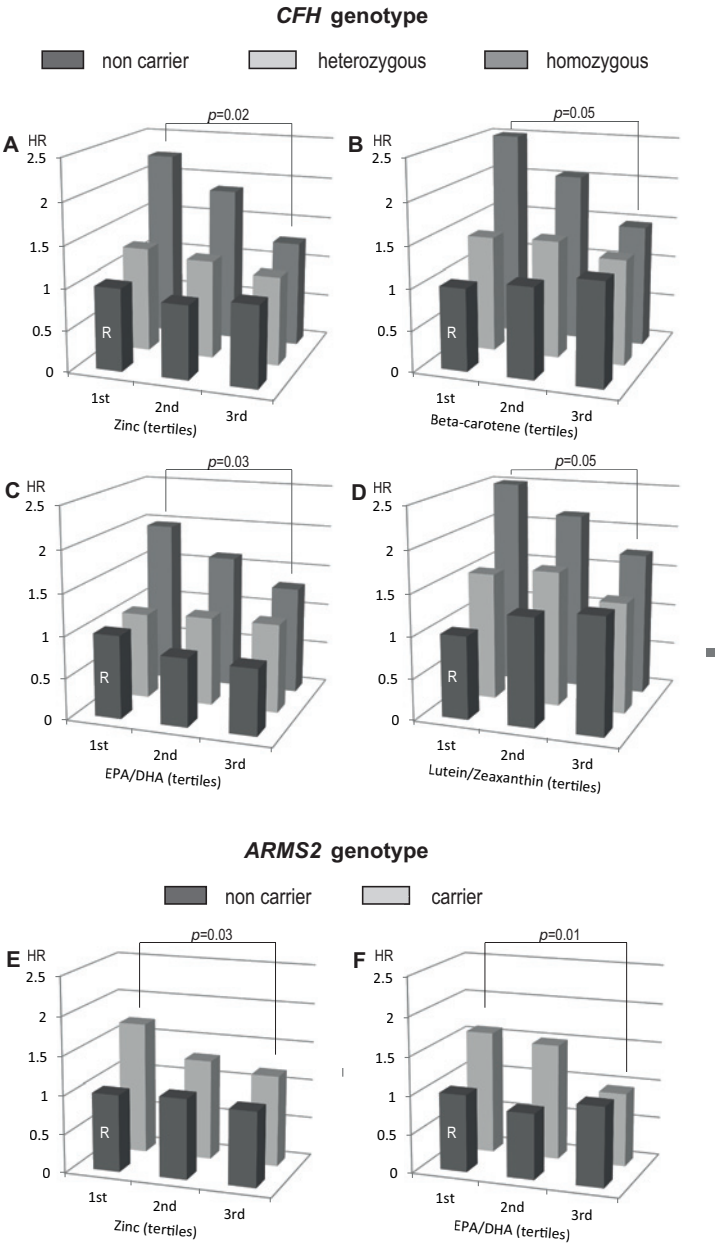

FIGURE 1 - Gene-environment interactions in the Rotterdam Study
A - D. Joint effect of dietary nutrient intake and *CFH* Y402H genotype on the risk of Early AMD;
E – F. Joint effect of dietary nutrient intake and *LOC387715 (ARMS2)* A69S genotype on the risk of Early AMD.
R is the common reference group.
HRs are estimates of the relative risk of Early AMD, and represent the risk of disease (Early AMD vs No AMD) in the various genetic-environmental risk groups divided by the risk of disease (Early AMD vs No AMD) in the common reference group (R). HRs are estimated with Cox regression analyses and included age, sex, smoking status, and atherosclerosis.

When lutein or lutein/zeaxanthin were supplemented in participants with early or even advanced AMD, these studies reported improvement of visual functions (e.g. visual acuity and contrast sensitivity), increase in serum lutein and zeaxanthin levels, and MPOD.[23,27] Since most carotenoids are lipid-soluble pigments, adding fatty acids to the oral supplementation could improve uptake of lutein/zeaxanthin in the gut. However, one study investigated this hypothesis and did not find a change of serum levels of lutein and zeaxanthin if omega-3 fatty acids were added to lutein/zeaxanthin supplementation.[28]

Only one randomized controlled trial compared supplementation of zeaxanthin (8 mg) to supplementation of lutein (9 mg), and to supplementation of zeaxanthin (8 mg) and lutein (9 mg) combined, versus placebo. MPOD was elevated in all supplementation groups, but was only significant in the groups supplemented with zeaxanthin or lutein alone. Zeaxanthin improved foveal cone-based visual parameters, while lutein enhanced those associated with gross detailed rod-based vision. In the lutein/zeaxanthin group, the two carotenoids were dosed equally rather than 10:1-2 which is the natural ratio. This may have led to duodenal, hepatic-lipoprotein or retinal competition between these nutrients, prohibiting efficient uptake and activity. [29]

Several design issues of the studies should be discussed. The majority of the studies on advanced cases of AMD investigated geographic atrophy (GA) and focused on visual acuity as the outcome event. An outcome as enlargement of surface area of the atrophic lesions would have been more objective. Another issue is the size of the studies. Most case-series and trials were very small (N= 5-136 participants). Contrary to other nutrients, there is no recommended daily allowance or upper level of intake (Table 2) for lutein/zeaxanthin, and harmful effects with unnatural high dosages are currently unknown.

Lycopene

This carotene can be found in tomatoes and other red vegetables (Table 1); it has no retinal activity. In most studies, dietary intake of lycopene was not associated with early or advanced AMD.[5,9,10] However, Morris et al. (2007) did find a trend for lycopene and pigmentary abnormalities; a history of higher intake of lycopene decreased the risk of pigmentary abnormalities (*P* trend = 0.02).

Serum levels of lycopene were inversely correlated with early and advanced AMD (RR 0.49; 95% CI 0.28-0.86 and RR 0.22; 95% CI 0.1-0.48 respectively).[8] A few small serum studies reported similar results for early, late and any AMD.[20,21,30]

TRACE ELEMENTS

Trace elements are dietary minerals and are needed in small amounts for normal cell function. Often a trace element is the core of enzymes. However, in large amounts trace elements are toxic.

Zinc

The retina contains high amounts of zinc suggesting a crucial role for this trace element. [31] Zinc has antioxidant functions and also acts as a cofactor in several enzymes including retinol dehydrogenase, an important enzyme in the vitamin A visual cycle.

TABLE 2 - Estimated Average Requirements (EAR), Recommended Dietary Allowances (RDA), Adequate Intakes (AI) and Tolerable Upper intake levels

	EAR				**RDA / *AI***				**Tolerable Upper intake**			
	Males		**Females**		**Males**		**Females**		**Males**		**Females**	
Nutrient	51-70 yrs	> 70 yrs	51-70 yrs	> 70 yrs	51-70 yrs	> 70 yrs	51-70 yrs	> 70 yrs	51-70 yrs	> 70 yrs	51-70 yrs	> 70 yrs
Choline (mg/day)*	ND	ND	ND	ND	*550*	*550*	*425*	*425*	3500	3500	3500	3500
Lutein and Zeaxanthin**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lycopene**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Retionol activity equivalents (RAEs)***	625	625	500	500	900	900	700	700	3000	3000	3000	3000
Omega-3 fatty acids**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Selenium (µg/day)	45	45	45	45	55	55	55	55	400	400	400	400
Vitamin C (mg/day)	75	75	60	60	90	90	75	75	2000	2000	2000	2000
Vitamin D (µg/day)****	10	10	10	10	15	20	15	20	100	100	100	100
Vitamin E (mg/day)*****	12	12	12	12	15	15	15	15	1000	1000	1000	1000
Zinc (mg/day)	9.4	9.4	6.8	6.8	11	11	8	8	40	40	40	40

Taken from United States Department of Agriculture

NOTE: Estimated Average Requirements (EAR) is the average daily nutrient intake level estimated to satisfy the needs of 50% of the healthy individuals in a group; Recommended Dietary Allowances (RDA) in ordinary type and Adequate Intakes (AI) in *italic type*; No Data (ND)

* Only adequate intake for choline has been set. No data available for betaine of methionine

** No RDA, AI or upper limit has been set for these nutrients

*** 1 RAE = 1µg retinol, 12 µg β-carotene, 24 µg α-carotene, 24 µg β-cryptoxanthin; Upper intake was not determined for carotenoids;

**** Vitamin D as cholecalciferol. 1 µg cholecalciferol = 40 IU vitamin D; also under the assumption of minimal sunlight

***** As α-Tocopherol. 1 mg α-Tocopherol = 1.5 IU α-Tocopherol.

Dietary zinc

Several observational studies have investigated the role of dietary zinc and early AMD. Two studies reported an inverse trend for dietary and supplementary intake of zinc approximately ten years before ophthalmic examination and pigmentary abnormalities.[6,7] However, no association was found for early AMD (OR 0.91; 95% CI 0.74-1.11). A significant interaction was found between dietary zinc intake and the major susceptibility genes: C*FH* (Y402H) and *AMRS2* (A69S), respectively. Carriers of risk variants had a higher risk of AMD in the lower tertile of zinc intake, but risks lowered dramatically when intakes increased (Figure 1).[15]

Supplementation of zinc

Zinc supplementation had an inverse effect on developing advanced AMD in participants with signs of early AMD in a meta-analysis (OR 0.73; 95% CI 0.58-0.93). Zinc was supplemented as zinc oxide (80mg/day; together with cupric oxide, 2 mg/day) in the Age-related Eye Disease Study (AREDS) trial, and as zinc sulphate (200 mg/day) in the other trials. These dosages are highly above the recommended upper level of zinc intake (Table 2). [32] Zinc sulphate is the most common zinc salt in diet and supplements; zinc oxide has the longest history as a medicine, especially for skin irritations and wounds. Supplements versus AMD genotype appeared less significant than diet versus genotype. In the AREDS trial, non-carriers of *CFH* (Y402H) showed a more decreased risk after zinc supplementation than carriers, contradicting the findings with diet. [33]

Zinc also has the capability to interact with the complement cascade, it is known to downregulate complement activation. Compliment activation is an established mechanism of AMD pathogenesis, hence, this explains the positive effect of high zinc intake. *ARMS2* (A69S) may influence mitochondrial function leading to increased complement activation, which again can be counteracted by zinc.[15] Zinc supplementation at high levels may lead to side effects and complications, including gastrointestinal symptoms, anemia and more severe genitourinary causes, therefore, caution is warranted.[32,34]

Selenium

Selenium has antioxidant and inflammatory capacities and has therefore been investigated in AMD. No positive associations have been found. [35] Selenium has been supplemented, but always in combination with other antioxidants, prohibiting the study of the single effect.

VITAMINS

Historically, all vitamins were thought to be amines, hence, in 1921 Kazimierz Funk put together the words 'vital' and 'amine', and composed 'vitamine'. Vitamins are needed only in limited amounts.

Vitamin A

This fat-soluble vitamin was discovered in the beginning of the 20th century in butterfat, and it appeared to be associated with yellow-plant pigments, the carotenoids. Later, vitamin A was found in the retinal tissue of rats, and was named 'retinol' after the retina.[36] Total vitamin A includes carotenoids, originating from plants, as well as retinol, derived from animals. Foods containing high levels of retinol are liver and butter (Table 1). Carotenoids have been discussed earlier in this chapter.

The influence of vitamin A on ophthalmological health has long been known; a vitamin A deficient diet leads to diseases of the cornea, i.e., xerolphthalmia and keratomalacia, and also to diseases of the retina causing nyctalopia and hemeralopia. Retinal, the active form of retinol, bonds with 'opsine' to form 'rhodopsine', the photosensitive molecule of rod photoreceptors. Aside from the visual

function, vitamin A is also needed for growth, survival, and immunity. Supplementation of vitamin A has been investigated since the 1920s and has been associated with a reduction of mortality and morbidity in different infectious diseases. [37,38]

Total vitamin A and retinol

Goldberg et al (1988) investigated the data from NHANES-I and suggested a negative association between dietary vitamin A and AMD (*P* trend = 0.058). Since then vitamin A has been investigated by others. A meta-analysis included three prospective cohort studies and could not find an association of dietary vitamin A intake and incident early AMD (OR 0.98; 95% CI 0.81-1.18).[5] Nevertheless, total vitamin A intake including supplement use appeared to be associated with a decrease of pigmentary abnormalities (*P* trend = 0.01).[7]

For advanced AMD, a trend for total vitamin A intake without supplements was found in a case control study (*P* trend = 0.05).[10] Inclusion of supplement use increased the association (*P* trend = 0.02). In this study, retinol per se did not have a significant effect. In the AREDS study, dietary Vitamin A intake including retinol had a beneficial effect on advanced AMD.[9]

Vitamin C

L-ascorbid acid, or vitamin C, is a powerful water-soluble antioxidant. Most mammals can synthesize vitamin C from glucose in their liver, except for some species like humans. These species lack the enzyme gulonolactone oxidase; as a consequence, diet is their only source for vitamin C. When diet does not include vitamin C, this can lead to scurvy, a lethal condition if not treated appropriately. Thus, vitamin C is needed to survive.[39]

Vitamin C may play a role in the pathogenesis of AMD. This potent antioxidant could potentially inhibit cellular damage from free radicals provoked by ultraviolet exposure in the retina. This hypothesis has been tested in many different study designs.

Dietary vitamin C

A meta-analysis has pooled the point estimates from four large, high quality cohort studies. The meta-analysis results, just like the single study results, showed no association vitamin C with incident early AMD (OR 1.11; 95% CI 0.84-1.46). No meta-analysis was carried out for advanced AMD, since not every study had investigated advanced AMD. Those studies that did, did not find an association of vitamin C with incident advanced AMD.[5] A case-control study found a trend for intake of vitamin C versus a lower risk of neovascular AMD (*P* trend = 0.03).[10]

Supplementation of vitamin C

There are almost no studies which investigated vitamin C as the only supplement use. Most studies combined vitamin C together with other antioxidants.[34] Recently, findings from a randomized double-masked, placebo-controlled trial showed that there was no association of 500 mg vitamin C supplementation with incident AMD (Hazard Ratio (HR), 0.99; 95% CI 0.75-1.31).[40] Supplementation of vitamin C mostly included 500 mg. Experiments have shown that plasma of subjects is saturated at doses of 400 mg daily.[39] Increasing the administered dose will probably provide the same results.

In conclusion, no strong association with AMD has been found for vitamin C, nor in diet, nor as supplementation.

Vitamin D

Vitamin D does not only play a role in bone mineralization, but also has anti-inflammatory and antiangiogenic properties.[41] Hence, a role for vitamin D has been suggested.

There are different forms of vitamin D. The two main forms are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol), which only differ from each other by one methyl-group.[42,43] Vitamin D2 is a plant-derived form and can be produced through ultraviolet exposure of foods. A food source naturally rich in vitamin D3 is fatty fish (Table 1).[42] Diet, however, only contributes 10% of total serum level of vitamin D.[44] The majority of vitamin D3 is from endogenous produced vitamin D3, which is synthesized in the skin under influence of ultraviolet light of the sun. In the liver, vitamin D3 is hydroxylated into 25-hydroxyvitamin D3 (25(OH)D), and in the kidney, it is hydroxylated to 1,25-dihydroxyvitamin D3 (1,25(OH)D), the active metabolite of vitamin D.[42]

Several studies investigated the role of vitamin D with any form of AMD, as well as early and advanced AMD, and studied the nutrient intake as sunlight exposure, serum levels 25(OH)D, and intake of vitamin D via diet or supplements.

Vitamin D through sunlight exposure

The majority of vitamin D is produced through sunlight exposure of the skin. This environmental factor has been analyzed since the beginning of AMD research, with contradicting results. The combination of a potential harmful effect by DNA damage as well as a beneficial effect through Vitamin D may be an explanation.

Serum levels 25(OH)D

Parekh et al. (2007) was the first to report an association of serum levels of 25(OH)D with AMD in white non-Hispanics. The three highest quintiles of serum level 25(OH)D were inversely associated with early AMD after adjustment for age and serum cotinine, a biomarker for exposure to tobacco (*P* trend = 0.003).[41] Among non-Hispanic Blacks and Mexican American individuals a similar, albeit non-significant, effect was found. This study also analyzed the different characteristics of early AMD, soft drusen and pigmentary changes on the entire population. Soft drusen, but not pigmentary changes were associated with 25(OH)D serum levels; *P* trend = 0.006 and 0.40 respectively. An association with advanced AMD was not found, most likely due to the small number of advanced cases.

An inverse association of 25(OH)D levels with early AMD, but not with advanced AMD, was found in postmenopausal women < 75yrs (*P* trend = 0.01).[44]

A few studies have analyzed hypovitaminosis D as a marker for AMD. No associations within these analysis has been found for neovascular AMD [45], but a significant association was reported for hypovitaminosis with any form of AMD.[46] Serum level of 25(OH)D lower than 50nmol/L increased the risk of AMD 3 times (OR 3.03; 95% CI 1.04-8.80). Although interesting, these results need to be interpreted carefully since the number of cases and controls was small (N=65), and the study has not yet been replicated.

Dietary vitamin D

A positive association with decreased intake was found by a twin study, which selected 28 discordant twin pairs: one twin was diagnosed with advanced AMD; the other twin had no or only signs of early AMD. Vitamin D intake appeared significantly higher in twins with no or only early AMD (*P* = 0.048).[47] In some countries, dairy products are enriched with vitamin D, such as liquid milk in the United States

of America. One study showed that milk consumption was positively correlated with serum vitamin D levels (Pearson correlation coefficient, 0.2; $P<0.001$), and more than weekly milk consumption was associated with lower odds for early AMD. But only soft drusen were significantly inversely associated with the consumption of milk. No association of advanced AMD with milk consumption was found.[41] Fatty fish is another food which is rich in vitamin D, and this may also attribute to the protective effect that has been found for fatty fish.[48,49]

Supplementation of vitamin D

Results of supplement use are inconclusive.[41,44] Most supplements contain vitamin D2, which is almost ten times less active than vitamin D3.[43] This may explain the difficulty in finding a strong association. Parekh et al. (2007) did not find an association in the total population, but, after excluding those with high intake due to milk consumption, he did. To disentangle the effects, it would have been more interesting if he analyzed the use of vitamin D supplements in the population not consuming any milk.

Vitamin E

Vitamin E refers to a group of tocopherols and tocotrienols, of which the former is the most profuse form in nature and has the highest biological activity. Vitamin E deficiency therefore mainly refers to α-tocopherol. Vitamin E is a fat-soluble chain-breaking antioxidant, and may protect the retina from damage caused by free-radicals. High levels of this nutrient can be found in corn and soybean oil (Table 1).[50]

Plasma levels α-tocopherol

In the POLA study, no significant association was found between plasma levels of α-tocopherol and advanced AMD ($P = 0.08$). When α-tocopherol plasma levels were standardized to plasma lipids like cholesterol and triglycerides, the ratio was negatively associated with advanced AMD ($P=0.004$). The ratio was also associated with any signs of early AMD, drusen or pigmentary changes, in subjects free of advanced AMD ($P=0.04$). All associations were adjusted for potential confounding factors.[51]

Dietary vitamin E

Several prospective cohort studies have investigated the role of dietary vitamin E and the risk of developing AMD. Of these studies, three were of high quality and results were pooled quantitatively using meta-analytic methods. An almost significant association was found for vitamin E and incident early AMD (OR 0.83; 95% CI 0.69-1.01).[5] For advanced AMD, an association was found in the long-term follow-up of one study; higher intakes of total vitamin E (including supplement use) predicted advanced AMD. [11] This was not confirmed by the other studies.

Supplementation of vitamin E

Recently, a meta-analysis was published concerning three large trials supplementing vitamin E to healthy subjects.[12] Vitamin E had been supplemented in different dosages of α-tocopherol, 50-402 mg = 75-600IU versus placebo for 4-10 years. The complete sample consisted of 40,887 participants of which 20,438 had received α-tocopherol supplementation; 20,449 had been assigned to placebo. In the supplemented group, 405 individuals developed early AMD, and 42 advanced AMD. In the placebo group, 458 progressed to early AMD; 31 to advanced AMD. Thus, no associations were found (any type of AMD RR 0.98; 95% CI 0.89-1.08; advanced AMD RR 1.05; 95% CI 0.80-1.39).

Many studies have investigated the role of vitamin E in chronic diseases. High-dose vitamin E supplements (≥ 268 mg=400 IU) have been linked to an increased risk of heart failure in people with vascular disease and diabetes. Supplements given in these trials were up to 15-fold higher than the maximum dietary intake in the cohort studies. Since a protective effect against AMD is dubious for vitamin E,[52] extra care should be taken not to prescribe supplementation to those with cardiovascular risk factors.

LIPIDS

Almost 20% of the dry weight of the retina is accountable to lipids. Over half of all the retinal fatty acids are unsaturated; the majority of these are polyunsaturated fatty acids (PUFAs). Docosahexaenoic acid (DHA) is a PUFA which can be found in the photoreceptor outer segments, and which has been shown to be a survival factor for photoreceptors. The high concentrations of this lipid in the retina and its anti-inflammatory properties [53] suggests a potential role for omega-3 fatty acids in retinal disease.

Dietary intake lipids

Reported outcomes of dietary omega-3 fatty acids, fish consumption (rich of omega-3 fatty acids) and nut consumption have all been shown to be protective against early and late AMD.[53] A meta-analysis showed an inverse effect of high intake of omega-3 fatty acids and late AMD (OR 0.62; 95% CI 0.48-0.82).[54] In contrast to omega-3 fat, high levels of trans-fat may potentially increase the risk of AMD, but consistent evidence on this notion is lacking.[53]

A large multi-centered case-control study found a link between the positive effect of omega-3 fatty acids and linolic acid, an omega-6 fatty acid. The beneficial effect of high intake of omega-3 fatty acids was particularly found in persons with a low intake of linolic acid.[55] Similar results have been described for fish intake.[48] This indicates that the ratio of omega-6/omega-3 fatty acids needs to be of the right balance. The most ideal ratio is 3:1 or 4:1, while in reality this is 10-50:1 for the average American.[56]

Interaction between AMD genes and intake of DHA and the other omega-3 lipid, eicopentaenoic acid (EPA), was studied in the Rotterdam Study. Homozygous carriers of the *CFH* variant Y402H could lower their risk of developing early AMD with high intake of DHA/EPA (*P* trend = 0.03). A same effect was found for carriers of the risk variant *ARMS2* A69S (*P* trend= 0.01).

A similar effect was found for fish intake and *CFH* Y402H carriers. Weekly consumption of fish was associated with reduced risk of late AMD for carriers of the risk variant.[49]

Supplementation of omega-3 fatty acids

Very little is known on supplementation of omega-3 fatty acids as a single nutrient and AMD. A pilot trial was carried out in 38 patients with drusenoid pigment epithelial detachment in one eye, and the effect of oral supplementation with EPA 720mg/day and DHA 480 mg day was compared to no treatment (control group). After 6 months of supplementation, a significant increase of serum levels and red blood cell membranes of EPA and DHA was found, while no change was found in the control group. Since no exudative AMD occurred during the short follow up time, no inferences on supplementation of EPA/DHA can be made.[57] Before long, the results of the AREDS2 trial are expected. In this trial the supplementation of lutein/zeaxanthin and/or omega-3 fatty acids and the risk of advanced AMD are being studied.[58]

EPIGENETICS AND NUTRIENTS

Epigenetics refers to functional changes of the genome without a change in DNA nucleotide sequence. This could explain phenotypical differences in diseased monozygotic twins.[59] Various nutrients may cause epigenetic changes. Among those reported to have this capacity, betaine (a choline derivate) and methionine have been associated with advanced AMD.[47] In the US Twin Study of Age-Related Macular Degeneration, Seddon et al. (2011) found high dietary intake of betaine to be inversely associated with grade of AMD (P = 0.009; adjusted for age, smoking and differences between twins), but not with drusen size, drusen area or pigment area. She found an inverse association for dietary methionine and drusen area (P =0.033; but not with AMD grade, drusen size or pigment area. There was no significant association for dietary choline intake with the macular phenotype.

Betaine, choline, methionine and homocysteine are involved in the one-carbon metabolism pathway, which occurs in DNA methylation. Choline is oxidized to betaine, which, together with homocysteine, will produce methionine under the influence of vitamin B12. Methionine is an essential amino acid for DNA methylation.[59] Dietary choline, betaine and methionine influence DNA methylation; higher intakes of choline and betaine showed lower plasma levels of homocysteine, and a reduction of inflammatory markers in serum.[59,60] Higher levels of homocysteine or inflammatory markers were found to be risk factors for AMD.[47] Further studies are needed to confirm these associations with AMD.

RESVERATROL

Resveratrol is a natural phenol produced by plants which may have anticancer and anti-inflammatory effects. It can be found in the skin of red grapes, and is also present in red wine, although in very low concentrations. Experiments have indicated that resveratrol may protect retinal pigment epithelial cells from oxidative stress in culture.[61] This nutrient could have a beneficial effect on eye health and help protecting the macula against AMD. We are not aware of any ongoing trials investigating resveratrol supplementation and AMD.

SUPPLEMENTATION WITH COMBINED NUTRIENTS

Many nutrients have been supplemented together, making it impossible to investigate the role of the individual nutrients and risk of AMD. Studies supplemented different combinations and dosages, further hampering comparison. Nevertheless, it appears that supplementation of several nutrients combined can be beneficial; they could enforce each other. In the AREDS trial, a double-masker clinical trial, participants were randomly subscribed to oral use of (1) antioxidant (vitamin C 500 mg; vitamin E 400 IU; ß-carotene 15 mg), (2) zinc (zinc oxide 80 mg and cupric oxide 2 mg), (3) antioxidants plus zinc or (4) placebo. Estimates of RR show that those who were taking antioxidant and zinc had a 25% risk reduction of advanced AMD, while the groups that were taking antioxidant alone or zinc alone had a reduction of 17% and 21%, respectively.[34]

However, the more the merrier does not account for al nutrients. Nutrients that are alike mostly use the same uptake and transportation routes throughout the body. High levels of supplementation could then lead to competition between these nutrients, and this could even lead to deficiencies. An example of this is cupper deficiency caused by increased zinc consumption.[32,34]

CONCLUSION

Nutrients and AMD have been widely studied. Not all prior questions have been answered, and new questions have already been launched. Nonetheless, from the current findings we conclude that a healthy diet for instance, as recommended by the United States Department of Agriculture in the food pyramid may help lower the risk of AMD with a special focus on carotenoids (lutein, zeaxanthin, lycopene), zinc, and omega-3 fatty acids (Table 3; Figure 2). Less firmly established, but also promising, appear a high intake of vitamin D and nutrients influencing epigenetics. Beneficial effects may be particularly present in those carrying a genetic risk of AMD.

A word of concern is at place. We need to keep in mind that the nutrients mentioned in this chapter are generally not consumed as single items, but accompanied by other nutrients. Furthermore, a healthy diet is correlated with other lifestyle factors, which makes it difficult to interpret the positive effect of a healthy diet by itself.

TABLE 3 - Overview of nutrients and risk of AMD

Nutrient	Early AMD	Late AMD	References
Astaxanthin	NA	NA	
Betaine	NA	↓?	Seddon et al. 2011
α Carotene	~	↑?	Chong et al. 2007; Zhou et al. 2011
β Carotene	↓?	~	Chong et al. 2007; Evans 2012; Ho et al. 2011
β Cryptoxanthin	~	~	Seddon et al. 1994; Chong et al. 2007
Lutein/zeaxanthin	↓↓	↓↓	Ma et al. 2012; Sabour-Pickett et al. 2012
Lycopene	↓↓	↓↓	Mares-Perlman et al. 1995 Simonelli et al. 2002; Cardinault et al. 2005; Morris et al. 2007
Methionine	NA	↓?	Seddon et al. 2011
Omega-3 fatty acids	↓↓	↓↓	Chong et al. 2008; Kishan et al. 2011
Omega-6 fatty acids	NA	↑?	Kishan et al. 2011
Resveratrol	NA	NA	
Selenium	NA	NA	
Vitamin A (total)	~	↓?	Seddon et al.1994; Chong et al. 2007; San Giovanni et al. 2007
Vitamin A (retinol)	~	~	Seddon et al. 1994; San Giovanni et al. 2007
Vitamin C	~	↓?	Seddon et al. 1994; Chong et al. 2007
Vitamin D	↓?	↓?	Parekh et al. 2007; Millen et al. 2011; Seddon et al. 2011; Graffe et al. 2012
Vitamin E*	~	~	Chong et al. 2007; Evans 2012
Zinc	↓?	↓↓	Chong et al. 2007; Evans 2008; Ho et al. 2011

* There are many different forms of vitamin E, the most common is α-tocopherol

↓↓ lowering
↓? possible lowering
~ questionable
↑? possible increase
↑↑ increase
NA Not able; no results available

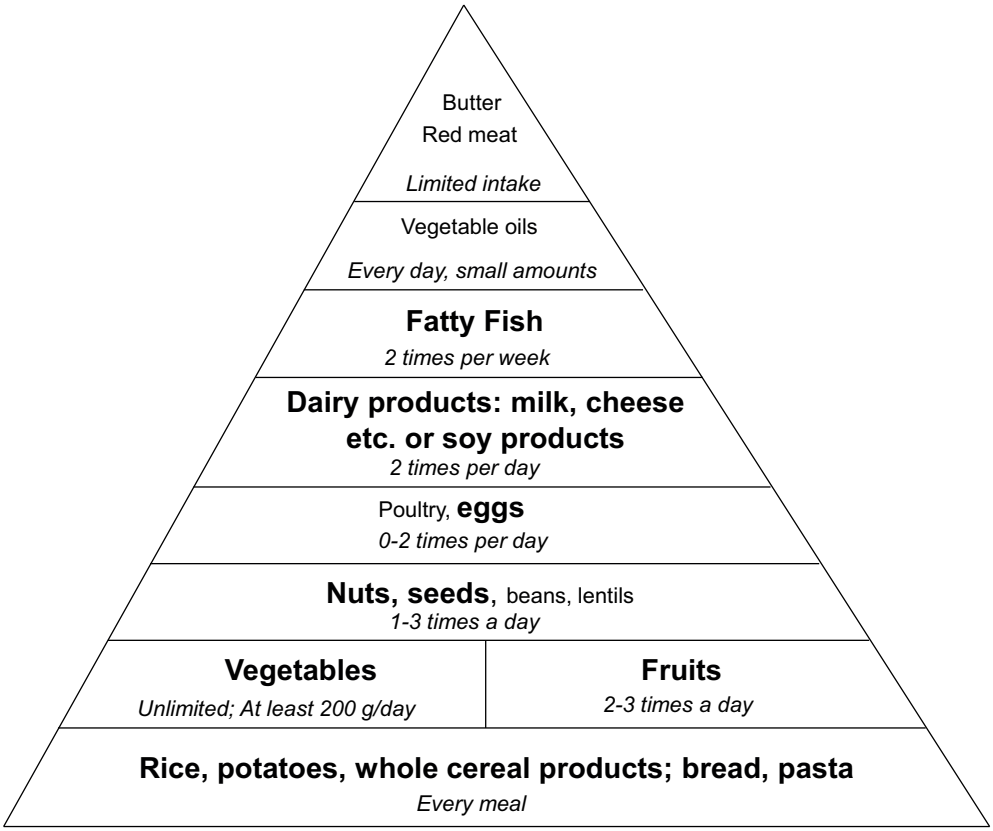

FIGURE 2 - Nutritional advice for lowering risk on AMD
This food pyramid gives a nutritional advice for lowering risk on AMD. All the foods in bold contain nutrients which have been associated with a lower risk on developing AMD.

Take-home messages

- Vitamins and minerals are essential for normal retinal physiology. Supplementation of these nutrients mostly has a dosage outside the recommended daily allowance, and therefore should be prescribed with thought.
- We consume foods, not nutrients.
- Dietary intake of carotenoids (lutein, zeaxanthin, lycopene) zinc and omega-3 fatty acids can help lower the risk of AMD
- Supplementation of lutein, zeaxanthin and zinc can help lower the risk of AMD.
- Vitamin D and nutrients influencing epigenetics are promising new topics for more in-depth research.
- Those at genetic risk should be made aware of their potential to lower the risk of AMD through diet.

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Chapter 4.3

Thyroid function and age-related macular degeneration: a prospective population-based cohort study: The Rotterdam Study

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ABSTRACT

Background In animal models, lack of thyroid hormone is associated with cone photoreceptor preservation while administration of high doses of active thyroid hormone leads to deterioration. The association between thyroid function and age-related macular degeneration (AMD) has not been investigated in the general population.

Methods Participants ≥55 years from the Rotterdam Study with thyroid-stimulating hormone (TSH) and/or free thyroxine (FT4) measurements and AMD assessment were included. We conducted age- and sex-adjusted Cox-proportional-hazards models to explore the association of TSH or FT4 with AMD, in the full range and in those with TSH (0.4-4.0 mIU/L) and/or FT4 in normal range (11-25 pmol/L). Cox-proportional-hazards models were performed for the association of TSH or FT4 with Retinal Pigment Alterations (RPA), as an early marker of retinal changes. Multivariable models additionally included cardiovascular risk factors and thyroid peroxidase antibodies positivity. We also performed stratification by age and sex. A bidirectional look-up in Genome-Wide Association Studies (GWAS) data for thyroid parameters and AMD was performed. Single Nucleotide Polymorphisms (SNPs) that are significantly associated with both phenotypes were identified.

Results We included 5573 participants with a median follow-up of 6.9 years (interquartile range 4.4-10.8 years). During follow-up 805 people developed AMD. TSH levels were not associated with increased risk of AMD. Within normal range of FT4, participants in the highest FT4 quintile had a 1.34-fold increased risk of developing AMD, compared to individuals in the middle group (95% confidence interval [CI] 1.07-1.66). Higher FT4 values in the full range were associated with a higher risk of AMD (Hazard Ratio 1.04, CI, 1.01-1.06 per 1 pmol/L increase). Higher FT4 levels were similarly associated with a higher risk of RPA. Restricting analyses to euthyroid individuals, additional multivariable models and stratification did not change estimates. We found a SNP (rs943080) in the *VEGF-A* gene, associated with AMD, to be significant in the TSH GWAS ($p=1.2x10^{-4}$). Adding this SNP to multivariable models did not change estimates.

Conclusion Higher FT4 values are associated with increased risk of AMD -even in euthyroid individuals- and increased risk of RPA. Our data suggest an important role of thyroid hormone in pathways leading to AMD.

BACKGROUND

Age-related macular degeneration (AMD) is a disease of the retina in the elderly which can lead to irreversible blindness and is characterized by drusen, pigmentary changes, choroidal neovascularization and geographic atrophy. While AMD is one of the leading causes of visual impairment worldwide and increasing in prevalence [1-7], the exact pathophysiology and pathways leading to AMD are not entirely understood.

Thyroid hormones are known to regulate various visual functions in experimental and human studies [8-10]. Human retinal pigment epithelial (RPE) cells express thyroid hormone receptors and seem to be a direct target for thyroid hormones [11]. Recently it has been shown that suppression of thyroid hormone signaling resulted in preservation of cone photoreceptors in mouse models of retinal degeneration [12]. In contrast, administration of active thyroid hormone leads to deterioration of cones. Thyroid dysfunction and subclinical thyroid dysfunction are common in the general population, with a prevalence up to 10% [13-16]. These thyroid disorders are associated with various cardiovascular risk factors, including alterations in lipid levels, atherosclerosis and hypertension [17-19], which are known predisposing factors for development and progression of AMD [20,21]. However, there are no studies in the general population assessing the association between thyroid function and the risk of AMD. Therefore, we aimed to assess the relation between thyroid-stimulation hormone (TSH), free thyroxine (FT4) and the risk of incident AMD in a prospective population-based cohort study and to study possible underlying genetic pathways through investigating an overlap in genome-wide significant hits (i.e. bidirectional genetic look-up).

METHODS

The Rotterdam Study

The Rotterdam Study is a prospective population-based cohort study that addresses determinants and occurrence of cardiovascular, neurological, ophthalmologic, psychiatric, and endocrine diseases in the elderly living in Ommoord, a suburb of Rotterdam. The aims and design of the Rotterdam study have been described elaborately elsewhere [22]. For this analysis we included participants from two independent cohorts from the Rotterdam Study. The Rotterdam Study Cohort I (RSI) started in 1989 and included a total of 7,983 participants (response rate 78 percent) aged 55 years and older. Baseline data were collected from 1990 until 1993 and four follow-up examinations were performed in 1993-1995, 1997-1999, 2002-2004 and 2009-2011. The second cohort is the Rotterdam Study Cohort II (RSII) and includes a total of 3,011 participants (response rate 67 percent) aged 55 years and older. Baseline data were collected from 2000-2001 and follow-up examinations were performed in 2004-2005 and 2011-2012.

Study Population

Participants from baseline study cohorts RSI (RSI-1) and RSII (RSII-1) were eligible for these analyses if they had TSH and/or FT4 measurements and had gradable fundus photographs at baseline and at least one follow-up eye examination. Since not all participants from RSI had thyroid measurements at baseline, additional baseline samples were drawn from RSI visit 3 (RSI-3). Participants with AMD at baseline (N=567) were excluded from further analyses. In total 5573 participants from these two cohorts were eligible to be included in our analyses (Supplementary Figure 1). The Medical Ethics Committee of the Erasmus University had approved the study protocols, and participants had given a written informed consent in accordance with the Declaration of Helsinki.

Assessment of thyroid function

For RSI-1, serum TSH (TSH Lumitest; Henning, Berlin, Germany), anti-TPOAb (ELISA; Milenia; Diagnostic Products Corp, Los Angeles, CA, USA) and free T4 levels (FT4; Vitros, ECI Immunodiagnostic System; Ortho-Clinical Diagnostics, Amersham, UK) were determined in a random subset of the baseline serum samples (n=1855). Thyroid function assessment was also performed in baseline serum samples for TSH and FT4 (The electrochemiluminescence immunoassay for thyroxine and thyrotropine, "ECLIA", Roche) for RSI-3 and RSII-1. The tests' TSH reference ranges did not differ substantially and had a good Spearman correlation co-efficient (0.96 for TSH, $p < 0.0001$and 0.81 for FT4, $p < 0.0001$). We determined the cut-off values for normal range TSH as 0.4-4.0 mIU/L according to national guidelines. The reference range for FT4 was 11-25 pmol/L and anti-TPOAb levels greater than 60 kU/mL were regarded as positive.

Diagnosis of age-related macular degeneration

All eligible participants underwent fundus photography after pharmacologic mydriasis. For visits RSI-1 to RSI-3 and RSII-1 a 35° film fundus camera was used (Topcon TRV-50VT, Topcon Optical Company, Tokyo, Japan) after which a 35° digital color fundus camera (Topcon TRC-50EX, Topcon Optical Company, Tokyo, Japan with a Sony DXC-950P digital camera; 0.44 megapixel, Sony Corporation, Tokyo, Japan) followed for visits RSI-4, RSI-5, RSII-2 and RSII-3. Fundus transparencies were graded according to the Wisconsin Age-Related Maculopathy Grading [23] and the modified International Classification System [24] by trained graders under the supervision of senior retinal specialists (J.R.V., C.C.W.K.). The eyes of each participant were graded and classified separately, and the eye with the more severe grade was used to classify the person. In the analyses incident early and late AMD combined was used as outcome variable. In the manuscript this is referred to as AMD. Besides AMD we also investigated AMD specific lesions as a separate outcome variable. These lesions included retinal pigmentary alterations, large drusen (≥125µm) and large drusen area (≥ 5331,820 µm2) [25].

Baseline measurements

Smoking was derived from computerized baseline questionnaires and categorized in current or non-current smokers. Blood pressure, systolic and diastolic, was calculated as the average of two consecutive measurements, using random-zero mercury sphygmomanometers. Hypertension was defined as having a systolic blood pressure ≥ 140 mmHg or a diastolic blood pressure ≥ 90mmHg or using anti-hypertensive medication at baseline. Cholesterol was measured at baseline by the CKCL (Centra Clinical Chemical Laboratory) of the Erasmus University Medical Center. A subgroup of measurements was carried out in the laboratory of the Department of Epidemiology & Biostatistics (Erasmus University Medical School). History of diabetes was defined by a repeated impaired fasting glucose ≥ 7 or use of anti-glycemic medication at baseline. Body-mass index (BMI) was calculated as weight kilograms divided by height squared in meters.

Statistical analysis

Participant baseline characteristics were compared using a χ^2 or t-test. Due to a skewed distribution, TSH was log-transformed for the statistical analyses. We used cox-proportional hazards model to calculate the relationship between TSH and FT4 at baseline and the risk of incident AMD, first including all participants and then including only those with normal range TSH and/or FT4 values. We performed a crude cox-model including only thyroid parameters after which we also included quadratic and cubic terms to explore possible non-linear relationships. We then performed additional models adjusting first for age and sex and second also adding smoking, hypertension, cholesterol, diabetes and BMI to the model. Hypertension, cholesterol, diabetes and BMI could act as

confounders and possible mediators depending on the presumed pathway through which thyroid function is related to AMD. These variables were included in the multivariable model as possible confounders of non-vascular pathways. We looked at the association between AMD and TSH or FT4 both continuously and in quintiles, as well as overall and within the normal range of TSH. The middle quintile was used as reference group as biologically it is expected to represent the subgroup with the most thyroid function within the euthyroid group. We performed pre-defined stratification by sex and age categories, using a cutoff 65 years as this is the median of the current population and the treatment threshold for subclinical thyroid dysfunction according to the European guidelines[26]. Further interaction terms were introduced to the model to explore possible differential risk patterns. We performed a sensitivity analysis excluding those using thyroid medication at baseline (levothyroxine and anti-thyroid drugs) and those with prior self-reported thyroid disease at baseline. We also performed FT4 and TSH analyses with specific AMD lesions defined as retinal pigment alteration, large drusen and large drusen area as separate outcome variable to examine possible early changes in underlying pathways. To address the issue of drop-out of individuals during follow-up that could possibly be not completely at random, we adjusted the model for inverse probability weights (IPW"s). These were calculated using possible baseline explanatory variables for drop-out such as smoking, BMI and medication use. Proportional hazards assumption was checked statistically using the Schoenfeld test and assessing the Schoenfeld plot. All statistical analyses were performed using SPSS version 21 (SPSS IBM, New York, U.S.A) except for the Schoenfeld tests and (Schoenfeld) plots which were performed in R (survival package, R-project, Institute for Statistics and Mathematics, R Core Team (2013), Vienna, Austria, version 3.0.2).

Bidirectional genetic look-up

Genome-Wide Association Studies (GWAS) have been performed for AMD [27] and thyroid function (TSH and FT4) [28,29]. These studies identified several single-nucleotide polymorphisms (SNPs) associated to these two phenotypes. Some of the genome-wide significant SNPs in the AMD GWAS might also play a role in thyroid function and vice versa. Overlap between common genetic polymorphisms can provide insight into possible shared genetic pathways. It might also elucidate a mediation effect between the two phenotypes, i.e. identify and explicate the process that underlies a possible observed relationship between thyroid function and AMD. To evaluate these potential genetic pathways, we conducted a bidirectional genetic look-up using the results of the above mentioned GWA Studies for AMD and thyroid function. We first extracted SNPs that reached genome-wide significance from the AMD GWAS performed by the AMD Gene consortium [27]. We then checked whether these were significantly associated with TSH or FT4 in the thyroid function GWAS performed by Porcu et al. [28]. Hereafter we extracted the genome-wide significant SNPs for TSH or FT4 from the thyroid function GWAS and checked whether they were associated with AMD in the AMD GWAS. For the significance level, we applied a multiple testing correction (Bonferroni Correction), using a p-value threshold of 0.05 divided by the amount of significant SNPs per GWAS. In case of a significant finding, we added the SNP to the multivariable model to evaluate a possible mediation effect.

RESULTS

We included 5573 participants with TSH and/or FT4 measurements at baseline and incident AMD data, with a median follow-up of 6.9 years (interquartile range [IQR] range of 4.4-10.8 years). Of these, 5572 had TSH and 5504 had FT4 baseline measurements. A total of 805 people developed AMD

(Early AMD N=725, Late AMD N=80) during follow-up with an incidence rate of 18 per 1000 person-years. The baseline characteristics for those with and without incident AMD during follow-up were comparable, except for proportion of diabetes (Table 1).

TABLE 1 - Baseline Characteristics of Included Participants from the Rotterdam Study Evaluating the Association between Thyroid Function and AMD*

Variable	No incident AMD N=4768	Incident AMD N=805	P-value**
Age, years	67.6 (7.6)	67.9 (7.1)	0.29
Sex % female	57.6	57.8	0.94
History of Diabetes %	10.8	8.4	0.04
BMI kg/m^2	26.9 (3.9)	26.6 (3.7)	0.07
Cholesterol mmol/L	6.1 (1.2)	6.1 (1.1)	0.23
Smoking % current	20.7	21.0	0.85
Hypertension %	63.0	58.7	0.17
TSH mIU/L median (IQR)	1.78 (1.15-2.69)	1.73 (1.17-2.67)	0.78
FT4 pmol/L	15.8 (2.6)	16.0 (3.2)	0.13
TPOAb kU/L	30.5 (95.1)	30.8 (96.2)	0.93

*Values are means and SD unless otherwise specified.
** For comparison a t-test was conducted, for TSH the log-transformed values were used.
Abbreviations: AMD = Age-related Macular Degeneration; BMI = body-mass index; TSH = thyroid-stimulating hormone, FT4 = free thyroxine; SD = Standard deviation; IQR = inter-quartile range; TPOAb = thyroid peroxidase antibodies.

Association between thyroid function and AMD

Although there was no association between TSH and AMD (hazard ratio [HR] 0.99; 95% confidence interval [CI] 0.91-1.07, Table 2), the risk of AMD was significantly increased in those with higher FT4 levels (Table 2). When categorizing the FT4 values within normal range quintiles, those in the highest FT4 quintile had an increased risk compared to the middle group with a HR of 1.34 (95% CI, 1.07-1.66) and a non-significant p for interaction (p=0.066) (Table 2). This association remained similar after additional adjustments for smoking, diabetes, hypertension, cholesterol, BMI, and TPOAb positivity (Figure 1). This association also remained similar after analyzing only those within the normal range of TSH and FT4, that is, those with normal thyroid function.

Excluding those with thyroid medication or thyroid disease at baseline as a sensitivity analysis, did not alter the association (Table 3). Stratifying for age and sex did not reveal any significant differential risk (Supplementary Table 1). The association between thyroid function and retinal pigment alterations for FT4 showed similar significant hazard ratios, with the exception of the risk estimates when looking at FT4 only in the normal range of TSH (Table 4). TSH and FT4 were not associated with large drusen or large drusen area (data not shown). Introducing quadratic and cubic terms for TSH and FT4 to the crude model, as an exploration of non-linearity, did not improve model performance. Taking possible non-random follow-up using IPW's did not change risk estimates. The proportional hazards assumption was checked statistically with the Schoenfeld test and Schoenfeld plot and met for both the TSH (p = 0.232) and FT4 (p = 0.154) analyses.

TABLE 2 - Association between TSH, FT4 and risk of AMD

Incident AMD vs no AMD	AMD *N*	Total *N*	HR (95% CI), model 1	HR (95% CI), model 2	HR (95% CI), model 3
TSH mIU/L	805	5572	0.99 (0.91-1.07)	0.99 (0.91-1.07)	0.99 (0.91-1.07)
TSH in normal range[a]	696	4756	1.06 (0.91-1.23)	1.09 (0.93-1.27)	1.08 (0.93-1.26)
Normal range TSH[a]					
Q1 0.40-1.10	148	1082	1.04 (0.82-1.32)	1.00 (0.79-1.28)	1.00 (0.79-1.28)
Q2 1.11-1.54	167	990	1.32 (1.04-1.66)	1.29 (1.02-1.62)	1.29 (1.02-1.62)
Q3 1.55-1.99	128	962	1	1	1
Q4 2.00-2.61	117	851	1.09 (0.85-1.40)	1.07 (0.83-1.37)	1.07 (0.83-1.38)
Q5 2.62-3.97	136	871	1.22 (0.96-1.56)	1.22 (0.95-1.55)	1.21 (0.94-1.54)
P interaction			0.648	0.485	0.517
Total	696	4756			
FT4 pmol/L	791	5504	1.04 (1.01-1.06)	1.04 (1.01-1.06)	1.04 (1.01-1.06)
FT4 in normal range[b]	765	5382	1.04 (1.01-1.07)	1.04 (1.01-1.07)	1.04 (1.01-1.07)
Normal range FT4[b]					
Q1 11.0-14.0	149	1090	1.03 (0.82-1.29)	1.04 (0.82-1.31)	1.04 (0.82-1.31)
Q2 14.0-15.1	152	1001	1.12 (0.89-1.41)	1.17 (0.92-1.47)	1.16 (0.92-1.47)
Q3 15.1-16.2	144	1094	1	1	1
Q4 16.2-17.5	134	1060	1.01 (0.80-1.28)	1.03 (0.81-1.30)	1.03 (0.81-1.31)
Q5 17.5-24.9	186	1137	1.34 (1.07-1.66)	1.35 (1.08-1.69)	1.35 (1.09-1.69)
P interaction	765	5382	0.066	0.088	0.08
Normal range FT4[b] in normal range TSH[a]	673	*4658*	1.04 (1.01-1.08)	1.04 (1.01-1.08)	1.04 (1.01-1.07)

[a] normal range of TSH defined as 0.4-4.0 mIU/L.
[b] normal range of FT4 defined as 11-25 pmol/L.
Model 1: Adjusted for sex and age.
Model 2: Model 1 + smoking, hypertension, cholesterol, diabetes, BMI.
Model 3: Model 2 + thyroid peroxidase antibodies positivity.
Abbreviations: AMD Age-related Macular Degeneration ; BMI body-mass index; CI confidence interval; FT4 free T4; HR hazard ratio; Q quintile; TSH Thyroid-Stimulating Hormone.

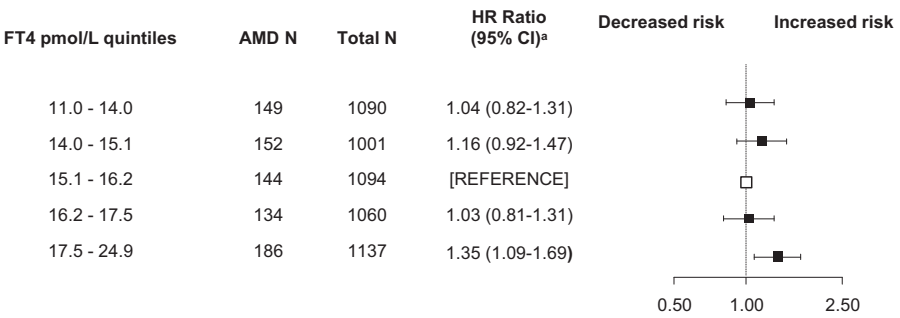

FIGURE 1 - Quintiles of FT4 within the normal range and risk of AMD.
The normal range of FT4 was defined as 11-25 pmol/L (Conversion 1 pmol/L=0.0777 ng/dL).

[a] Analyses were adjusted for sex, age, smoking, hypertension, cholesterol, diabetes, body-mass index and thyroid peroxidase antibodies positivity.
Abbreviations: AMD Age-related Macular Degeneration; FT4 free thyroxine; HR hazard ratio

Bidirectional genetic look-up

In the thyroid function GWAS, 20 SNPs were associated with TSH and 6 with FT4 [28]. The AMD GWAS revealed 19 genome-wide significant SNPs related to the phenotype. None of the SNPs from the thyroid function GWAS were significant in the AMD GWAS. One SNP (rs943080) in the Vascular Endothelial Growth Factor A (*VEGFA*) gene that is related to AMD, was also significantly associated with TSH ($p=1.2x10^{-4}$, significance threshold =0.0026) (Supplementary Table 2). Within our study population, GWAS data were available for a total 4646 participants. Additionally correcting for the rs943080 SNP in the most adjusted model in these participants, resulted in similar risk estimates for the FT4 analysis (HR 1.04, CI 95% 1.01-1.07). Stratifying for this SNP did show risk differences between the different genotypes but not significantly (Supplementary Table 1).

TABLE 3 - Sensitivity Analyses excluding participants with Thyroid Medication or Thyroid Disease at baseline

Incident AMD vs no AMD	AMD N	Total N	HR (95% CI), model 1	HR (95% CI), model 2	HR (95% CI), model 3
Excluded medication thyroid[a]					
Free T4	752	5225	1.03 (1.01-1.06)	1.03 (1.01-1.06)	1.03 (1.01-1.06)
TSH mIU/L	778	5417	0.99 (0.91-1.08)	1.00 (0.92-1.09)	1.00 (0.91-1.09)
Excluding baseline thyroid disease[b]					
Free T4	751	5237	1.04 (1.01-1.08)	1.04 (1.01-1.07)	1.04 (1.01-1.08)
TSH mIU/L	764	5300	0.98 (0.89-1.07)	0.98 (0.89-1.07)	0.97 (0.89-1.07)

[a] 155 participants had thyroid medication (ie. thyroid hormone use) at baseline
[b] 272 participants had self-reported thyroid disease at baseline
Model 1: Adjusted for sex and age.
Model 2: Model 1 + smoking, hypertension, cholesterol, diabetes, BMI.
Model 3: Model 2 + thyroid peroxidase antibodies positivity
Abbreviations: BMI body-mass index; CI confidence interval; FT4 free thyroxine; HR hazard ratio; TSH thyroid-stimulating hormone

TABLE 4 - Association between FT4 and TSH with Retinal Pigment alterations[a]

Incident pigment alterations vs no pigment alterations	Cases N	Total N	HR (95% CI), model 1	HR (95% CI), model 2	HR (95% CI) , model 3
TSH mIU/L	729	5401	0.98 (0.90-1.06)	0.97 (0.90-1.06)	0.96 (0.88-1.04)
Normal range TSH[b]	618	4591	1.02 (0.87-1.20)	1.05 (0.89-1.23)	1.04 (0.88-1.22)
FT4 pmol/L	720	5338	1.04 (1.01-1.07)	1.04 (1.01-1.07)	1.04 (1.01-1.07)
Normal range FT4[c]	697	5226	1.04 (1.01-1.07)	1.04 (1.01-1.07)	1.04 (1.01-1.07)
Normal range FT4[c] in normal range TSH[b]	601	4500	1.03 (1.00-1.07)	1.03 (0.99-1.06)	1.03 (0.99-1.06)

[a] participants with late AMD were excluded from this analysis
[b] normal range of TSH defined as 0.4-4.0 mIU/L.
[c] normal range of FT4 defined as 11-25 pmol/L.
Model 1: Adjusted for sex and age.
Model 2: Model 1 + smoking, hypertension, cholesterol, diabetes, BMI.
Model 3: Model 2 + thyroid peroxidase antibodies positivity
Abbreviations: BMI body-mass index; CI confidence interval; FT4 free thyroxine; HR hazard ratio; TSH thyroid-stimulating hormone

DISCUSSION

In this prospective cohort study we investigated the association between thyroid function and incidence of AMD. Higher FT4 values were associated with an increased risk of developing AMD, even within the normal range of TSH and FT4 (i.e. euthyroid subjects), while there was no association between TSH and AMD. The similar findings between higher levels FT4 and retinal pigment alterations might suggest that thyroid hormone plays a role in the development of AMD rather than just act as a promoter of disease. To our knowledge, this is the first prospective population-based cohort study to look at the association between thyroid function and AMD.

A limited number of studies investigating thyroid disease and AMD have been published, all lacking laboratory assessment of thyroid function. Bromfield et al. reported an increased risk of AMD in subjects with self-reported hypothyroidism [30]. A case-control study by Anand et al. reported an association between thyroid hormone use and a higher risk of AMD with geographic atrophy [31], but no data were reported on the number of patients that were over- or undertreated. Similarly, the Beaver Dam Eye study also reported an association between thyroid hormone use and early AMD [32], but this was not confirmed by Douglas et al. [33]. As mentioned previously, none of these studies had laboratory assessment of thyroid function nor did they investigate the association in a time-to-event analysis. In our study, excluding all subjects using thyroid medication did not alter risk estimates, supporting a potential intrinsic effect of thyroid hormone.

There are several pathophysiological explanations for the relationship between thyroid hormones and AMD. In a mouse model of retinal degeneration, suppression of thyroid hormone signaling resulted in preservation of cone photoreceptors [12]. The same study found that stimulating thyroid hormone signaling, by administering the active thyroid hormone triiodothyronine, deteriorates cones in mouse models with a slow progressive and moderate degeneration phenotype [12]. In addition, mice lacking type 3 deiodinase, the enzyme responsible for the degradation of thyroid hormones, have decreased survival and disturbed maturation of cone photoreceptors [34]. The findings of these studies suggest that thyroid hormone may lead to a higher turnover of photoreceptors and in retinal degeneration this leads to deterioration of photoreceptors. Beside photoreceptors, thyroid hormone might also have an influence on the retinal pigment epithelial cells [11]. In the healthy retina the turnover of photoreceptors is extremely high. Every day the photoreceptors shed the ends of their outer segments resulting in full renewal every ten days. These shedded parts of the outer segments are fagocytosed by the retinal pigment epithelium (RPE) cell [35]. Increase of the turnover of the photoreceptors by thyroid hormone may bring additional stress to the process. RPE cells at distress may change resulting in pigmentary alterations in the macular area. The RPE cells may also be targeted directly by the thyroid hormone resulting in these changes [11]. These results may provide an explanation for the findings in our study.

Thyroid dysfunction has been linked to cardiovascular risk factors and disease, including effects on the vascular function, lipids and atherosclerosis [36]. As some of these risk factors are also linked to AMD [20,21], one could speculate about a joint vascular pathway leading to both thyroid dysfunction and AMD or perhaps that the relation between thyroid dysfunction and AMD could be mediated through this pathway. We were not able to confirm these hypotheses. First of all, these cardiovascular risk factors are mainly seen in hypothyroidism, (i.e. high TSH and low FT4), whereas our data show an association between high FT4 and AMD. Also, correcting for some of these risk factors (for example hypertension) that could act as confounders and possible mediators did not change risk estimates, suggesting that the effect of thyroid function is not through these pathways. Lastly, *VEGFA* gene was

found to be significant in the look-up for the TSH GWAS and not the FT4 GWAS. However, our results suggest a higher risk of AMD in higher levels of FT4 and not in TSH. Furthermore, the association did not change by adding this SNP to the multivariable model.

We find an effect with FT4 but not with TSH. This however does seem to be in line with previous literature from cohort studies in elderly populations investigating the relation between thyroid function and several other endpoints [37,38]. Regulation of serum thyroid hormone levels is controlled by the hypothalamus-pituitary-thyroid axis. The set point of this feedback mechanism is defined individually, with thyroid hormone levels showing a much greater inter-individual than intra-individual variability [39]. The individual set point can be modulated by several pathophysiological (for example critical illness) and physiological (for example ageing) mechanisms[40]. This could be one of the explanation why in this elderly and ageing population we do find an association with FT4 but not with TSH, especially in the euthyroid range. Furthermore, previous literature showed an increase in TSH with increasing age, suggesting higher TSH levels are needed to keep thyroid hormone levels within the desired range[38]. We only have thyroid function measures at baseline and are therefore not able to investigate whether changes in thyroid function over time is an explanation for the discordant association between TSH, FT4 and AMD.

Important strengths of our study are the assessment of thyroid function at baseline through laboratory testing as well as the elaborate assessment of AMD at baseline and follow-up. Also, we were also able to investigate the association between thyroid function and specific AMD lesions like retinal pigment alterations to examine possible early changes in underlying pathways. The availability of genetic data gave us the opportunity to explore possible genetic pathways. The bidirectional genetic look-up, revealed one SNP in the *VEGFA* gene to be significant in the TSH GWAS but not for FT4. Adding this SNP to the multivariate model did not alter risk estimates. An explanation for the absence of overlapping genome-wide significant SNP's could be that these GWA studies were underpowered for this association.

A limitation of our study is that thyroid parameters were measured once at baseline. Therefore, the evolution of thyroid hormone levels could not be taken into account. Also, residual confounding cannot be excluded, even with the large number of covariates included in these analyses. Lastly, this study is conducted in a mainly Caucasian population of 55 years and older and may not be generalizable to other populations.

CONCLUSION

We find an increased risk of incident AMD in subjects with higher FT4 levels, even in those with a normal thyroid function and when excluding thyroid medication users. This implies an intrinsic (that is. not exogenous) deleterious effect of thyroid hormone on AMD. We also find an association between higher FT4 levels and retinal pigment alterations, suggesting that thyroid hormone could even play a role in the early stage of development of AMD. Functional and clinical studies could provide more evidence for a true causal relationship.

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Chapter 4.4

Antiplatelet and anticoagulant drugs do not affect visual outcome in neovascular age-related macular degeneration in the BRAMD trial

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ABSTRACT

Purpose To determine if use of antiplatelet or anticoagulant (AP/AC) medication influences visual acuity in patients with active neovascular age-related macular degeneration (N-AMD).

Design Retrospective analysis of data from a randomized controlled trial

Methods
Setting: Multi-center
Study Population: 330 patients with active N-AMD from the BRAMD study, a comparative trial between bevacizumab and ranibizumab in the Netherlands.
Observation Procedures: Patients underwent an extensive ophthalmic examination. Visual acuity was categorized into functional vision (best corrected visual acuity (BCVA) >=0.5), visual impairment (BCVA < 0.5), and severe visual impairment (BCVA<0.3). Fundus photographs were graded for presence of retinal or subretinal hemorrhages. Information on AP/AC medication was obtained through interview. Logistic regression analysis was used to determine associations between AP/AC medication and outcomes. Frequency of hemorrhages in users and non-users stratified for visual acuity categories was analyzed with ANCOVA.
Main Outcome Measures: BCVA and presence of hemorrhages.

Results In total, 40.9% of the patients used AP/AC medication, of which 73.3% was aspirin. AP/AC use was not associated with visual impairment (adjusted odds ratio (OR) 0.79 (95% confidence interval (CI) 0.43-1.44), or severe visual impairment (adjusted OR 0.75 (95% CI 0.40-1.43). Patients on AP/AC presented with comparable frequencies of hemorrhages (27% versus 32%, *P*=0.32, respectively). Similar results were found when analyses were restricted to aspirin users only.

Conclusion In our study, use of AP/AC medication was neither associated with visual decline nor with the occurrence of hemorrhages in patients with active N-AMD.

INTRODUCTION

Recently, several population based-studies have shown an association of aspirin use with an increased risk of choroidal neovascularization (CNV) in age-related macular degeneration (AMD).[1-3] These findings were not unequivocal as randomized controlled trials[4,5] and meta-analyses were inconclusive.[6-9] These contrasts have renewed the discussion on the use of aspirin and other antiplatelet or anticoagulant drugs in persons with neovascular AMD. While these therapies are mostly prescribed for their cardiovascular and cerebrovascular protective effects, they may have adverse events due to an increased risk of bleeding.[10-12] The antithrombotic characteristics may deteriorate the outcome of neovascular AMD, leading to more severe hemorrhages and increased fibrovascular scarring, both jeopardizing visual acuity.[13] At this time, it is still unclear whether antiplatelet or anticoagulant medication should be discontinued for patients with neovascular AMD.

We investigated the effect of antiplatelet and anticoagulant medication on visual acuity in patients with active CNV. We studied visual acuity, presence of retinal and subretinal hemorrhages, total CNV lesion size and retinal thickness in relation to the use of antiplatelet and anticoagulant medication before and after anti-vascular endothelial growth factor (anti-VEGF) therapy.

METHODS

BRAMD Study participants

BRAMD is a Dutch comparison study investigating Bevacizumab versus Ranibizumab in AMD, and was designed as a triple masked, randomized, clinical non-inferiority multicenter trial. Details on the study design have been described elsewhere.[14] In short, 330 patients aged 60 years or older from five academic medical centers in the Netherlands were included in the study. Inclusion criteria was a diagnosis of primary or recurrent active sub- or juxtafoveal choroidal neovascularization (CNV) secondary to AMD with a total area of CNV <12 disc areas and a best corrected visual acuity (BCVA) score between 20 and 78 letters on an Early Treatment Diabetic Retinopathy Study like chart (ETDRS) in the study eye. Further details on in- and exclusion criteria of this study are provided in Supplemental Table 1. After baseline assessment, all patients received monthly injections with bevacizumab or ranibizumab for 12 months. The institutional ethical review boards from the participating medical centers approved the BRAMD study. The study adhered to the tenets of the Declaration of Helsinki and was registered at the Dutch trial register (Nederlands trial register) (NTR1704). Participants who had a full ophthalmic examination including imaging, disclosed medication use, and a signed informed consent were included in the current analysis. In total, 330 eyes from 330 participants were included.

Ophthalmic data collection

All participants underwent a standardized full ophthalmic examination and imaging including, fluorescein angiogram, 30° color digital fundus photographs and spectral domain optical coherence tomography (SD-OCT). Diagnosis, size of lesion quantified as optic disc area (DA), and presence of active choroidal neovascularization due to AMD was confirmed by trained independent graders, masked for patient information such as the use of antiplatelet or anticoagulant drugs, at the UK Network of Ophthalmic Reading Centers. The presence of retinal or subretinal hemorrhage in the posterior pole was determined on fundus photographs. Retinal thickness was measured in the fovea using a built-in caliper on SD-OCTs at baseline and during last visit. Presence of pseudophakia was determined using slit lamp observation.

Best corrected visual acuity (BCVA) was assessed using an ETDRS chart and categorized in 3 groups:

	ETDRS chart letters	Snellen decimal equivalent	Snellen Foot equivalent
Functional vision	BCVA ≥ 70	BCVA ≥ 0.50	BCVA ≥ 20/40
Moderate visual impairment	70 > BCVA ≥ 59	0.50 > BCVA ≥ 0.30	20/40 > BCVA ≥ 20/66
Severe visual impairment	59 > BCVA ≥ 20	0.30 >BCVA ≥ 0.05	20/66 > BCVA ≥ 20/400

Assessment of variables

At baseline, detailed information was obtained from the participants concerning demographic characteristics, medical history, and medication use. Medical history included information about cardiovascular diseases such as myocardial infarction, congestive heart failure, stroke, transient ischemic attack and hypertension. Information on use of antiplatelet and anticoagulant (AP/AC) drugs including name of the drug, dosage, frequency and start date was obtained at baseline. Blood pressure was measured at baseline. Hypertension was defined as systolic blood pressure ≥ 140 mmHg or a diastolic blood pressure ≥90 mmHg or if the participant was using anti-hypertensive medication at baseline. Smoking habits were assessed by interview and categorized into never/past/current smoking.

Statistical analysis

Primary study outcomes were visual impairment (moderate and severe visual impairment combined) and severe visual impairment. Secondary outcomes were presence of retinal hemorrhages, foveal retinal thickness, and CNV lesion size in the study eye. Several determinants had missing values. Lens data were missing in 16% of study subjects, smoking data in 18.8%. Missing data were randomly distributed, and not significantly associated with visual impairment (analysis of covariance (ANCOVA) adjusted for age, sex P=1.00 for lens; P=0.47 for smoking). We used the iterative Markov chain Monte Carlo algorithm (5 steps) for multiple imputations. These were calculated using possible predictable baseline values such as age, sex, diabetes, and smoking for lens data; age, sex, history of cardiovascular disease, hypertension, and CNV presence in the fellow eye for smoking data. Analyzing the dataset excluding the multiple imputations for the variables lens status and smoking did not change risk estimates or associations observed in this study.

We first analyzed baseline characteristics between users of AP/AC and nonusers; continuous variables were compared using ANCOVA and categorical variants using logistic regression. After this we plotted users and nonusers of AP/AC with visual acuity and with retinal hemorrhages. Groups were compared using ANCOVA adjusted for age, sex, and study center. We then performed binary logistic regression analysis for the use of AP/AC and visual impairment, severe visual impairment, retinal hemorrhages, and CNV lesions size. Foveal retinal thickness measurements were also analyzed using ANCOVA. All analyses were adjusted for age, sex, and study center, and subsequently for smoking, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization in the fellow eye, lens status. All statistical analyses were performed using SPSS version 21 (SPSS IBM, New York, U.S.A.).

RESULTS

General characteristics of the 330 study participants from the BRAMD study are presented in Table 1. AP/AC users were slightly older (mean age 79.4 years for AP/AC users versus 76.4 years for nonusers; *P* = 0.001) and presented more often with diabetes mellitus (17.0% versus 8.7%; *P* = 0.01). Although users were more often diagnosed with hypertension (81.9% versus 52.0%; *P* <0.0001), no significant differences were found for blood pressure measurements (*P* = 0.37 for systolic and *P* = 0.54 for diastolic blood pressure). History of cardiovascular diseases was more common in users (34.1% versus 4.6%; *P* <0.0001). 40.9% of the study participants used any type of AP/AC drug. In those using AP/AC medication, antiplatelet drugs, with aspirin in particular, were the type of medication mostly used; 76.3% and 73.3%, respectively. Other baseline characteristics did not differ significantly between both groups.

We investigated use of AP/AC drugs and risk of visual impairment (Table 2). Use of AP/AC was not associated with visual impairment (Odds' Ratio (OR) 0.74 (95% Confidence Interval (CI) 0.44-1.24), adjusted for age, sex, and study center) and (OR 0.79 (95% CI 0.43-1.44), additional adjustment for smoking, lens status, medical history for cardiovascular diseases, diabetes, hypertension and presence of CNV in the fellow eye). AP use was significantly associated with a lower risk of visual impairment (OR 0.58 (95% CI 0.34-0.99). After adjustment for additional confounders, the association was no longer significant (OR 0.61 (95% CI 0.34-1.09)). For aspirin use, estimates were in the same direction, but no significant association was found. Associations with AC drugs were not significant in either model. The analyses with severe visual impairment as outcome showed similar effects, but none of the associations were significant. We also analyzed visual acuity from the last visit of the BRAMD study. Estimates were in the same direction, but no significant association was found (Supplemental Table 2).

The distribution of baseline functional vision and visual impairment was plotted for the presence of retinal or subretinal hemorrhages in users and nonusers of AP/AC medication (Supplemental Figure 1). Visual acuity was significantly lower in those with retinal or subretinal hemorrhages (*P* = 0.008). However, users or nonusers of AP/AC with hemorrhages did not differ in visual acuity (*P*=0.77).

The association between AP/AC use and the presence of retinal or subretinal hemorrhages is shown in Table 3. Users of AP/AC medication had a lower risk of hemorrhages. The association became significant after additional adjustment for covariates, OR 0.76 (95% CFI 0.46-1.25) and OR 0.47 (95% CI 0.26-0.83), respectively. This trend was also observed for AP; OR 0.52 (95% CI 0.30-0.90) and OR 0.34 (95% CI 0.18-0.62), respectively. Aspirin use became significantly associated with a lower risk of hemorrhages after additional adjustment; OR 0.61 (95% CI 0.34-1.08) and OR 0.36 (95% CI 0.18-0.71), respectively. No association was found between AC and hemorrhages.

Foveal retinal thickness differed significantly between aspirin users and nonusers at baseline (*P* = 0.04). This was not observed during the last visit (*P* =0.84). With respect to other groups, no significant difference was found between users and nonusers at baseline (Supplemental Table 3) or during their last visit (Supplemental Table 4). AP/AC was not associated with CNV lesions size at baseline (Supplemental Table 5).

TABLE 1 - Baseline characteristics of the study population

	BRAMD study N=330		
	Nonusers N=195	**AP/AC users N=135**	***P* Value[a]**
Age, yrs (sd)	76.4 (8.9)	79.4 (6.5)	0.001
Sex, % males	42.1	47.4	0.29
Smokers, % former and current	65.6	68.4	0.47
Retinal or subretinal hemorrhage, %	32.3	26.7	0.32
Best corrected visual acuity study eye, %			0.35
Functional vision	25.1	28.1	
Moderate visual impairment	32.3	28.9	
Severe visual impairment	42.6	43.0	
Pseudophakic eyes, %	37.0	21.5	0.49
CNV in the fellow eye, %	22.6	23.8	0.63
Retinal foveal thickness, µm (sd)	380 (123)	396 (120)	0.27
CNV lesion size, %			0.36
0-1 disc area	36.6	32.6	
≥2 disc area	63.4	67.4	
Diabetes Mellitus, %	8.7	17.0	0.01
Hypertension, %	46.2	83.7	<0.0001
Systolic blood pressure, mmHg (sd)	153 (21)	151 (25)	0.37
Diastolic blood pressure, mmHg (sd)	81 (10)	80 (12)	0.54
History of cardiovascular diseases, %	4.6	34.1	<0.0001
Myocardial infarction	3.1	11.9	<0.0001
Congestive heart failure	1.0	4.4	0.16
Stroke	1.0	3.7	0.26
Transient ischaemic attack	1.0	18.5	<0.0001
Antiplatelet or anticoagulant drugs, %	0	100	
Antiplatelet drugs, %	-	76.3	
Aspirin	-	73.3	
Clopidogrel	-	5.7	
Anticoagulant drugs, %	-	27.4	
Acenocoumarol	-	21.0	
Fenprocoumon	-	4.8	

For continuous variables means were calculated

[a] *P* values were calculated using analysis of covariance for continuous variables and logistic regression for discrete variables, adjusted for age, sex and study center

Abbreviations: AP/AC = antiplatelet or anticoagulant, CNV = choroidal neovascularization, sd = standard deviation, yrs = years

TABLE 2 - Association between antiplatelet or anticoagulant medication and risk of visual impairment at baseline

Risk of visual impairment	Functional vision eyes (N=87)	Visual impaired eyes (N=243)	OR (95% CI) Model 1	OR (95% CI) Model 2
Use of antiplatelet or anticoagulant drug				
No	49	146	1	1
Yes	38	97	0.74 (0.44-1.24)	0.79 (0.43-1.44)
Use of antiplatelet drugs				
No	54	173	1	1
Yes	33	70	0.58 (0.34-0.99)	0.61 (0.34-1.09)
Use of anticoagulant drugs				
No	81	212	1	1
Yes	6	31	1.88 (0.75-4.70)	2.00 (0.77-5.18)
Type of antiplatelet or anticoagulant drugs				
None	49	146	1	1
Aspirin	31	68	0.64 (0.37-1.12)	0.76 (0.40-1.44)
Acenocoumarol	5	24	1.42 (0.51-3.98)	1.26 (0.40-3.99)
Risk of severe visual impairment	**Functional vision eyes (N=87)**	**Severe visual impaired eyes (N=141)**	**OR (95% CI) Model 1**	**OR (95% CI) Model 2**
Use of antiplatelet or anticoagulant drug				
No	49	83	1	1
Yes	38	58	0.74 (0.42-1.30)	0.75 (0.40-1.43)
Use of antiplatelet drugs				
No	54	97	1	1
Yes	33	44	0.64 (0.36-1.15)	0.66 (0.35-1.26)
Use of anticoagulant drugs				
No	81	124	1	1
Yes	6	17	1.63 (0.61-4.41)	1.57 (0.57-4.33)
Type of antiplatelet or anticoagulant drugs				
None	49	83	1	1
Aspirin	31	44	0.71 (0.39-1.30)	0.75 (0.38-1.50)
Acenocoumarol	5	14	1.34 (0.44-4.09)	1.18 (0.36-3.85)

Model 1: adjusted for age, sex and study center
Model 2: model 1 including smoking, lens status, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: CI = confidence interval, OR = Odds' ratio

TABLE 3 - Association between antiplatelet or anticoagulant medication and the risk of retinal or subretinal hemorrhage at baseline

	No hemorrhage (N=231)	retinal or subretinal hemorrhage (N=99)	OR (95% CI) Model 1	OR (95% CI) Model 2
Use of antiplatelet or anticoagulant drug				
No	132	63	1	1
Yes	99	36	0.76 (0.46-1.25)	0.47 (0.26-0.83)
Use of antiplatelet drugs				
No	150	81	1	1
Yes	77	22	0.52 (0.30-0.90)	0.34 (0.18-0.62)
Use of anticoagulant drugs				
No	210	83	1	1
Yes	21	16	2.07 (1.02-4.20)	1.71 (0.82-3.58)
Type of antiplatelet or anticoagulant drugs				
None	132	63	1	1
Aspirin	77	22	0.61 (0.34-1.08)	0.36 (0.18-0.71)
Acenocoumarol	14	15	2.37 (1.06-5.31)	1.28 (0.51-3.20)

Model 1: adjusted for age, sex and study center
Model 2: model 1 including smoking, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: CI = confidence interval, OR = Odds' ratio

DISCUSSION

In the BRAMD study, we showed that AP/AC use did not increase the risk of visual impairment in patients with neovascular AMD, nor did this medication increase the risk of retinal hemorrhages. Subgroup analysis identified aspirin as the major contributor to these associations. AP/AC use was not associated with CNV lesion size. For foveal retinal thickness, however, aspirin use was borderline significantly associated with a thicker retina. After monthly anti-VEGF injections given for one year, no differences were observed between users and nonusers for the different outcomes.

This study has strengths and limitations. An important benefit was that our study was a case only study of neovascular AMD, resulting in a larger similarity of disease characteristics than population-based studies, which compare AMD versus no AMD. Factors such as smoking, visual acuity in the study eye, disease activity in the fellow eye, and pseudophakia were comparable between users and non-users. Other strengths are the strict inclusion criteria of the BRAMD trial requiring multimodal imaging to diagnose active CNV, homogenizing the CNV cases entering the study. Among the limitations are the lack of data on medication use prior to the onset of CNV, the exclusion of severe cases, and the relatively small sample size. This limited our ability to perform detailed subgroup analyses, and may hamper extrapolation of findings. We observed a higher age and frequency of diabetes and cardiovascular disorders among users of AP/AC in concordance with the indication for these medications. Nevertheless, true confounding by indication is unlikely: the beneficial effect for AP/AC users found in our study is not in line with the higher risk of AMD for cardiovascular disorders suggested by some studies.[15,16]

Our study investigated the effect of AP/AC medication on visual acuity, while most other studies focused on onset of early and late stages of AMD, or on the occurrence of hemorrhages only. With respect to AMD, the majority of population-based studies found an increased risk of CNV. The cross-sectional EUREYE study observed a two times increased risk of CNV for aspirin users, which sustained after correction for indication.[1] The Beaver Dam Eye Study (BDES) and Blue Mountains Eye Study (BMES) found a similarly increased risk of CNV, however, BDES observed this increase only for those with at least 10-year intake.[2] The association found by BMES was no longer significant after adjustment of additional cardiovascular risk factors.[3] Case studies and clinical trials did not support an increased risk of AMD. The Women's Health Study found a lower, albeit nonsignificant, risk of visually significant AMD for those randomized to aspirin.[5] The Physician Health Study found a similar trend for the entire group of aspirin users, and a significantly protective effect in men with hypertension.[4] A case study performed by Wilson et al. also found a protective effect of aspirin: fewer AMD patients on this medication developed CNV.[17] The Age-Related Eye Disease Study 2 reported this trend as well. This study observed a protective effect of aspirin use with the presence of AMD, including CNV, and with progression of AMD.[18,19] Meta-analyses and reviews have been published evaluating studies of various designs.[6-9,20-23] Overall risk estimates varied from no to a small increased risk (overall risk ratio < 2) for CNV as well as for other forms of AMD. Nevertheless, the inconsistency of study results was striking.

To evaluate whether AP/AC medication influenced the macular anatomy, we compared foveal retinal thickness and CNV lesion size between users and nonusers. We found a borderline significantly thicker foveal retinal thickness for aspirin users, compared to non-users. This association was found after additional adjustment for frequency of diabetes, hypertension, cardio vascular history and CNV in the fellow eye, all variables which could influence foveal retinal thickness. Unfortunately, we did not have morphology on OCT images to our disposal, which may have helped to explain the observed differences. Foveal retinal thickness was comparable between the aspirin users and the nonusers after treatment with anti-VEGF medication. For the other AP/AC groups, no significant association as found for both foveal retinal thickness and CNV lesions size at baseline and after anti-VEGF treatment. Animal studies showed equivalent results: aspirin treated mice did not differ in laser induced CNV lesion size from the control group.[24]

AP/AC use has been associated with an increased risk of bleeding, such as cerebral and gastrointestinal bleeding.[10] Whether this also accounts for ocular hemorrhages is still unclear. This study provides evidence that in particular aspirin users do not have an increased risk of retinal or subretinal hemorrhages. Several other studies focused on AP/AC and hemorrhages in CNV.[25-35] None of these studies found a protective effect. Tilanus et al.[29] found a higher risk of large hemorrhages, in particularly for AC users, while Kuhli-Hattenbach et al.[27,33] and Kiernan et al.[25] found an increased risk of large hemorrhages for all AP/AC users. By contrast, the Macular Photocoagulation Study[26] did not find a significant association between aspirin use and hemorrhages, and the Comparison of AMD Treatment Trials (CATT) study[30] only found a significant association for aspirin and clopidogrel bisulfate after stratification for hypertension. Stratification for hypertension did not influence our results (Supplemental Table 6). What could explain these differences in results, in particularly those from the CATT Study? The BRAMD study used very similar inclusion criteria as CATT, however, patients with >70% lesion area covered by blood were excluded in our study. We do not think that this is an explanation to the data, as the proportion of patients with >2 DA of hemorrhage in CATT was small (<5%). A factor that could be held accountable is the eligibility of patients with past treatments for

AMD in BRAMD (13.3%). In CATT, only naïve patients were eligible and hemorrhages had therefore not undergone prior treatment. Reports on patients treated with anti-VEGF reported only 0.6% macular hemorrhages and these hemorrhages were not associated with AP/AC use.[36]

Aspirin appeared to be the major driver of the beneficial effect of AP/AC in our study. Fourty percent of the population used AP/AC medication, of which three fourth was aspirin. Aspirin is a nonsteroidal anti-inflammatory drug (NSAID) and the only NSAID that irreversibly inactivates both isoforms of the cyclooxygenase (COX) enzymes.[12,23] It can suppress the production thromboxanes and prostaglandins by inhibiting the catalytic activity of COX-1 and COX-2, thereby reducing platelet aggregation, inflammation, and angiogenesis, and promoting apoptosis.[23,24,37] A recent study in mice showed that inhibition of COX-2 reduced angiogenesis and subretinal fibrosis in CNV.[38] Mechanisms independent of COX can also induce cell apoptosis.[37] Aspirin has antioxidant properties and provides a protective effect against lipid peroxidation, upregulates other antioxidants, and inhibits mitochondrial oxidative phosphorylation reducing reactive oxygen species.[24,37,39] Finally, aspirin modulates DNA transcription through inhibition of nuclear factor kappa B, thereby influencing many biological processes, including inflammation and apoptosis.[23,37] Taken together, aside from the anti-platelet aggregation and apoptosis, these actions of aspirin are in line with a favorable effect on AMD.

In summary, the BRAMD trial suggests that AP/AC use did not increase the risk of visual impairment or the occurrence of hemorrhages. Validation of our data is expected from the large-scale 'ASPirin in Reducing Events in the Elderly' (ASPREE) trial, an ongoing study which investigates the use of low-dose daily aspirin on AMD phenotype.[40] Our findings are of clinical importance, because they imply that patients with neovascular AMD can continue their prescribed use of AP/AC medication without negative effects on AMD outcome.

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SUPPLEMENTAL TABLE 1 - In- and exclusion criteria of the BRAMD study

Inclusion criteria
Patients 60 years of age or higher.
Patients with primary or recurrent sub-, juxta- or extrafoveal CNV secondary to AMD, including those with RAP, that may benefit from anti-VEGF treatment in the opinion of the investigator.
The total area of CNV (including both classic and occult components) encompassed within the lesion must be more or equal to 30% of the total lesion area.
The total lesion area should be < 12 disc areas.
A best corrected visual acuity (BCVA) score between 78 and 20 letters (approximately 0,63–0,05 Snellen equivalent) in the study eye.
Exclusion criteria
Ocular treatment with anti-angiogenic drugs in the last 2 months or Triamcinolone in the last 6 months.
Laser photocoagulation (juxtafoveal or extrafoveal) in the study eye within one month preceding baseline.
Patients with angioid streaks or precursors of CNV in either eye due to other causes, such as ocular
histoplasmosis, trauma, or pathologic myopia.
Spherical equivalent of refractive error in the study eye demonstrating more than– 8 diopters of myopia.
Cataract extraction within three months preceding baseline.
IOP >25 mm Hg.
Active intraocular inflammation in the study eye.
Vitreous hemorrhage obscuring view of the posterior pole in the study eye.
Presence of a retinal pigment epithelial tear involving the macula in the study eye.
Subretinal hemorrhage in the study eye if the size of the hemorrhage is > 70% of the lesion.
Subfoveal fibrosis or atrophy in the study eye.
History of hypersensitivity or allergy to fluorescein.
Inability to obtain fundus photographs, fluorescein angiograms or OCT's of sufficient quality to be analyzed and graded by the Central Reading Centre.
Systemic disease with a life expectancy shorter than the duration of the study.
Inability to adhere to the protocol with regard to injection and follow-up visits.
Legally incompetent adult.
Refusal to give written informed consent.

SUPPLEMENTAL TABLE 2 - Association between antiplatelet or anticoagulant medication and risk of visual impairment during last visit

Risk of visual impairment	Functional vision eyes (N-178)	Visual impaired eyes (N=148)	OR (95% CI) Model 1	OR (95% CI) Model 2
Use of antiplatelet or anticoagulant drug				
No	105	87	1	1
Yes	73	61	0.90 (0.57-1.42	0.97 (0.58-1.65)
Use of antiplatelet drugs				
No	117	106	1	1
Yes	61	42	0.69 (0.42-1.12)	0.71 (0.42-1.19)
Use of anticoagulant drugs				
No	163	127	1	1
Yes	15	21	1.69 (0.83-3.45)	1.81 (0.86-3.82)
Type of antiplatelet or anticoagulant drugs				
None	105	87	1	1
Aspirin	57	42	0.80 (0.48-1.32)	0.92 (0.52-1.63)
Acenocoumarol	11	17	1.63 (0.71-3.74)	1.90 (0.73-4.96)
Risk of severe visual impairment	**Functional vision eyes (N-178)**	**Severe visual impaired eyes (N=94)**	**OR (95% CI) Model 1**	**OR (95% CI) Model 2**
Use of antiplatelet or anticoagulant drug				
No	105	52	1	1
Yes	73	42	1.02 (0.60-1.71)	1.04 (0.57-1.88)
Use of antiplatelet drugs				
No	117	64	1	1
Yes	61	30	0.81 (0.47-1.39)	0.81 (0.45-1.48)
Use of anticoagulant drugs				
No	163	80	1	1
Yes	15	14	1.78 (0.81-3.92)	1.73 (0.76-3.91)
Type of antiplatelet or anticoagulant drugs				
None	105	52	1	1
Aspirin	57	30	0.96 (0.54-1.70)	1.23 (0.53-1.94)
Acenocoumarol	11	12	1.96 (0.80-4.83)	2.04 (0.72-5.75)

Model 1: adjusted for age, sex and study center
Model 2: model 1 including smoking, lens status, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: CI = confidence interval, OR = Odds' ratio

SUPPLEMENTAL TABLE 3 - Association between antiplatelet or anticoagulant medication and foveal retinal thickness on OCT at baseline

	User		Nonuser			
	N	**FRT**	***N***	**FRT**	***P* value***	***P* value****
Antiplatelet or anticoagulant drug	135	396 (120)	195	380 (123)	0.27	0.08
Antiplatelet drugs	103	401 (128)	227	380 (118)	0.19	0.06
Anticoagulant drugs	37	390 (95)	293	386 (125)	0.73	0.73
Type of anticoagulant						
Aspirin	99	405 (129)	195	380 (123)	0.15	0.04
Acenocoumarol	29	399 (96)	195	380 (123)	0.37	0.29

FRT is in µm and values are mean (SD)
P values obtained using ANCOVA
* adjusted for age, sex and study center
** adjusted for age, sex, study center, smoking, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: FRT = foveal retinal thickness, OCT = optical coherence tomography, SD = standard deviation

SUPPLEMENTAL TABLE 4 - Association between antiplatelet or anticoagulant medication and foveal retinal thickness on OCT during last visit

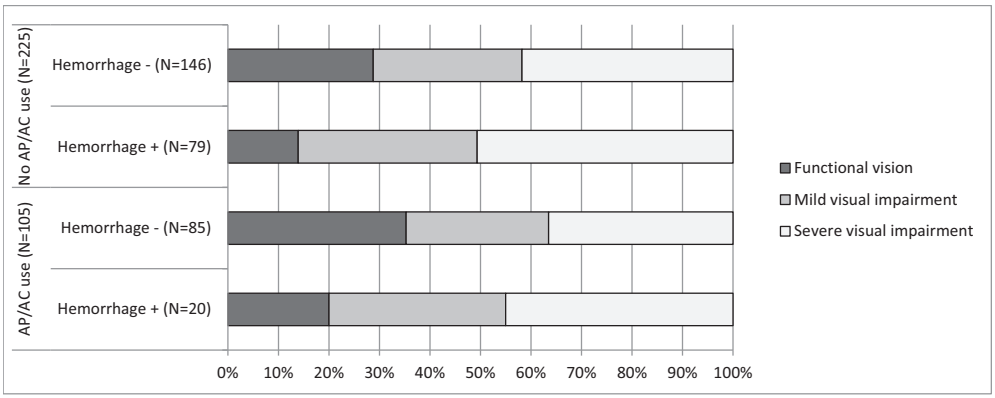
	User		Nonuser			
	N	**FRT**	**N**	**FRT**	**P value***	**P value****
Antiplatelet or anticoagulant drug	133	247 (67)	193	248 (61)	0.73	0.91
Antiplatelet drugs	103	247 (70)	223	248 (60)	0.77	0.98
Anticoagulant drugs	35	247 (62)	291	248 (64)	0.99	0.98
Type of anticoagulant						
Aspirin	99	246 (71)	193	248 (61)	0.61	0.84
Acenocoumarol	28	248 (67)	193	248 (61)	0.91	0.56

FRT is in µm and values are mean (SD)
P values obtained using ANCOVA
* adjusted for age, sex and study center
** adjusted for age, sex, study center, smoking, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: FRT = foveal retinal thickness, OCT = optical coherence tomography, SD = standard deviation

SUPPLEMENTAL TABLE 5 - Association between antiplatelet or anticoagulant medication and CNV lesion size at baseline

	Lesion size < 2 DA (N=109)	Lesion size ≥ 2 DA (N=203)	OR (95% CI) model 1	OR (95% CI) model 2
Use of antiplatelet or anticoagulant drug				
No	67	116	1	1
Yes	42	87	1.26 (0.77-2.06)	1.47 (0.83-2.60)
Use of antiplatelet drugs				
No	75	138	1	1
Yes	34	65	1.09 (0.65-1.82)	1.18 (0.67-2.06)
Use of anticoagulant drugs				
No	100	178	1	1
Yes	9	25	1.58 (0.70-3.55)	1.66 (0.72-3.86)
Type of drugs				
None	67	116	1	1
Apirin	30	65	1.37 (0.79-2.35)	1.68 (0.90-3.15)
Acenocoumarol	7	20	1.67 (0.65-4.25)	1.83 (0.64-5.20)

Model 1: adjusted for age, sex and study center
Model 2: model 1 including smoking, medical history cardiovascular diseases, diabetes, hypertension, choroidal neovascularization fellow eye
Abbreviations: CI = confidence interval, CNV = choroidal neovascularization, DA = disk area, OR = Odds' Ratio



SUPPLEMENTARY FIGURE 1 - Distribution of visual acuity in those with or without retinal or subretinal hemorrhage for antiplatelet or anticoagulant medication

On the x-axis the frequency in percentages is plotted, on the y- axis the groups indicating the users and nonusers of antiplatelet or anticoagulant medication and these group were further stratified for presence of retinal or subretinal hemorrhage (+) or no presence hemorrhage (-).
Abbreviations AC = anticoagulant, AP = antiplatelet.

SUPPLEMENTAL TABLE 6 - Association between antiplatelet or anticoagulant medication and risk of retinal or subretinal in hemorrhages at baseline in those with and without hypertension

	With hypertension (N= 203)				Without hypertension (N= 127)			
	No hemorrhage (N= 131)	retinal or subretinal hemorrhage (N=72)	OR (95% CI) Model 1	OR (95% CI) Model 2	No hemorrhage (N=100)	retinal or subretinal hemorrhage (N=27)	OR (95% CI) Model 1	OR (95% CI) Model 2
Use of antiplatelet or anticoagulant drug								
No	52	38	1	1	80	25	1	1
Yes	79	34	0.57 (0.32-1.02)	0.49 (0.26-0.93)	20	2	0.42 (0.09-1.98)	0.36 (0.06-2.14)
Use of antiplatelet drugs								
No	67	51	1	1	83	26	1	1
Yes	64	21	0.41 (0.22-0.76)	0.36 (0.18-0.69)	17	1	0.26 (0.03-2.11)	0.23 (0.02-2.36)
Use of anticoagulant drugs								
No	113	57	1	1	97	26	1	1
Yes	18	15	1.75 (0.81-3.76)	1.76 (0.81-3.85)	3	1	1.18 (0.12-12.24)	0.97 (0.09-10.82)

Model 1: Adjusted for age, sex, study center
Model 2: Model 1 including smoking, history of cardiovascular disease, diabetes, choroidal neovascularization fellow eye
Abbreviations: CI = confidence interval, NA = not able, OR = Odds' ratio

Chapter 5.1

Genetics and gene-environment interactions in age-related macular degeneration

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SUMMARY

The genetics of age-related macular degeneration (AMD) has been an exciting research area during the last decade. The advances in molecular genetic techniques paved the way for great progress in the discovery of genes and led to identification of many disease-associated risk variants. Several genes have been associated with AMD and the two major AMD genes are *CFH* and *ARMS2-HTRA1* . Other genes associated with AMD are complement (*CFB/C2, C3, CFI*), lipid (*APOE*, *LIPC, CETP*, suggested genes *LPL* and *ABCA1*), and collagen genes (*COL8A1* and *COL10A*), implicating a role for these in the pathogenesis of AMD.

Interactions of AMD genes with life style factors such as smoking and anti-oxidant intake have been reported and are of interest to manage AMD risk.

GENETIC FACTORS

Although familial occurrence had been known for many years, major advances in the identification of genetic factors for AMD were achieved after the start of Genome Wide Association Studies (GWAS). We will discuss the currently known AMD-associated genes and their importance to the disease. A summary of the genes can be found in Table 1.

The Complement Pathway Genes

Complement Factor H (CFH)

CFH is one of the two major AMD genes. The CFH protein is a key regulator of the complement pathway – it inhibits the activation of complement component C3 to C3b and degrades C3b, thereby limiting the amplification phase of the alternative complement cascade.[1] CFH is present in serum, not membrane-bound, expressed in the retinal pigment epithelium and can be found in drusen.[2,3]

First reports on an association between *CFH* and AMD stem from 2005,[4,5,6] and since then this finding has been replicated by numerous studies in different populations.[7-38] The well-known risk allele Y402H is common in Caucasians and Africans (~36%), but much less so in Asians (~7–15%) and Hispanics (~17%).[39] Functionally, this and other risk alleles have been shown to alter CFH binding, thereby impairing the regulatory function of CFH, increasing complement activation, and subsequently causing an inflammatory response and cell death.[3-6,13,40-42]

CFH is located in a large region of linkage disequilibrium. Apart from Y402H, many other variants have been shown to be associated with increased risk of AMD. A non-coding variant (rs1410996) was found to have an even stronger association than Y402H.[43-44] In particular in Asian populations, the Y402H variant was not significantly associated with AMD, whereas other variants including rs1410996 were.[24,35] The genes in the vicinity of *CFH,* such as *CFHR1-5,* have gene functions similar to *CFH,* and have also been associated with AMD. A haplotype carrying a deletion of *CFHR1* and *CFHR3* (delCFHR1/3) was reported to have a protective effect, and occurred in 20% of controls and 8% of cases.[45-46] The proteins encoded by these genes are absent in serum of persons who are homozygous for delCFHR1/3. [45] *CFHR1* and *CFHR3* contain a C3-binding site and deletion of these genes may reduce competition for the binding of CFH to C3b, enhancing inhibitory activity by CFH. DelCFHR1/3 was more frequent in African Americans (16%), and less common in Hispanics (6.8%) and European Americans (4.7%).[47]

Complement Factor B (CFB)/Complement Component 2 (C2)

Complement factor B (CFB) and complement component 2 (C2) are activators of the alternative and classical pathways, respectively. Four variants in the *CFB* and *C2* gene located on chromosome 6p21 have been shown to have a strong protective effect: *CFB* R32Q, which is in nearly complete linkage disequilibrium with *C2* IVS10, and *CFB* L9H, which is in nearly complete linkage disequilibrium with *C2* E318D.[44,48-54]

Genetic and functional data suggest that *CFB* variants rather than *C2* variants are likely to have caused the observed protection. Only the *CFB* R32Q variant results in inferior C3b binding affinity, leading to a lower potential to amplify complement activation.[55-56] Moreover, the majority of proteins of the alternative pathway (e.g., CFH, CFB) are present in drusen, whereas proteins from the classical pathway (e.g., C2) are not.[57-58] Good epidemiologic analysis with adjustment for confounders showed that the association with *C2* R32Q was robust (OR, 0.21; 95% CI, 0.11–0.39), while the association

with *C2* E318D became insignificant (OR, 0.60; 95% CI, 0.25– 1.47).[49] These data suggest that the *C2* variants show residual association with AMD originating from their high linkage disequilibrium with *CFB*.

Complement Component 3 (C3)

Complement component C3 is the convergence point of all complement pathways (classical, lectin, and alternative). Activation of C3 is crucial for the formation of membrane attack complexes that leads to cell lysis.[59] The *C3* gene is located on chromosome 19p13.3–13.2. The amino acid changes caused by the *C3* variants R102G and P314L may alter the binding capacity of C3 to pathogenic cell surfaces or other complement proteins.[59-61] A causal relation with AMD is plausible, since C3 mRNA is present in neural retina, choroids, and retinal pigment epithelium[57]; its cleavage product C3a is present in drusen,[1,62] and C3a can induce vascular endothelial growth factor expression and promote choroidal neovascularization.[63]

The two functional variants, R102G (rs2230199) and P314L (rs1047286), are in high linkage disequilibrium. They have both been identified as genetic risk factors for AMD.[64-72] R102G has also been implicated in the progression from the earlier stages of AMD to late AMD.[51] The two initial investigations as well as later studies concluded that R102G is more significant in AMD causality than P314L.[64-65,67-69,72-73]

An allele-dose effect for R102G was observed in the various Caucasian studies with an increased risk of 1.4–1.7 for heterozygotes and 1.8–3.3 for homozygotes. The Rotterdam Study found associations of the *C3* variants with early as well as late AMD, and reported that the risk increase was most prominent for the mixed type of AMD (both geographic atrophy and neovascular AMD present).[68] The effect of the *C3* alleles is independent from *CFH* Y402H and *ARMS2* A69S.[68-69]

Complement Factor I (CFI)

The CFI protein is regulated by CFH and functions as a cofactor for the cleavage and inactivation of C3b. Recently, several variants near the *CFI* gene have been associated with risk of AMD in Caucasian as well as Asian populations[74-79] In a Japanese study, rs10033900 had a protective effect with OR 0.28 (95% CI, 0.11–0.69) for homozygous carriers of the minor allele. No association was found for heterozygous carriers (OR, 0.99; 95% CI, 0.61–1.62). A recent genome-wide association study found that the major allele of rs2285714 was associated with an increased risk of 1.31 (95% CI, 1.18–1.45). Ennis et al. reported significantly ($P < 0.05$) protective effects for rs11728699, rs6854876, rs7439493, and rs13117504 with ORs ranging from 0.68 to 0.74 ($P < 0.05$), and these SNPs also tagged significant protective (GCAG, OR 0.69) and causative (TGGC, OR 1.34) haplotypes.[75-77]

The ARMS2-HTRA1 (10q26) Locus

Linkage studies had already identified a susceptibility locus at chromosome 10q26. GWA studies conformed this locus as the second major contributor to the pathogenesis of AMD.[7,81-98] As this region contains many genes in high linkage disequilibrium (*Pleckstrin Homology Domain-containing Protein family A member 1 (PLEKHA1)*, *LOC387715 (or Age-related maculopathy susceptibility gene 2, ARMS2)* and *high temperature requirement factor A1* (*HTRA1*) gene), controversy exists about which gene is the AMD susceptibility gene.

In the *ARMS2* gene, rs10490924 has repeatedly been reported to increase risk of AMD up to 15 times.[7,85-86,89-92,94,98-97] This functional SNP causes an A69S change, and has been described as the causal SNP that by itself could explain the bulk of the association between the 10q26 region and

AMD.[91] The precise function of *ARMS2* in AMD remains to be elucidated. Disorganized mitochondrial membranes, as well as decreased number of mitochondria in retinal pigment epithelium cells of AMD donors have provided evidence of mitochondrial dysfunction in AMD.[99-100] This suggests that *ARMS2* may jeopardize mitochondrial function, and consequently lead to the formation of reactive oxygen species, apoptosis, and AMD.[94,99-103] Moreover, immunohistochemical studies located the ARMS2 protein to the mitochondrial outer membrane, in particular of rods and cones.[91,94] However, its presence has also been reported in the cellular cytosol[104] and the extracellular matrix.[105]

Meta-analyses of the *HTRA1* gene reported an increased risk of AMD for homozygous (OR, 9.26; 95% CI, 7.27–11.91) as well as heterozygous (OR, 2.33; 95% CI, 2.01–2.71) carriers of the rs11200638 risk allele.[106] Stratified analyses revealed that rs11200638 was significantly associated with CNV but not with GA, and that the causative effect was stronger in Caucasians than in Asians.[107-108] Also for this gene, various lines of evidence support involvement in AMD. The rs11200638 risk allele has been associated with higher levels of *HTRA1* mRNA and protein in some studies,[87,98,109-110] although two other studies with larger datasets could not validate this finding in heterologous expression systems.[91,111] Furthermore, HTRA1 may inhibit signaling of TGF-ß proteins, which have been reported to act as negative growth regulators in the retina and RPE.[112-114] In addition, *HTRA1* may stimulate the degradation of extracellular matrix through enhanced expression of matrix metalloproteases. Consequently, overexpression of *HTRA1* may affect the integrity of Bruch's membrane and RPE contributing to AMD development. Recently, Richardson et al. found rs3793917 (located between *ARMS2* and *HTRA1*) to be most associated with AMD (OR, 3.45; 95% CI, 2.36–5.05), and indicated that the intergenic region between this SNP and *HTRA1* rs11200638 was most likely to confer AMD risk.[96] However, they could not distinguish rs3793917 from rs11200638 and rs10490924 to explain causality since they were all in high linkage disequilibrium.

Common haplotypes encompassing both the *ARMS2* and the *HTRA1* genes have also been linked to AMD. Gibbs et al. described a common haplotype TAT tagged by rs10490924, rs11200638, and rs2293870 that significantly predisposed to AMD ($P = 2.70 \times 10–9$), and a haplotype GGG that was significantly protective against AMD ($P = 0.003$).[95] Yang et al. also found a haplotype T-G-Wt-G tagged by rs2736911, rs10490924, in/del/Wt, and rs11200638, which was protective in Caucasian as well as Chinese populations ($P < 0.007$).[98] They also observed that the in/del or the rs11200638 risk allele by itself was insufficient to alter HTRA1 expression, and found that a common disease haplotype including both the in/del and rs11200638 leads to upregulation of *HTRA1*. Hence, they proposed a binary model where downregulation of *ARMS2* and concomitant upregulation of *HTRA1* best explained the risk associated with the 10q26 locus. Further functional analyses in larger datasets are warranted to conclude what the key genetic contributors in the 10q26 locus are.

The Lipid-Related Genes

Apolipoprotein E (APOE)

Apolipoprotein E is a key regulator of lipid and cholesterol transport in the central nervous system,[115] and has been linked to various neurodegenerative and cardiovascular diseases (e.g., Alzheimer's disease and stroke).[116-118] In the eye, APOE is expressed in photoreceptor cells, retinal ganglion cells, Müller cells, retinal pigment epithelium, Bruch's membrane, choroid, and in the disease-associated lesions: drusen and basal laminar deposits.[57,58,119-123] There are three common allelic variants of the *APOE* gene: ε2, ε3, and ε4, with ε3 being the most prevalent.[124-125] The majority of studies support a protective effect of the *APOE* ε4 allele against AMD,[119,126-138] though in some reports, this inverse association failed to reach statistical significance.[127,131,133,137-138] Stratification of late AMD into GA and

CNV showed that the greatest protective effect for the ε3ε4 genotype was in individuals with GA (OR 0.35, 95% CI 0.13–0.92).[132] The *APOE* ε2 allele has mainly been associated with a non-significant but increased risk of AMD.[119,126,129-132,134,137]

Several studies reported that ε4 carriers have significantly lower CRP levels than noncarriers, especially compared to ε2 carriers. CRP level reportedly decreases in a dose-dependent order of ε2/ ε2, ε2/ε3, ε3/ε3, ε2/ε4, ε3/ε4, and ε4/ε4.[140-145] In addition, *APOE* ε2 appears to enhance expression by RPE cells of the vascular endothelial growth factor and fibroblast growth factor,[146] whereas their expression is reportedly suppressed by *APOE* ε4.[136,147] This indicates that *APOE* ε2 induces neovascularization by altering angiogenic cytokines, whereas *APOE* ε4 limits this process. And in contrast to ε2, *APOE* ε4 has positive charges which diminish hydrophobicity of Bruch's membrane, and results in better clearance of debris. Moreover, ε4 carriers reportedly have 36% lower risk of hypertension than noncarriers.[148] Another interesting finding is that APOE ε4 levels seem to decrease with advancing age,[145] which may reduce transport of lipids and cell debris, culminating in a higher rate of AMD in older age. APOE also plays an important role in the maintenance of retinal membrane cell: Lipids are released from the degenerating cell membrane and astrocytes react by synthesizing APOE to bind the free cholesterol and lipids and to distribute them for reuse in cell membrane biosynthesis.[149-151] Based on the cumulative empirical evidence and pooled data outlined above, it can be proposed that the *APOE* ε4 offers a reduced risk for onset, severity, and progression rate of AMD, in contrast to *APOE* ε2.

Hepatic Lipase (LIPC)

Two parallel published genome-wide association studies (GWAS) reported causative variants for the *LIPC* gene.[76,152] *LIPC* has been associated with high-density lipoprotein cholesterol (HDL-c) levels in blood[153-154] and is involved in mediating the uptake of HDL-c at the cell surface.[155] LIPC is expressed in the retina and modification of HDL-related efficiency could influence the risk of AMD, because HDL is an important transporter of lutein/zeaxanthin.[152,156-157] The common allele of rs493258 near the *LIPC* gene on chromosome 15q22 (OR, 1.14; 95% CI, 1.09-1.20; frequency in controls ~0.56; P = 1.3 × 10-7) increased the risk of AMD, whereas the minor allele of rs10468017, a functional promoter variant, (OR, 0.82; 95% CI, 0.77-0.88; frequency in controls ~0.30; P = 1.34 × 10-8) had a protective effect. However, confirmation of the protective variant was achieved after targeted examination of the suggestive markers of the GWAS peformed by Neale et al.[152]

Cholesterylester Transfer Protein (CETP)

The rare allele of rs3764261 at the *CETP* gene on chromosome 16q21 (OR, 1.19; 95% CI, 1.12-1.27; frequency in controls ~0.32; P = 7.4 × 10-7) in associated with an increased risk of AMD[76] and has recently been replicated by Yu et al.[158] CETP plays an important role in the production and degradation of HDL-c and is expressed in the retina.[152,156]

Lipoprotein Lipase (LPL)

Chen et al. (2010) reported also the variant rs12678919 at *LPL* on chromosome 8p22 (OR, 1.38; 95% CI, 1.11-1.43; P = 3 × 10-3). This variant was not significant but consistent with the hypothesis that HDL metabolism is associated with AMD pathogenesis; LPL plays, like CETP, an important role in the production and degradation of HDL-c.[152,156] Recently, an association, although not significant, between *LPL* rs12678919 and late AMD was suggested by Peter et al.(OR, 0.5, 95% CI, 0.2—1.2, P = 0.10) for carriers of 1 or 2 G alleles compared to non-carriers.[79] The G allele increases serum HDL levels (P < 10-10, 2.44 mg/dl increase per G allele), confirming the role of LPL in the HDL metabolism.[153]

ATP-Binding Cassette Subfamily A Member 1 (ABCA1)

ABCA1 is involved in mediating the uptake of HDL-c at the cell surface and have been shown to be expressed in the retina.[152,155-156] The variant rs1883025 near *ABCA1* on chromosome 9q22 (OR, 1.15; 95% CI, 1.07-1.23; P = 5.6 × 10-4) has been suggested by Chen et al. to be associated with AMD.[76] Several other studies confirmed this suggestion and showed a significantly higher risk allele frequency in AMD patients compared with control individuals (P = 0.00027).[152,158-160]

With increasing age, lipids and cholesterol accumulate underneath the RPE and are constituents of drusen.[57,161] The HDL-c associated variants might affect the formation of drusen and subsequently the development of AMD. The 'non risk' TT-genotype of ABCA1 rs1883025 had a significant protective effect for intermediate and large drusen; (OR, 0.48; 95% CI; 0.27-0.85), (OR, 0.41; 95% CI, 0.23-0.74), respectively.[160]

Collagen related genes

Alpha chain of type VIII collagen (COL8A1)

The *COL8A1* gene on chromosome 3 encodes for one of the alpha chains of type VIII collagen, a major component of the multiple basement membranes in the eye, including Bruch's membrane and the choroidal stroma.[152] The intronic variant rs13095226 was associated with a slight increased risk of AMD (OR, 1.24; 95% CI, 1.13-1.35; P = 2.50 × 10-6).[162]

Alpha chain of type X collagen (COL10A1)

Recently, a genome-wide association study (GWAS) has published a novel loci, rs1999930 near the *COL10A1* gene (OR, 0.87; 95% CI, 0.83-0.91; P = 1.1 × 10-10).[158] COL10A1 is a short-chain collagen expressed by hypertrophic chondrocytes during endochondral ossification. Although no relation of COL10A1 with the retina has been found, the previous finding of the collagen gene *COL8A1*, implicates a role for collagen in a causal pathway for AMD.

Other genes

Tissue Inhibitor of Metalloproteinases-3 (TIMP3)

Candidate gene analyses initially found no evidence of linkage or association between AMD and TIMP3 on chromosome 22q12.1–13.2.[163,164] Recently, a genome-wide association study (GWAS) found the region near TIMP3 to be a susceptibility locus,[76] which was previously reported by one linkage study.[165] TIMP3 is a metalloproteinase involved in degradation of the extracellular matrix in the retina,[166] and is mutated in Sorby's fundus dystrophy.[167] The common variant rs9621532, nearby TIMP3 was associated with increased risk of AMD (OR, 1.41; 95% CI, 1.27-1.57; frequency in controls ~0.95; P = 1.1 × 10-11).[76]

Vascular Endothelian Growth Factor A (VEGFA)

VEGFA is a member of the VEGF family and functions to increase vascular permeability, angiogenesis, cell growth and migration of endothelial cells. VEGFA is also a target in the treatment of CNV with anti-VEGF therapy. Haines et al found a strong association for the variant rs2010963 with linkage-analysis (LOD score = 1.32, P= 0.0001) in early and late AMD, but a moderate result in a later case-control setting (P = 0.02).[168] Recently a new variant near the *VEGFA* gene was published.[158] The variant rs4711751 on 6p12 near *VEGFA* (OR, 1.15; 95% CI, 1.10-1.21; P=8.7 × 10-9) was not in LD with the earlier found variant, indicating a novel region associated with AMD. Unfortunately the variant found by Haines et al could not be replicated.

TABLE 1 - Overview of genes associated with AMD

Gene	Chrom1	Variant	Effect allele	EAF	OR (95%CI) het*	OR (95% CI) hom**	Function	References, meta-analyses
CFH	1	rs10661170 (Y402H)	C	0.349	2.50 (1.96-3.30)	6.32 (4.25-9.48)	Key regulator of the alternative complement pathway and inhibits C3 activation	Thakkinstian et al. 2006
C3	19	rs22330199 (R102G)	G	0.193	1.44(1.33-1.56)	1.88 (1.59-2.23)	Activation of C3 is crucial for the formation of membrane attack complexes that leads to cell lysis	Thakkinstian et al. 2011
		rs1047286 (P314L)	A	0.196	1.27(1.15-1.41)	1.70 (1.27-2.11)		
CFI	4	rs10033900	C	Controls: 0.39	0.99 (0.61-1.62)	0.28 (0.11-0.69)	CFI is regulated by CFH and functions as a cofactor for the cleavage and inactivation of C3b	Kondo et al. 2010
ARMS2	10	rs10490429 (A69S)	T	Controls: 0.25	2.35 (2.07-2.67)	7.51 (5.71-9.66)	ARMS2 may be involved in mitochondrial function in the retinal pigment epithelium cells	Tang et al. 2009
HTRA1	10	rs11200638	A	Controls: 0.32	2.33 (2.01-2.71)	9.26 (7.27-11.91)	HTRA1 may inhibit signalling of TGF-b proteins	
C2/CB	6	rs641153 (R32Q)	A	Controls: 0.10	0.29 (0.17-0.48)		C2 and CB are activators of the alternative and classical pathway	Spencer et al. 2007
APOE	19	ε4	-	Controls: ~0.16	0.54 (0.41-0.70)		APOE is a key regulator of lipid and cholesterol transport in the central nervous system	Schmidt et al. 2002
TIMP3	22	rs9621532	A	Controls: 0.95	1.41 (1.27-1.57)		TIMP3 is involved in degradation of the extracellular matrix in the retina	Chen et al. 2010
LIPC	15	rs493258	C	Controls: 0.56	1.14 (1.09-1.20)		LIPC is involved in mediating the uptake of HDL-c at the cell surface	Chen et al. 2010
		rs10468017	T	Controls: 0.30	0.82 (0.77-0.88)			Neale et al. 2010
CETP	16	rs3764261	A	Controls: 0.32	1.19 (1.12-1.27)		CETP plays an important role in the production and degradation of HDL-c	Chen et al. 2010
COL8A1	3	rs13095226	C	0.12	1.24 (1.13-1.35)		Collagene type VIII is a major component of Bruch's membrane and the choroidal stroma	Neal et al. 2010
COL10A1	6	rs1999930	T	Controls: 0.30	0.87 (0.83-0.91)		Collagene type X is expressed by hypertrophic chondrocytes	Yu et al. 2011b
VEGFA	6	rs4711751	T	Controls: 0.30	1.15 (1.10-1.21)		VEGFA increases vascular permeability, angionesis, cell growth and migration of endothelial cells	Yu et al. 2011b

Abbreviations: Chrom = chromosome, EAF = Effect allele frequency, OR = Odds' Ratio
* OR het = Odd's Ratio for heterozygotes, carriers of one risk allele
** OR hom = Odd's Ratio for homozygotes, carriers of two risk alleles

Tumor necrosis factor receptor superfamily 10 a (TNRSF10A)

TNRSF10A encodes for TRAILR1, a TRAIL receptor, which is broadly expressed in human adult RPE.[166] Arakawa et al reported a variant, rs13278062 near *TNRSF10A* on chromosome 8p21 (OR, 0.73; 95% 0.67-0.80; P = 1.03 × 10-12) to be associated with exudative AMD in a Japanese population.[169]

GENE-GENE AND GENE-ENVIRONMENT INTERACTIONS

CFH Y402H

The Rotterdam Study (RS) reported interaction between *CFH* Y402H and smoking, C-reactive protein level, and erythrocyte sedimentation rate (ESR),[13] meaning that the joint effect of each determinant with Y402H was significantly greater than the sum of the independent effects. Compared to persons with the homozygous non-risk (TT) genotype and normal ESR levels, persons with the homozygous risk (CC) genotype and elevated ESR levels had a risk of 20.2 (95% CI, 9.5–43.0) for late AMD. Higher serum CRP levels in persons with the CC-genotype augmented AMD risk to 27.7 (95% CI, 10.7–72.0) compared to persons with the lowest CRP levels and the TT-genotype.

Current smokers with the CC-genotype had an OR of 34.0 (95% CI, 13.0–88.6) for late AMD relative to individuals with the TT-genotype who never smoked. Other studies also observed stronger effects of *CFH* Y402H among smokers.[18,20,22,170-172] DeAngelis et al. (2007) further specified that smoking ten pack-years or more and having the CC-genotype was estimated to increase risk of CNV 144-fold compared with smoking less than ten pack-years and having the CT- or TT-genotype.[22]

AREDS reported a significant interaction between *CFH* Y402H and BMI.[18] Higher BMI (≥25) did not increase the risk of AMD for persons with the TT-genotype (OR 0.7; 95% CI 0.4–1.2), whereas it did increase risk for those with the CT- (OR 2.2; 95% CI 1.3–4.0) and CC-genotype (OR 5.9; 95% CI 3.1–11.4).

Gold et al. reported that the protection conferred by *C2* and/or *CFB* was strongest in persons with the *CFH* CC-genotype (OR = 0.27), intermediate in persons with the CT-genotype (OR = 0.36), and weakest in persons with the TT-genotype (OR = 0.44).[48] However, the confidence intervals of all these estimates overlapped.

Two studies have examined interaction between genetic variants and antioxidants in the development of late AMD.[173,174] AREDS calculated the risk of progression to late AMD for the CFH Y402H and *ARMS2* A69S genotypes in various antioxidant treatment arms.[173] A high zinc dosage was most protective against AMD in persons with the homozygous non-risk *CFH* genotype, but produced the greatest, albeit non-significant, protection in persons carrying the risk variant of *ARMS2*. The Blue Mountains Eye Study (BMES) found that high fish intake resulted in greater protection against late AMD in homozygous carriers of Y402H than in non-carriers.[174] In addition, the RS showed that higher dietary intake of zinc, w-3 fatty acids, b-carotene, and lutein/zeaxanthin can attenuate the incidence of early AMD in those carrying these genetic risk variants (Figure 1).[175]

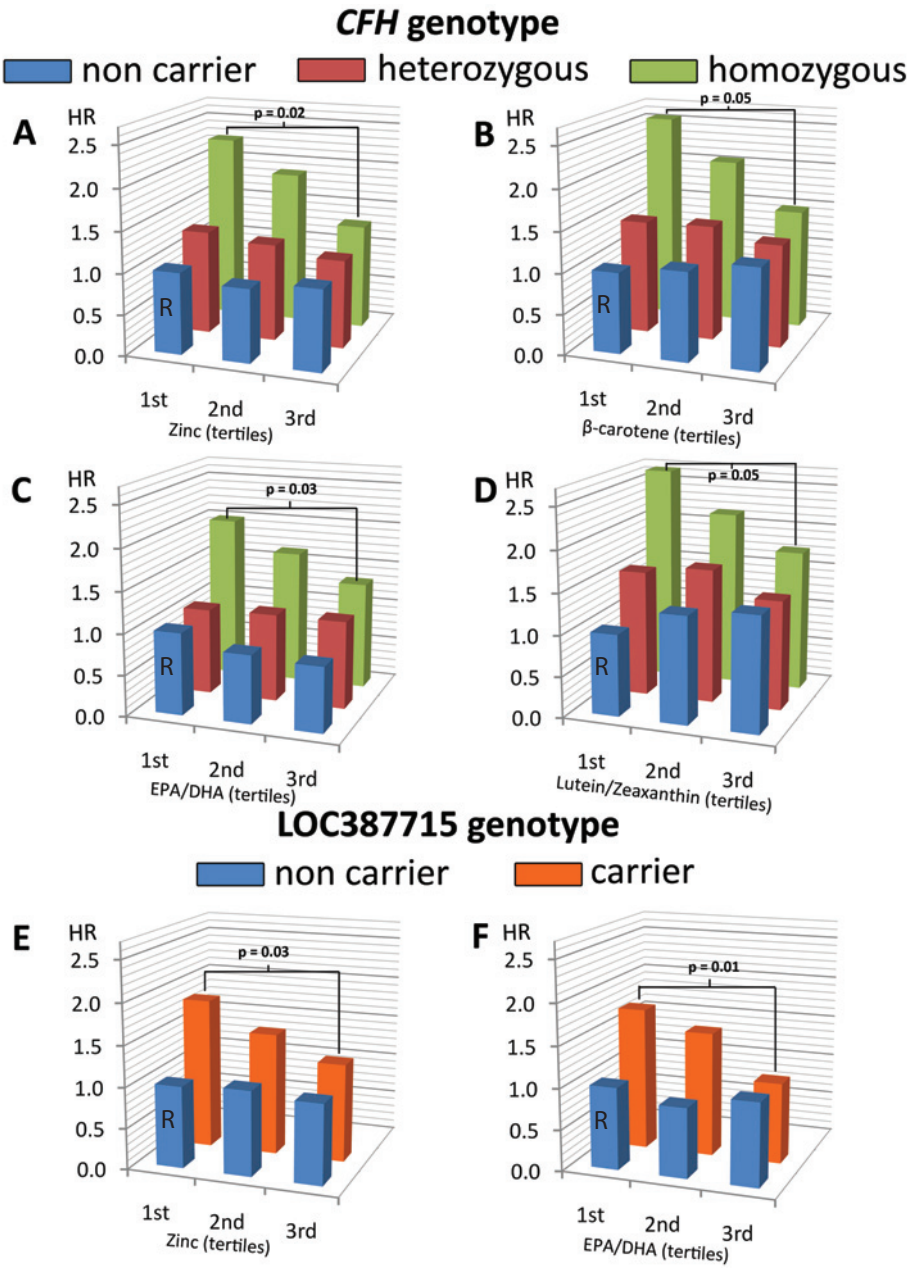

FIGURE 1 - Risks for AMD stratified for genotype and nutrient intake

A - D. Joint effect of dietary nutrient intake and *CFH* Y402H genotype on the risk of Early AMD.

E – F. Joint effect of dietary nutrient intake and *LOC387715 (ARMS2)* A69S genotype on the risk of Early AMD.

R is the common reference group.

HRs are estimates of the relative risk of Early AMD, and represent the risk of disease (Early AMD vs No AMD) in the various genetic-environmental risk groups divided by the risk of disease (Early AMD vs No AMD) in the common reference group (noncarriers - 1st tertile of nutrient intake). HRs are estimated with Cox regression analyses and included age, sex, smoking status, and atherosclerosis.

ARMS2-HTRA1

Although not all studies reported statistical interaction, the majority supported a strong combined effect of smoking and *ARMS2* A69S in AMD susceptibility.[84,170,176-182] Interaction analyses by Schmidt et al. between number of pack-years of smoking and A69S genotypes revealed that in affected persons, the frequency of the homozygous risk (TT) genotype linearly increased with increasing pack-years irrespective of age and gender, with a corresponding decrease in the homozygous non-risk (GG) genotype frequencies ($P < 0.05$).[181] When comparing current smokers to never smokers, risks for heterozygotes (GT) increased 3- to 6-fold, while for the homozygotes (GG), risk increased 10- to 27-fold.[170,176]

Combined effects on the likelihood of early or late AMD were demonstrated by the BMES for the A69S GT- and TT-genotypes with the marker's high-sensitivity CRP (ORs, 1.2 for the highest tertile alone, 1.6 for GT- and TT-genotypes alone, and 2.2 for both GT and TT-genotypes plus the highest tertile, compared with the GG-genotype with the two lower tertiles), IL-6 (corresponding ORs, 1.1, 1.6, and 2.2), sICAM-1 (ORs, 1.0, 1.5, and 2.3, respectively), and PAI-1 (ORs, 1.3, 1.7, and 2.3, respectively), but not with WCC, fibrinogen, homocysteine, and von Willebrand factor.[179]

Interaction with anti-oxidants and *ARMS2* was studied within the same settings as *CFH*, and resulted in similar effects (Figure 1).

Risk of AMD due to the Combined Effect of CFH and ARMS2/HTRA1 SNPs

Several studies have investigated the combined effect of *CFH* Y402H and *ARMS2* A69S/*HTRA1* rs11200638.[7,38,87,170,178,183-185,188] Persons with homozygous risk genotypes at both loci (*CFH* CC –*ARMS2* TT) compared to the non-risk genotype (TTGG) had ORs ranging from 27 in a Finnish case-control study[178] to 228 in the Caucasian clinic-based AREDS.[176] For persons with the homozygous risk genotype for both *CFH* Y402H and *HTRA1* rs11200638, the combined ORs ranged from 8 in a Japanese case-control study[184] to 193 in AREDS relative to persons with no risk alleles at either locus.[184] In addition to the combined risk conferred by *CFH* Y402H and *ARMS2* A69S, Schmidt et al. also observed an extra risk of AMD caused by smoking.[181] Compared to the nonsmoker/TT(Y402H)/GG(A69S) combination, the OR for individuals with the CC-genotype at Y402H and the TT-genotype at *ARMS2* increased from 10.2 for nonsmokers to 34.5 for smokers. Seitsonen et al. also found that smoking caused an extra risk for AMD, but only in connection with sex and *C3* genotype.[178] The univariate ORs for carrying at least one risk allele of *CFH* Y402H was 5.45 (95% CI, 2.18–16.83), of *ARMS2* A69S was 4.89 (95% CI, 1.73–16.43), of *C3* R102G was 2.12 (95% CI, 0.52–8.70), and for smoking was 3.22 (95% CI 1.81–6.09), while the joint OR for the three loci and smoking was 74.3 (95% CI, 10.81–2123.6).

APOE gene

Debate remains regarding the gender-specific role of the *APOE* alleles in the development or progression of AMD. Schmidt et al. found significant interaction between ε2-carrier status and sex.[130] The ε2-allele conferred a risk of 0.74 (95% CI, 0.52–1.06) in women, and of 1.54 (95% CI, 0.97–2.45) in men. Hence, the authors suggested that an increased risk of AMD due to the ε2-allele may only be conferred to men. Conversely, Baird et al. found that ε2-carriers had a significant 4.8-fold (95% CI, 1.19–19.09) increased risk of AMD progression compared to ε4-carriers and a nearly significant 2.8-fold (95% CI, 0.96–19.09) increased risk compared to ε3-carriers.[132] Since this finding was only present in women, the authors suggested that there may be a gender-specific role in progression of AMD in persons with an ε2-allele. Fritsche et al. could not corroborate any gender-specific role of the *APOE*-alleles.[186]

Schmidt et al.suggested a modifying effect of *APOE* genotypes on the smoking-associated risk of AMD, particularly for CNV.[128,187] The effect of smoking was most deleterious for *APOE* ε2 carriers, compared to *APOE* ε4 carriers and persons with the *APOE* ε3/ε3 genotype. The increase in CNV risk due to smoking was greatest in *APOE* ε2 carriers, with genotypespecific risks increasing from 1.9 for *APOE* ε4 carriers (P = 0.11) to 2.2 for *APOE* ε3/ε3 homozygotes (P = 0.007) to 4.6 (P = 0.001) for *APOE* ε2 carriers, compared to non-smoking *APOE* ε3/ε3 persons. In other studies, the sample sizes of each subgroup were too small to determine statistical significance.[134,139]

CONCLUSION

Since the first assumption of a familial component to AMD, 15 genes associated with the disease have been identified. These genes have shed light on the pathogenesis of AMD, and have increased our knowledge on the causes of AMD enormously.

Most of the genetic risk is explained by only two genes, *CFH* and *ARMS2/HTRA1*. The risk variants in these genes occur at a much higher frequency in the general population than the actual disease does, provoking the view that life style factors ultimately determine whether these genes will have a deleterious effect. Interaction with life style factors such as smoking and BMI has been difficult to prove, but the first reports on the complexity of gene-environment modulations have appeared.

Future genetic research will make use of the new molecular methodology such as exome and whole genome sequencing. This will undoubtedly lead to finding more risk variants, and more information on causal pathways for AMD. Large genetic epidemiologic collaborations will be able to address the interaction with environmental factors better than single studies can, and they will also help elucidate AMD pathogenesis. It is expected that these developments will open up new avenues for long-lasting and successful treatments for AMD.

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Chapter 5.2

Seven New Loci Associated with Age-Related Macular Degeneration

The AMD Gene Consortium
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ABSTRACT

Age-related macular degeneration (AMD) is a common cause of blindness in older individuals. To accelerate understanding of AMD biology and help design new therapies, we executed a collaborative genome-wide association study, examining >17,100 advanced AMD cases and >60,000 controls of European and Asian ancestry. We identified 19 genomic loci associated with AMD with $p<5x10^{-8}$ and enriched for genes involved in regulation of complement activity, lipid metabolism, extracellular matrix remodeling and angiogenesis. Our results include 7 loci reaching $p<5x10^{-8}$ for the first time, near the genes *COL8A1/FILIP1L, IER3/DDR1, SLC16A8, TGFBR1, RAD51B, ADAMTS9/MIR548A2,* and *B3GALTL*. A genetic risk score combining SNPs from all loci displayed similar good ability to distinguish cases and controls in all samples examined. Our findings provide new directions for biological, genetic and therapeutic studies of AMD.

INTRODUCTION, RESULTS AND DISCUSSION

AMD is a progressive neurodegenerative disease that leads to loss of central vision through death of photoreceptors[1,2]. In developed countries, AMD is the leading cause of blindness in those >65 years[3]. Genes in the complement pathway[4-11] and a region of chromosome 10[12,13] have now been implicated as the major genetic contributors to disease. Association has also been demonstrated with several additional loci[14-20], each providing an entry-point into AMD biology and potential therapeutic targets.

To accelerate the pace of discovery in macular degeneration genetics, 18 research groups from across the world formed the AMD Gene Consortium in early 2010, with support from the National Eye Institute (Table 1, Supplementary Table 1, Supplementary Note). To extend the catalog of disease associated common variants, we first organized a meta-analysis of genomewide association scans (GWAS) – combining data for >7,600 cases with advanced disease (geographic atrophy, neovascularization, or both) and >50,000 controls. Each study was first subject to GWAS quality control filters (customized taking into account study specific features as detailed in Supplementary Table 2) and standardized to the HapMap reference panel and statistical genotype imputation[22-25]. Results were combined through meta-analysis26 and thirty-two variants representing loci with promising evidence of association were genotyped in an additional >9,500 cases and >8,200 controls (Supplementary Tables 1–3; summary meta-analysis results available online). Our overall analysis of the most promising variants thus included >17,100 cases and >60,000 controls.

TABLE 1 - Summary of the samples used in genome-wide discovery and targeted follow-up analyses
For additional details, including a breakdown of the number of cases and controls in individual samples, see Supplementary Table 1. N_{cases} includes only cases with geographic atrophy, choroidal neovascularization, or both.

Analysis	Contributing study groups	N_{cases}	% Female	% Neovascular disease	$N_{controls}$	% Female
Genome-wide discovery	15	7,650	53.9	59.2	51,844	45.2
Targeted follow-up	18	9,531	56.3	57.8	8,230	53.8
Overall	33	17,181	55.2	58.4	60,074	46.3

Our meta-analysis evaluated evidence for association at 2,442,884 SNPs (Figure 1). Inspection of Q-Q plots (Supplementary Figure 1) and the genomic control value (ëGC=1.06) suggest that unmodeled population stratification does not significantly impact our findings (Supplementary Table 4 for details). Joint analysis of discovery and follow-up studies[27] resulted in 19 loci reaching $p<5x10^{8}$ (Figure 1, Table 2, Supplementary Table 5). These 19 loci include all susceptibility loci previously reaching $p<5x10^{-8}$, except the 4q12 gene cluster for which association was reported in a Japanese population. In addition, the set includes seven loci reaching $p<5x10^{-8}$ for the first time.

TABLE 2 - Summary of loci reaching genome-wide significance.

						Discovery		Follow-up		Combined	
SNP	**Risk allele**	**Chr.**	**Position**	**Nearby genes**	**EAF**	***P***	**OR**	***P***	**OR**	***P***	**OR(95% CI)**
Loci previously reported at $P < 5 \times 10^{-8}$											
rs10490924	T	10	124.2 Mb	*ARMS2-HTRA1*	0.30	4×10^{-353}	2.71	2.8×10^{-190}	2.88	4×10^{-540}	2.76 (2.72-2.80)
rs10737680	A	1	196.7 Mb	*CFH*	0.64	1×10^{-283}	2.40	2.7×10^{-152}	2.50	1×10^{-434}	2.43 (2.39-2.47)
rs429608	G	6	31.9 Mb	*C2-CFB*	0.86	2×10^{-54}	1.67	2.4×10^{-37}	1.89	4×10^{-89}	1.74 (1.68-1.79)
rs2230199	C	19	6.7 Mb	*C3*	0.2	2×10^{-26}	1.46	3.4×10^{-17}	1.37	1×10^{-41}	1.42 (1.37-1.47)
rs5749482	G	22	33.1 Mb	*TIMP3*	0.74	6×10^{-13}	1.25	9.7×10^{-17}	1.45	2×10^{-26}	1.31 (1.26-1.36)
rs4420638	A	19	45.4 Mb	*APOE*	0.83	3×10^{-15}	1.34	4.2×10^{-7}	1.25	2×10^{-20}	1.30 (1.24-1.36)
rs1864163	G	16	57.0 Mb	*CETP*	0.76	8×10^{-13}	1.25	8.7×10^{-5}	1.17	7×10^{-16}	1.22 (1.17-1.27)
rs943080	T	6	43.8 Mb	*VEGFA*	0.51	4×10^{-12}	1.18	1.6×10^{-5}	1.12	9×10^{-16}	1.15 (1.12-1.18)
rs13278062	T	8	23.1 Mb	*TNFRSF10A*	0.48	7×10^{-10}	1.17	6.4×10^{-7}	1.14	3×10^{-15}	1.15 (1.12-1.19)
rs920915	C	15	58.7 Mb	*LIPC*	0.48	2×10^{-9}	1.14	0.004	1.1	3×10^{-11}	1.13 (1.09-1.17)
rs4698775	G	4	110.6 Mb	*CFI*	0.31	2×10^{-10}	1.16	0.025	1.08	7×10^{-11}	1.14 (1.10-1.17)
rs3812111	T	6	116.4 Mb	*COL10A1*	0.64	7×10^{-8}	1.13	0.022	1.06	2×10^{-8}	1.10 (1.07-1.14)
Loci reaching $P < 5 \times 10^{-8}$ for the first time											
rs13081855	T	3	99.5 Mb	*COL8A1-FILIP1L*	0.10	4×10^{-11}	1.28	6.0×10^{-4}	1.17	4×10^{-13}	1.23 (1.17-1.29)
rs3130783	A	6	30.8 Mb	*IER3-DDR1*	0.79	1×10^{-6}	1.15	3.5×10^{-6}	1.16	2×10^{-11}	1.16 (1.11-1.20)
rs8135665	T	22	38.5 Mb	*SLC16A8*	0.21	8×10^{-8}	1.16	5.6×10^{-5}	1.13	2×10^{-11}	1.15 (1.11-1.19)
rs334353	T	9	101.9 Mb	*TGFBR1*	0.73	9×10^{-7}	1.13	6.7×10^{-6}	1.13	3×10^{-11}	1.13 (1.10-1.17)
rs8017304	A	14	68.8 Mb	*RAD51B*	0.61	9×10^{-7}	1.11	2.1×10^{-5}	1.11	9×10^{-11}	1.11 (1.08-1.14)
rs6795735	T	3	64.7 Mb	*ADAMTS9*	0.46	9×10^{-8}	1.13	0.006	1.07	5×10^{-9}	1.10 (1.07-1.14)
rs9542236	C	13	31.8 Mb	*B3GALTL*	0.44	2×10^{-6}	1.12	0.0018	1.08	2×10^{-8}	1.10 (1.07-1.14)

All results reported here include a genomic control correction for individual studies and also for the final meta-analysis[51]. A summary of all gene name abbreviations used in this table and elsewhere in the manuscript is provided in Supplementary Table 5. EAF is the allele frequency of the risk-increasing allele. Chr., chromosome.

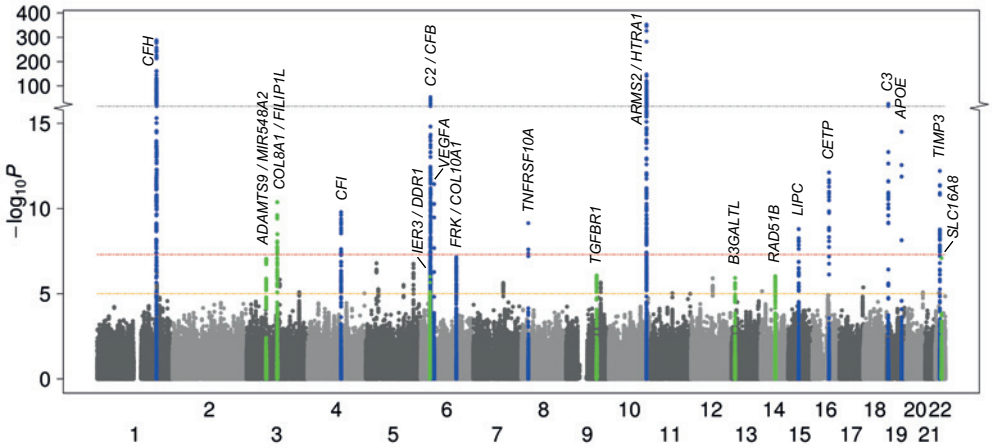

FIGURE 1 - Summary of genomewide association scan results. Summary of genomewide association scan results in the discovery GWAS sample. Previously described loci reaching p < 5x10-8 are labeled in blue; new loci reaching p < 5x10-8 for the first time after follow-up are labeled in green.

We evaluated heterogeneity between studies using the I2 statistic, which compares variability in effect size estimates between studies to chance expectations28. We observed significant (p<.05/19) heterogeneity only for loci near *ARMS2* (I^2=75.7%, p<1x10^{-6}) and near *CFH* (I^2=85.4%, p<1x10^{-6}). Although these two loci were significantly associated in every sample examined, the magnitude of association varied more than expected. To explore sources of heterogeneity, we carried out a series of sub-analyses: we repeated the genomewide meta-analysis adding an age-adjustment, separating neovascular (NV) and geographic atrophy (GA) cases, in men and women, and in European- and Asian-ancestry samples separately (Figure 2, Supplementary Figure 2). These sub-analyses of the full GWAS dataset did not uncover additional loci reaching p<5x10-8; furthermore heterogeneity near *CFH* and *ARMS2* remained significant in all sub-analyses (I^2>65%, p <0.001). Consistent with previous reports[17,29,30], separate analysis of NV and GA cases showed *ARMS2* risk alleles preferentially associated with risk of NV (OR_{NV}=2.97, OR_{GA}=2.50, $p_{difference}$=.0008) whereas *CFH* risk alleles preferentially associated with risk of GA (OR_{NV}=2.34, OR_{GA}=2.80, $p_{difference}$=.0006). We also observed large differences in effect sizes when stratifying by ethnicity, with variants near *CFH* exhibiting stronger evidence for association among Europeans (p=0.0000001) and those near *TNFRSF10A* among East Asians (p=0.002). Potential explanations include differences in linkage disequilibrium between populations or differences in environmental or diagnostic factors that modify genetic effects.

Identifying the full spectrum of allelic variation that contributes to disease in each locus will require sequencing of AMD cases and controls. To conduct an initial evaluation of the evidence for multiple AMD risk alleles in the nineteen loci described here, we repeated genome-wide association analyses conditioning on the risk alleles listed in Table 2. We then examined each of the 19 implicated loci for variants with independent association (at p<0.0002, corresponding for an estimate of ~250 independent variants per locus). This analysis resulted in the identification of the previously well documented independently associated variants near *CFH* and *C2/CFB*[8,10,31,32] and of additional independent signals near *C3*, *CETP, LIPC, FRK/COL10A1*, *IER3/DDR1*, *RAD51B* (Supplementary Table

6). In four of these loci, the independently associated variants mapped very close (within <60kb) to the original signal. This shows each locus can harbor multiple susceptibility alleles, encouraging searches for rare variants that elucidate gene function in these regions[33,34].

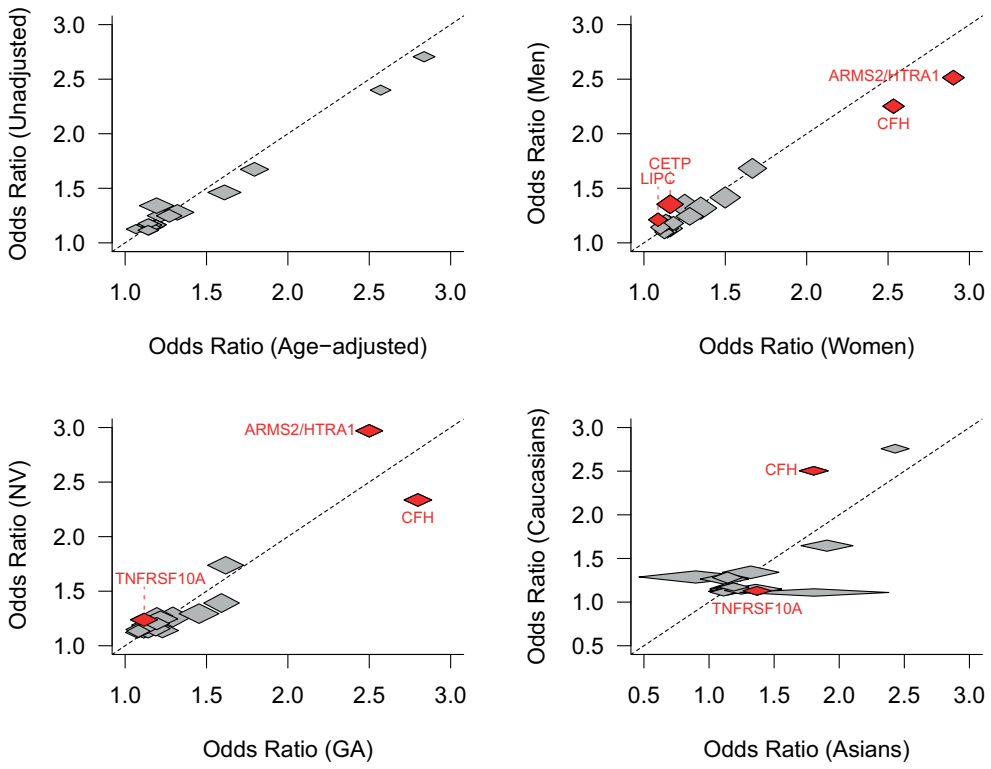

FIGURE 2 - Sensitivity analysis. The top left panel compares estimated effect sizes for the original analysis and for an age-adjusted analysis (where age was included as a covariate and samples of unknown age were excluded). The top right panel compares analyses stratified by sex. The bottom left panel evaluates stratification by disease subtype. The bottom right panel evaluates stratification by ethnicity. The size of each marker reflects confidence intervals (with height reflecting confidence interval along the Y axis and width reflecting confidence interval along the X axis). Comparisons reaching $p < 0.05$ are labeled and colored in red.

To prioritize our search for likely causal variants, we examined each locus in detail (see LocusZoom[35] plots in Supplementary Figure 3) and investigated whether AMD risk alleles were associated with changes in protein sequence, copy number variation or insertion deletion polymorphisms. One quarter of associated variants altered protein sequence, either directly (N=2) or through linkage disequilibrium (r^2>.6; N=3) with a nearby non-synonymous variant (Supplementary Table 7). Some coding variants point to well-studied genes (*ARMS2*, *C3* and *APOE*) while others help prioritize nearby genes for further study. In chromosome 4q25, index SNP rs4698775 is in strong linkage disequilibrium (r^2=.88) with a potentially protein damaging variant in *CCDC109B*[36], a coiled coil domain containing protein that may be involved in the regulation of gene expression. In chromosome 6q22, index SNP rs3812111 is a perfect proxy for a coding variant in *COL10A1*, a collagen protein that could be

important in maintaining the structure and function of the extra-cellular matrix. Interestingly, Y402H was not in disequilibrium with rs10737680, the most strongly associated SNP in the *CFH* region but, instead, was tagged by a secondary and weaker association signal (Supplementary Tables 6&7). This is consistent with prior haplotype analyses of the locus[10,31,32,34,37].

We used publicly available data[38,39] to check whether any of our index SNPs might be proxies for copy number variants or insertion-deletion polymorphisms (indels), which are hard to directly interrogate with genotyping arrays. This analysis identified a single strong association (r^2=.99), between rs10490924, a coding variant in the *ARMS2* gene which is the peak of association in 10q26, and a 3' UTR indel polymorphism associated with *ARMS2* mRNA instability[40]. Because index SNP rs10490924 is also in strong disequilibrium (r^2=.90) with a nearby SNP, rs11200638, that regulates *HTRA1*41, our data does not directly answer whether *HTRA1* or *ARMS2* is the causal gene in this locus. Although a common deletion of the *CFHR1* and *CFHR3* genes has been proposed[42,43], there was only modest signal in this study which is likely due to linkage disequilibrium with our most significantly associated variants in the locus (r^2=.31 between rs10737680 and 1000 Genomes Project MERGED_DEL_2_6731) as previously suggested[34].

Using RNA-sequencing[44], we examined mRNA levels of 85 genes within 100 kb of our index SNPs in post-mortem human retina (Supplementary Table 8). Of 19 independent risk loci, three had no genes with expressed transcripts in either old or young retina. Two genes showed differential expression between post-mortem retina of young (ages 17–35) and elderly (ages 75 and 77) individuals: *CFH* (p=0.009) and *VEGFA* (p=0.003), both with increased expression in older individuals. Using previously published data[45], we also examined the expression of associated genes in fetal and adult retinal pigment epithelium (RPE). This revealed increased *C3* expression in adult RPE compared to fetal RPE (p=0.0008). *CFH*, *VEGFA* and *C3* are thus up-regulated with aging, and their role in AMD may indicate an accelerated aging process. In addition to *C3* and *CFH*, all the complement genes with detectable expression in the retina or RPE experiments showed higher expression levels in older tissue.

To identify biological relationships among our genetic association signals, we catalogued the genes within 100kb of the variants in each association peak (r^2>0.8 with the index SNP listed in Table 1). Ingenuity Pathway Analysis (Ingenuity Systems, Redwood, CA) highlighted several biological pathways, particularly the complement system and atherosclerotic signaling, to be enriched in the resulting set of 90 genes (Table 3, Supplementary Table 9). To account for features of genome-wide association studies (such as the different number of SNPs in each gene), we carried out two additional analyses. First, we repeated our analysis for 50 sets of 19 control loci drawn from the National Human Genome Research Institute (NHGRI) GWAS catalog[46]. In these 50 control sets, Ingenuity enrichment p-values for the complement system and for atherosclerosis signaling genes were exceeded 16% and 32% of the time respectively (although these two specific pathways were never implicated in a control dataset). Second, we repeated our enrichment analyses using the Interval-based Enrichment Analysis Tool for Genome-Wide Association Studies (INRICH)[47], which is specifically designed for the analysis of GWAS but accesses a more limited set of annotations. The INRICH analyses showed enrichment for genes encoding collagen and extra-cellular region proteins (both with $p=1x10^{-5}$ and after adjustment for multiple testing p_{adjust}=0.0006), complement and coagulation cascades (p=0.0002, p_{adjust}=0.03), lipoprotein metabolism (p=0.0003, p_{adjust}=0.04), and regulation of apoptosis (p=0.0009, p_{adjust}=0.09) (Supplementary Table 10).

TABLE 3 - Pathway analysis

	Enrichment analysis			
Ingenuity canonical pathways	**Nominal *P* value**	**FDR *q* value**	**Molecules**	**Pathway size (N_{genes})**
Complement system	0.000012	0.0015	*CFI, CFH, C3, CFB[a],C2[a], C4A[a], C4B[a]*	35
Atherosclerosis signaling	0.00014	0.009	*PLA2G12A, APOC1[b], APOE[b], APOC2[b], APOC4[b], TNFS14,COL10A1, PLA2G6*	129
VEGF family ligand-receptor interactions	0.0042	0.150	*VEGFA, PLA2G12A, PLA2G6*	84
Dendritic cell maturation	0.0046	0.150	*RELB, ZBTB12, DDR1, COL10A1*	185
Phospholipid degradation	0.0058	0.151	*PLA2G12A, LIPC, PLA2G6*	102
MIF-mediated glucocorticoid regulation	0.0088	0.153	*PLA2G12A, PLA2G6*	42
Inhibition of angiogenesis by TSP1	0.0093	0.153	*VEGFA, TGFBR1*	39
FcεRI signaling	0.0098	0.153	*VAV1, PLA2G12A, PLA2G6*	111
p38 MAPK signaling	0.011	0.153	*PL2G12A, TGFBR1, PLA2G6*	106

Abbreviations: FDR = false discovery rate.
[a]All flank rs429608 and are thus counted as a single hit when determining the significance of enrichment.
[b]All flank rs4420638 and are thus counted as a single hit when determining the significance of enrichment.

To explore the connections between our genetic association signals, we tested for interaction between pairs of risk alleles – looking for situations where joint risk was different than expected based on marginal effects. This analysis resulted in 171 tests of interaction, of which 9 were nominally significant ($p<0.05$, see Supplementary Table 11), consistent with chance expectations. The strongest observed interaction involved risk alleles at rs10737680 (near *CFH*) and rs429608 (near *C2/CFB*), the only association that remained significant after adjusting for multiple testing ($p=0.000052$, $<0.05/171=0.00029$). Individuals carrying risk alleles at both these loci where at slightly higher risk of disease than expected.

The proportion of the variability in the risk of AMD that is due to genes, or heritability, has been estimated at 45–70% [2]. Estimating the proportion of disease risk explained by the susceptibility loci identified[48] depends greatly on the disease prevalence, which is difficult to estimate in our sample, as it includes cases and controls of different ages and collected through a variety of ascertainment schemes. Using a model that assumes an underlying normally distributed but unobserved disease risk score or liability[49], the nineteen loci described here account for between 10% (if AMD prevalence is close to 1%) and 30% (if AMD prevalence is closer to 10%) of the variability in disease risk (corresponding to 15–65% of the total genetic contribution to AMD). The variants representing the peak of association at loci previously reaching genome-wide significance account for the bulk of this variability: the new loci identified here account for 0.5–1.0% of the total heritability of AMD whereas secondary signals at novel and known loci account for 1.5–3.0% of the total heritability.

We report here the most comprehensive genetic association study of macular degeneration yet conducted, involving 18 international research groups, and a large set of cases and controls. Our data reveal 19 susceptibility loci, including 7 loci reaching $p<5x10^{-8}$ for the first time, nearly doubling the number of known AMD loci outside the complement pathway. Our results show some susceptibility alleles exhibit different association across ethnic groups and may be preferentially associated with specific subtypes of disease. As with other GWAS meta-analysis, differences in genotyping methods, quality control steps and imputation strategies between samples might have a minor effect in our

results – future studies may document that more uniform approaches across larger sample sizes might uncover more signals. A conundrum of macular degeneration genetics remains that the loci identified to date contribute to both GA and NV, two different phenotypes of advanced disease. In our sample, subtype specific GWAS analyses considering GA or NV cases only did not identify additional loci. Consistent with observations for other complex diseases[39], the majority of common disease susceptibility alleles do not alter protein sequences and are not associated with insertions or deletions of coding sequence or with copy number variation. We expect that the loci identified here will provide an ideal starting point for studies of rare variation[33,34].

In contrast to most other complex diseases, a risk score combining information across our 19 loci, can distinguish cases and controls relatively well (Figure 3, area under the ROC curve [AUC]=0.52 including only new loci or AUC=0.74 including new and previously reported loci; Supplementary Figure 4). It may be possible to use similar scores to identify and prioritize at risk individuals so they receive preventative treatment prior to the onset of disease[50]. Monotherapies are increasingly utilized to manage neovascular disease, but offer only a limited repertoire of treatment options to patients. Identification of novel genes and pathways enables us to pursue a larger range of disease-specific targets for development of new therapeutic interventions. We expect that future therapies directed at earlier stages of the disease process will allow patients to retain visual function for longer periods, improving the quality of life for individuals with AMD.

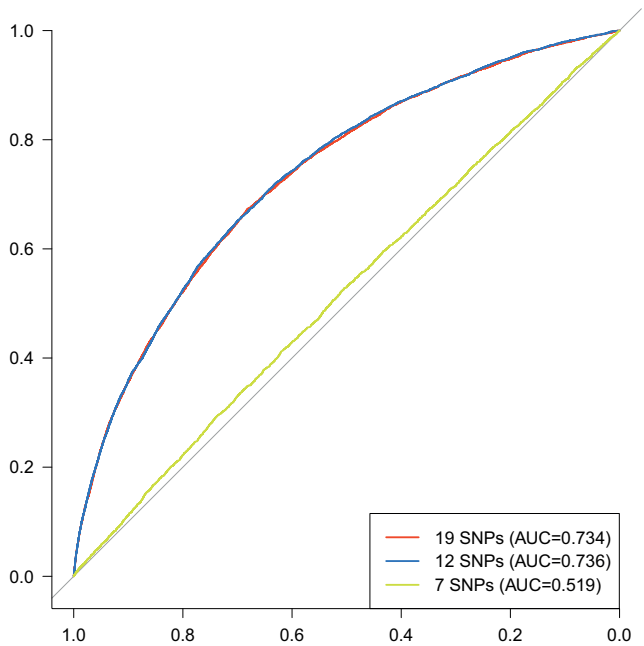

FIGURE 3 - Risk score analysis. We calculated a risk score for each individual, defined as the product of the number of risk alleles at each locus and the associated effect size for each allele (measured on the log-odds scale). The plot summarizes the ability of these overall genetic risk scores to distinguish cases and controls. Analyses were carried out using 19 SNPs that reached $P < 5 \times 10^{-8}$ here, the 12 SNPs previously reaching this threshold and the 7 new variants.

METHODS

Genome-wide scan for late AMD association including follow-up

Study-specific association analysis for discovery: Genotyping was performed on a variety of different platforms summarized in Supplementary Table 2. Each group submitted results from association tests using genotyped and imputed data where the allelic dosages were computed with either MACH[25], IMPUTE[23], BEAGLE[24], or snpStats[52] using the HapMap2 reference panels. The CEU panel was used as a reference for imputation-based analyses for most samples (predominantly of European ancestry), with two exceptions: for the JAREDS samples (predominantly of East Asian ancestry), the CHB+JPT panel was used as a reference; for the VRF samples (predominantly of South Asian ancestry) the combined CEU and CHB+JPT panels were used[22,53]. For most data sets association tests were run under a logistic regression model using either Plink[54], Mach2dat[25], ProbABEL[55], or snpStats[52], though for one dataset containing related individuals the generalized estimating equations algorithm[56] as implemented in R[57,58]. In addition to the primary analysis which tested for SNP associations with advanced AMD unadjusted for age, an age-adjusted sensitivity analysis was conducted by each group with available age. Each group also provided stratified results by sex or AMD subtype (GA or NV) as long as the sample size per stratum exceeded 50 subjects. For all analyses, study-specific control for population stratification was conducted (Supplementary Table 4).

Study-specific association analysis for follow-up: Genotyping of the selected SNPs was performed on different platforms; the same models, sensitivity and stratified analyses were computed by each follow-up partner, while SNPs with insufficient call rate were excluded based on study-specific thresholds. If the index SNP could not be genotyped, a highly correlated proxy was used whenever possible (Supplementary Tables 2&3).

Quality control before meta-analysis: Before meta-analysis, all study-specific files underwent quality control procedures to check for completeness and plausible descriptive statistics on all variables as well as for compliance of allele frequencies with HapMap[59]. In addition, we excluded SNP results of a study into meta-analysis (i) for discovery: if imputation quality measures were too low (MACH & PLINK <0.3; SNPTEST <0.4) or if effect sizes (|beta|) or standard errors were too extreme (≥5) indicating instability of the estimates, (ii) for follow-up: if Hardy-Weinberg equilibrium was violated ($p<0.05/32$).

Meta-analyses: For both discovery and follow-up, we performed meta-analyses using the inverse variance weighted fixed effect model, which pools the effect size and standard error of each participated GWAS. Using an alternative weighted z-score method, which is based on a weighted sum of z-score statistics, we obtained a very similar set of test statistics (correlation of –log10(p-value) >0.98). All analyses were performed using METAL[26] and R. For the discovery, we applied two rounds of genomic control corrections to each individual GWAS and the combined meta results, respectively[51]. All results were analyzed and validated among four independent teams.

Extended analyses for the identified AMD loci: Extended analyses were conducted on the identified loci and particularly on the top SNP of each locus.

Second signal analysis: To detect potential independent signals within the identified AMD loci, each study partner with genotypes for all identified SNPs available re-analyzed their data for all SNPs in the respective loci (index SNP ±1Mb) using a logistic regression model containing all identified index SNPs. Quality control procedures were performed as before. The beta estimates for each SNP were meta-analyzed applying the effective sample size weighted z-score method and two rounds of genomic control correction. The significance threshold ($p<0.05$) for an independent association

signal within any of the identified loci was Bonferroni-adjusted using the average effective number of SNPs involved across the identified loci determined by SNPSpD[60]. To this analysis, 13 studies contributed including 7,489 cases and 51,562 controls.

Interaction analysis: Utilizing a pre-specified R-scripts (see supplementary material), GWAS partners performed 171 logistic regression analyses modeling the pair-wise interaction of the 19 index SNPs assuming an additive model for main and interaction effects. Study-specific covariates were included to the model if required. Per study, quality control included a check for consistency of SNP main effects between discovery and interaction analysis. SNPs with low imputation quality measures and pairs with |beta|>5 or standard errors >5 were excluded before meta-analyzing the interaction effects with the inverse variance weighted fixed effect model in METAL. To this analysis, 12 studies contributed including 6,645 cases and 49,410 controls.

Genetic risk score

The meta-analyzed effect sizes,, for each of the 19 SNPs were calculated in the meta-analysis described above and normalized by:

$$\hat{\beta}_j = \beta_j / \sum_{k=1}^{19} \beta_k$$

where *j*=1,…,19. Using these values as weights, each study partner with data available for all 19 SNPs computed the genetic risk score for an individual as a normalized weighted sum of the AMD risk increasing alleles among the identified SNPs, with

$$S_i = \sum_j \hat{\beta}_j x_{ij}$$

where x_{ij} is the phenotype of the *i*th individual at the *j*th SNP, so ranges from 0 to 2. Data for these calculations were available from 12 studies including 7,195 cases and 49,149 controls.

For each study, we used a leave-one-out cross-validation to access the prediction of the risk score. For the *k*th subject, we fitted a logistic regression model from all subjects in the study excluding the *k*th subjects as:

$$\log(\frac{y_i}{1-y_i}) = \alpha + \gamma S_i,\ i != k$$

where *a* is the intercept and *y* is the effect of the genetic risk score. The fitted probability of the *k*th subject was then estimated.

$$\hat{y}_k = 1/\left(1+e^{-(\hat{\alpha}+\gamma S_k')}\right)$$

We sorted the fitted probabilities and calculated sensitivity and specificity by varying the risk threshold (the value compared with the fitted probability to dichotomize the subject into case or control) from 0 to 1. These were utilized to compute the area-under-the curve (AUC) of the receiver-operating-curve (ROC).

Identification of correlated coding variants and tagged non-SNP variation

LD estimates were calculated using genotype data of the identified risk loci (index SNPs ±500kb) of individuals with European ancestry from the 1000 Genomes Project (March 2012 release)[61] or from HapMap (release #28)[59]. Variants correlated (r^2>0.6) with one of the GWAS index SNPs were identified using PLINK[54]. To filter coding variants, all correlated variants were mapped against RefSeq transcripts using ANNOVAR[62].

Gene expression

We evaluated expression of genes within 100kb of one of the 19 index SNPs, as well as of several retina-specific, RPE-specific and housekeeping genes unrelated to AMD for comparison in retina (RNA-sequencing data from three young [17–35 yrs age] and two old individuals [75 and 77 yrs age]) as well as in fetal and adult retinal pigment epithelium (RPE; published data in the Gene Expression Omnibus database[45]; GSE18811). Expression was analyzed using previously described protocols[44] (Supplementary Table 8).

Pathway analysis

Functional enrichment analysis was performed using the Ingenuity Pathway Analysis software (IPA, Ingenuity® Systems). Any gene located within 100kb of a SNP in high LD (r2>0.8) with one of the index SNPs was considered a potential AMD risk associated gene and considered for subsequent pathway enrichment analysis. LD estimates were calculated as described above. Applying the above inclusion filters, 90 genes appear to be implicated by our 19 replicated AMD SNPs (Supplementary Table 8). Because genes with related function sometimes cluster in the same locus, we trimmed gene lists during analysis so that only one gene per locus was used to evaluate enrichment for each pathway. The P-value of the association between our implicated gene list and any of the canonical pathways and/or functional gene sets as annotated by IPA's Knowledge Base was computed using a one-sided Fisher's exact test. The Benjamini-Hochberg method was used to estimate False Discovery Rates. To evaluate significance of observed enrichment, we repeated our Ingenuity analysis starting with 50 lists of 19 SNPs randomly drawn from the NHGRI GWAS catalog[46] and, again, using the INRICH tool63. When using INRICH, we used gene sets defined in the Broad's Molecular Signatures database[47] (ver3.0) representing manually curated canonical pathway, Gene Ontology biological process, cellular component and molecular function gene sets (C2:CP, C5:BP, C5:CC and C5:MF). We provided INRICH with our full GWAS SNP list and allowed it to carry out 100,000 permutations, matching selected loci in terms of gene count, SNP density and total number of SNPs.

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Chapter 5.3

Genetic susceptibility, dietary antioxidants and long-term incidence of age-related macular degeneration in two populations

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ABSTRACT

Objective To examine effect modification between genetic susceptibility to age-related macular degeneration (AMD) and dietary antioxidant or fish consumption on AMD risk.

Design Pooled data analysis of population-based cohorts

Participants Participants from the Blue Mountains Eye Study (BMES) and Rotterdam Study (RS).

Methods Dietary intakes of antioxidants (lutein/zeaxanthin (LZ), β-carotene and vitamin C), long-chain omega-3 polyunsaturated fatty acids and zinc were estimated from food frequency questionnaires. AMD genetic risk was classified according to number of risk alleles of *CFH* (rs1061170) and/or *ARMS2* (rs10490924) as low (none or 1 risk allele) or high (≥2 risk alleles). Interactions between dietary intake and genetic risk levels were assessed. Associations between dietary intake and AMD risk were assessed comparing the highest versus the two lower intake tertiles by genetic risk subgroups using discrete logistic regression, conducted in each study separately, and next in pooled data. Participants without AMD lesions at any visit were controls. We adjusted for age and sex in analyses of each cohort sample, and additionally adjusted for smoking status and study site in pooled-data analyses.

Main Outcome Measures All 15-year incident late AMD cases were confirmed by chief investigators of the Beaver Dam Eye Study, BMES and RS. Inter-grader reproducibility was assessed in an early AMD subsample, with 86.4% agreement between BMES and RS graders, allowing for a 1-step difference on a 5-step AMD severity scale.

Results In pooled data analyses we found significant interaction between AMD genetic risk status and LZ intake (p=0.0009) but non-significant interactions between genetic risk status and weekly fish consumption (p=0.05) for risk of any AMD. Among participants with high genetic risk, the highest intake tertile of LZ was associated with >20% reduced risk of early AMD, and weekly consumption of fish was associated with a 40% reduced risk of late AMD. No similar association was evident among participants with low genetic risk. No interaction was detected between β-carotene or vitamin C and genetic risk status.

Conclusions Protection against AMD from greater LZ and fish consumption in persons with high genetic risk based on two major AMD genes raises the possibility of personalized preventive interventions.

INTRODUCTION

Genetic predisposition for susceptibility to age-related macular degeneration (AMD) has been confirmed[1] with estimated heritability ranging from 46% to 71% and environmental exposures explaining some proportion of risk variance.[2]

The Age-Related Eye Disease Study (AREDS), a randomized controlled trial (RCT), documented that high dose zinc and antioxidant vitamin supplementation slowed AMD progression in advanced early AMD cases.[3] Another RCT conducted in persons with geographic atrophy demonstrated that a lutein supplement over 12 months improved visual function.[4] Although evidence about the protective association between dietary intake or serum levels of these carotenoids and AMD has been inconsistent, findings from a systematic review and meta-analysis support an association of dietary lutein/zeaxanthin (LZ) intake and reduced risk of late AMD.[5]

Previously, Blue Mountains Eye Study (BMES) and Rotterdam Study (RS) investigators independently documented that high dietary intake of LZ was associated with a reduced long-term risk of AMD.[6;7] In addition, in the BMES cohort weekly consumption of fish was associated with reduced risk of late AMD only in participants with the *CFH* risk (CC) genotype.[8] In the RS cohort high dietary intake of antioxidants reduced the risk of early AMD in persons with high genetic risk for AMD.[9] The apparent protective effect observed in persons with genetic susceptibility to AMD[8;9] suggests an effect modification between known AMD genetic variants and dietary long-chain omega-3 polyunsaturated fatty acids (ω-3 PUFAs) or antioxidants.

Joint contributions and interactions between AMD-related genetic variants and other AMD risk factors (eg. smoking,[10;11] inflammatory markers[12;13]) have been documented previously, including an effect modification of dietary docosahexaenoic fatty acids (DHA, a component of ω-3 PUFAs) on risk of geographic atrophy in persons with the risk genotype of the *ARMS2* gene.[14] The interplay between nature and nurture may provide a better understanding of why some, but not all, persons with AMD-related genetic risk variants develop this condition. Using pooled longitudinal data from two population-based cohorts, we aimed to assess the consistency of the suggested effect modification between AMD genetic susceptibility and dietary intake of antioxidants or fish in relation to the incidence of early, late and any (early and late) AMD.

METHODS

The BMES and RS are population-based cohort studies with follow-up periods of 15 years. Participants were predominantly white.

Blue Mountains Eye Study (BMES)

In 1992-4, 3654 residents (82.4% of those eligible) aged 49+ years, living in two postcode areas west of Sydney, Australia, participated in baseline examinations; 2335, 1952 and 1140 were re-examined after 5 (1997-9), 10 (2002-4) and 15 years (2007-10), respectively. There were 2452 baseline participants followed-up at least once. Each study visit was approved by the University of Sydney and the Sydney West Area Health Service Human Research Ethics Committees, and written, informed consent was obtained.

After pharmacological mydriasis, 30º stereoscopic color transparencies of the macula and optic disc, and non-stereoscopic color transparencies of another four subfields were taken, using a Zeiss FF3 fundus camera (Carl Zeiss, Oberkochen, Germany) for baseline, 5- and 10-year follow-up visits, and a 40° digital camera (Canon CF-60 DSi with a Canon EoS 1DS Mark II camera body, Canon Inc., Tokyo, Japan) for the 15-year follow-up examination.

Rotterdam Study (RS)

At baseline (1990-93), 7983 (77.7% participation rate) eligible persons aged 55+ years were interviewed and examined. Ophthalmological examinations and retinal photography were performed on 6419 participants. Of these, 4977, 3637, 2674 and 1452 were re-examined at the second (1993-95), third (1997-99), fourth (2002-04) and fifth (2009-11) visits, respectively. Overall, 3579 participants had genetic and baseline dietary data together with follow-up information, were free of late AMD at baseline, and were included. In order to correspond with the BMES follow-up visit intervals (each 5 years), participants of the second follow-up visit (1993-95) were excluded, except for incident late AMD cases that were included as incident AMD cases at the third visit (1997-99). Each visit was approved by the Erasmus Medical Center Ethics Committee and complied with the Declaration of Helsinki. All participants gave written informed consent prior to participation.

After pharmacological mydriasis, 35° stereoscopic color transparencies of the macula (Topcon TRV-50VT fundus camera, Topcon Optical Co, Tokyo, Japan) were taken in each of the first three visits, and 35° digital images (Topcon TRC 50EX fundus camera with the Sony DXC- 950P digital camera, Topcon Optical Co, Tokyo, Japan) were taken in the fourth and fifth visits.

Age-related Macular Degeneration Phenotype Definitions and Harmonization

In both studies, retinal photographs of both eyes were graded by trained graders of each study initially,[15;16] following the Wisconsin Age-related Maculopathy Grading System. Phenotype harmonization was performed within the Three Continent AMD Consortium.[17] In brief, all late AMD incident cases detected from each study were initially adjudicated and confirmed by the retinal specialists of the corresponding study team, and then were confirmed by chief investigators of the BMES, RS and Beaver Dam Eye Study (BDES). A five-step severity scale (levels 10-50) was developed (Table 1, available at http://aao.journal.org). A subsample of 60 eyes covering various severity levels of early AMD was selected from the BDES and sent to BMES and RS teams to be graded independently. Exact agreement on the 5-step severity scale was 61.0% between BMES and RS graders; allowing a 1-step difference increased agreement to 86.4%.

Assessment of Dietary Intake

In the BMES, a validated[18] 145-item, semi-quantitative food frequency questionnaire (FFQ), modified from an early FFQ by Willett et al[19], was used. The FFQ was completed and returned by 3267 baseline participants (89.4%), of which 2900 (88.8%) were considered usable.[18]

The electronic version of the Australian Tables of Food Composition 1990[20] was used to calculate the intake of most nutrients. . The intake of ω-3 PUFA was calculated by adding dietary consumption of eicosapentaenoic (20:5, n-3) and docosahexaenoic (22:6, n-3) fatty acids. Information on fish consumption was obtained from the FFQ and regular fish consumption was defined as ≥1 serving per week, compared to <1 serving per week.

In the RS, baseline dietary information was collected in 2 stages. First, participants completed a checklist at home. Second, a face-to-face interview was conducted by a trained dietitian at the research center, using a 170-item validated semi-quantitative FFQ.[21] Using the computerized Dutch Food Composition Table, these dietary data, including intakes of vitamins and zinc, were converted to total energy and nutrient intakes per day.[21] Intake of specific fatty acids was based on a food composition database derived from the TRANSFAIR Study.[22] Beta-carotene and LZ were updated using an additional database of the Netherlands Institute of Public Health and Environmental Protection (personal communication, YCJ Vollebregt and EJM Feskens, unpublished observations, 1993).[23]

Genotyping BMES

In the BMES, genotyping was performed using the Illumina Human 670-Quadv1 custom genotyping array at the Wellcome Trust Centre for Human Genetics, Sanger Institute, Cambridge, as part of the Wellcome Trust Case Control Consortium 2. After quality checking,[24] genotypes of 2534 participants (544,802 single-nucleotide polymorphisms (SNPs)) were used for imputation. Genotypes were imputed from the 1000 Genomes (Version 1) reference using IMPUTE software (https://mathgen.stats.ox.ac.uk/impute/impute.html, accessed June 4, 2013).

In addition, genotype data were obtained previously for rs1061170 in *CFH,* and rs10490924 in *ARMS2* for participants who attended the 5-year follow-up visit.[8] We therefore used the genotyped SNPs of these two whenever available. The concordance rate between typed and imputed genotypes was 99.61% for rs1061170 and 99.26% for rs10490924 based on participants who had both typed and imputed data of these two SNPs.

Genotyping RS

In the RS, genotyping was performed using TaqMan assays (Applied Biosystems, Foster City, California, USA). The two SNPs (rs1061170 in *CFH* and rs10490924 in *ARMS2*) were successfully genotyped in 6345 and 6411 participants, respectively, and 6260 had both SNPs typed.[9] In addition, for participants without genotype data, imputed data of these two SNPs were obtained from a genome-wide association scan dataset, genotyped using the Illumina Infinium II HumanHap5. Imputation was performed using Markov Chain Haplotyping package version 1.0.15 software (http://www.sph.umich.edu/csg/abecasis/MACH/, Ann Arbor, Michigan, USA, accessed June 4, 2013) and HapMap CEU data (NCBI build 36, release 22, The International HapMap Project). There were 6478 participants with both SNPs either typed or imputed.

Statistical Analysis

Of the 2534 BMES participants with dietary and genotype data available, 680 participated in the BMES Extension Study (1999-2000) who had not been followed, leaving 1854 included in this report. Of the 3579 RS baseline participants with follow-up information, dietary and genotype data available, 2778 were followed at the third, fourth and/or fifth visits and thus included. Characteristics between participants who were included and excluded were compared by each study (Table 2, available at http://aao.journal.org). Distributions of baseline AMD risk factors by incident AMD categories are shown in Table 3.

TABLE 3 - Baseline Characteristics of Blue Mountains Eye Study and Rotterdam Study Populations by Incidence of Age-related Macular Degeneration (AMD)

	Incident Age-related macular degeneration (AMD)							
	Blue Mountains Eye Study				Rotterdam Study			
Characteristics	**Controls (level 10)**	**Early (levels 20-40)**	**Late (levels 50)**	**Any (levels 20-50)**	**Controls (level 10)**	**Early (levels 20-40)**	**Late (levels 50)**	**Any (levels 20-50)**
Total numbers (N)	**723**	**467**	**88**	**555**	**2006**	**657**	**115**	**772**
Mean age in years (SD)	61.3 (7.6)	64.9 (7.7)	70.6 (7.1)	65.8 (7.9)	65.01 (6.41)	65.57 (6.46)	69.67 (6.55)	66.18 (6.63)
Gender (% of men)	45.1	39.4	31.8	38.2	42.1	41.3	38.3	40.8
Smoking status (%)								
never	47.9	55.1	51.1	54.5	33	33.2	29.8	32.7
past	36.7	34.4	29.1	33.6	45.4	46.2	43.9	45.8
current	15.4	10.5	19.8	11.9	21.6	20.6	26.3	21.4
Regular fish consumption (%)								
At least weekly	64.1	60.4	44.3	57.8	39.9	40.9	37.4	40.4
Genotype (%)								
CFH (rs1061170)								
TT	42.3	34.9	21.8	32.8	46.4	33.8	22.6	32.1
CT	46.2	46.9	50.6	47.5	43.9	49.7	44.4	48.9
CC	11.5	18.2	27.6	19.7	9.8	16.5	33	19
ARMS2 (rs10490924)								
GG	64.9	57.7	49.4	56.3	65.2	56.7	40	54.2
TG	30.8	37.2	42.5	38.1	31.3	38.4	52.2	40.5
TT	4.3	5.1	8.1	5.6	3.5	4.9	7.8	5.3
Genetic risk level (% with 0, 1 or 2 risk alleles from the *CFH and ARMS2 genes*)								
0	27.5	18.9	9.3	17.3	29.2	20.8	5.2	18.5
1	41.5	42.9	36.1	41.7	45.1	38	37.4	37.9
2	31	38.2	54.7	40.9	25.7	41.2	57.4	43.6
Phenotype (%)*								
Early AMD	–	–	61.4	–	–	–	62.6	–
Large soft drusen	–	–	46.3	–	–	–	47	–
Retinal pigmentary abnormality	–	–	47.6	–	–	–	31.3	–

AMD = age-related macular degeneration; CFH = complement factor H; ARMS2 = age-related maculopathy susceptibility 2; SD = standard deviation.
*Among participants who developed late AMD over the follow-up period, proportions with early AMD or early AMD lesions at study baseline.

We compared dietary intakes between Australian and Netherlandish people using national survey data of the two countries (Table 4, available at http://aao.journal.org). The most recent published food survey data available are from the 1995 Australian National Survey,[25;26] and the Dutch National Food Consumption Survey 2007-2010.[27] Main macronutrient intakes were also compared between BMES and RS baseline populations (Table 5, available at http://aao.journal.org).

We used energy-adjusted dietary intakes of antioxidants (LZ, β carotene, vitamin C), ω-3PUFA, zinc and fish consumption as dietary exposures, and incident early, late or any (early and late combined) AMD as outcome variables. Controls were participants who had no early or late AMD lesions at all visits (level 10 on the severity scale; Table 1, available at http://aao.journal.org). Given the substantial differences in estimates for population means of dietary antioxidant intake between the two studies, we used population-specific tertiles in analyses, and examined tertile distributions of dietary antioxidant intake by incident early, late and any AMD (Table 6). We initially examined the associations of these dietary intakes with risk of AMD across three tertiles, and detected a threshold between the highest and the two lower intake tertiles. We therefore decided to compare the highest vs the two lower tertiles in all models, referenced to controls.

To assess if there were effect modifications between the selected dietary intakes and genetic susceptibility to AMD, using the two variants of major AMD-related genes (*CFH,* rs1061170 and *ARMS 2,* rs10490924) we grouped participants' genetic risk of AMD into three levels: 1) having no risk alleles of either *CFH* (rs1061170) or *ARMS2* (rs10490924); 2) having 1 risk allele from either of the two genes; and 3) having ≥2 risk alleles from either or both of these genes. We tested for statistical interactions between genetic risk status according to the above grouping and the dietary exposures by adding product terms of the gene risk levels with one dietary exposure at a time, together with the genetic risk status and the dietary exposure in each of the discrete logistic regression models. We adjusted for age, sex and smoking in analyses performed within each study, and additionally adjusted for study site in analyses of pooled data of the two studies (Table 7, available at http://aao.journal.org).

We further compared the highest intake tertile versus the two lower tertiles in subgroups stratified by the three AMD genetic risk levels, with 15-year incident early, late or any AMD as the dependent variable and time-to-event as the discrete variable in logistic regression models. We adjusted for age and sex in all models, one model for each dietary exposure, in analyses of data from each study separately (Table 8), and adjusted for age, sex, smoking status and study indicator in pooled data analyses (Table 9).

Risk estimations are presented as odds ratios (OR) and 95% confidence intervals (CIs). SAS (Version 9.2, SAS Institute, Cary, NC) was used for all analyses. OR above (or below) 1 indicated an increase (or decrease) in risk associated with the highest intake tertile relative to the risk associated with the lower intake tertiles.

TABLE 6 - Incident Age-related Macular Degeneration Cases and Controls by Tertiles of Baseline Nutrient Intake in the Blue Mountains Eye Study and Rotterdam Study Cohorts

	Blue Mountains Eye Study				Rotterdam Study			
Dietary Antioxident Intake/day	**Population Mean (SD)**	**First tertile**	**Second tertile**	**Third tertile**	**Population Mean (SD)**	**First tertile**	**Second tertile**	**Third tertile**
Lutein/zeaxanthin (µg), mean (range)	912 (490)	442 (0-642)	810 (642-1005)	1425 (1005-4870)	2365 (1070)	1478 (101-1918)	2252 (1919-2610)	3362 (2610-32645)
% of persons by tertile:								
Controls		30.7	32	37.3		31.2	34.4	34.4
Incident early AMD		28.9	36.8	34.3		30.9	33.5	35.6
Incident late AMD		31.8	39.8	28.4		33.9	38.3	27.8
Incident any AMD		29.40%	37.3	33.3		31.3	34.2	34.5
Beta carotene (µg), mean (range)	7311 (4156)	3290 (0-5159)	6703 (5159-8299)	11512 (8299-43699)	4009 (2227)	2400 (200-3193)	3754 (3193-4363)	5871 (4364-55376)
% of persons by tertile:								
Controls		33.3	34	32.6		32.3	33.3	34.4
Incident early AMD		29.1	34.9	36		32.9	32.7	34.4
Incident late AMD		25	34.1	40.9		32.2	38.3	29.6
Incident any AMD		28.5	34.8	36.8		32.8	33.5	33.7
Vitamin C (mg), mean (range)	182 (88)	98 (0-136)	167 (136-201)	272 (201-999)	120 (53)	70 (0-95)	113 (95-133)	177 (133-659)
% of persons by tertile:								
Controls		30.6	32.9	36.5		33.2	32.9	33.9
Incident early AMD		31.7	34.7	33.6		29.2	36.4	34.4
Incident late AMD		31.8	34.1	34.1		30.4	38.3	31.3
Incident any AMD		31.7	34.6	33.7		29.4	36.7	33.9
Omega-3 PUFA (g), mean (range)	0.22 (0.25)	0.05 (0-0.11)	0.16 (0.11-0.22)	0.45 (0.22-3.30)	0.15 (0.21)	0.024 (0-0.05)	0.1 (0.05-0.15)	0.32 (0.15-0.64)
% of persons by tertile:								
Controls		31.5	32.9	35.6		32.3	32.7	35
Incident early AMD		33	34.5	32.5		33.6	33	33.3
Incident late AMD		29.6	37.5	32.9		32.2	36.5	31.3
Incident any AMD		32.4	35	32.6		33.4	33.6	33
Zinc (mg), mean (range)	12 (2.3)	9.6 (4.4-10.8)	11.6 (10.8-12.5)	14.2 (12.5-31.4)	11 (2)	8.5 (3.7-9.7)	10.6 (9.7-11.4)	12.8 (11.4-24.9)
% of persons by tertile:								
Controls		33.3	33.2	33.5		32.9	32.6	34.6
Incident early AMD		28.9	34.3	36.8		28.9	35.8	35.3
Incident late AMD		21.6	34.1	44.3		41.7	29.6	28.7
Incident any AMD		27.8	34.2	38		30.8	34.8	34.3

Abbreviations: AMD = age-related macular degeneration; LZ = lutein/zeaxanthin; PUFA = polyunsaturated fatty acid; SD = standard deviation

TABLE 8 - Associations of dietary antioxidants with incidence of early and late stage age-related macular degeneration by subgroups of the population with none, one only, or two or more risk alleles of the *CFH* and *ARMS2* genes combined.

	Blue Mountains Eye Study			Rotterdam Study		
	Genetic Risk Group = 0 risk allele from CFH or ARMS2					
Risk Factors	**Early AMD (n=80)**	**Late AMD (n=8)**	**Any AMD (n=88)**	**Early AMD (n=136)**	**Late AMD (n=6)**	**Any AMD (n=142)**
Age (per 10yrs)	2.26 (1.61, 3.17)	11.55 (2.86, 46.72)	2.36 (1.71, 3.27)	1.67 (1.23, 2.26)	9.24 (2.40, 35.65)	1.80 (1.34, 2.41)
Female	1.41 (0.86, 2.32)	-	1.53 (0.94, 2.48)	0.98 (0.68, 1.41)	0.39 (0.07, 2.25)	0.94 (0.66, 1.35)
Current smoking	1.54 (0.67, 3.57)	-	1.53 (0.66, 3.54)	1.05 (0.66, 1.65)	1.41 (0.15, 13.66)	1.07 (0.68, 1.67)
Fish (≥1 serving/week)†	1.12 (0.68, 1.85)	1.79 (0.36, 8.80)	1.10 (0.68, 1.78)	1.20 (0.84, 1.72)	0.35 (0.04, 3.10)	1.16 (0.81, 1.65)
Lutein/zeaxanthin*	0.99 (0.60, 1.65)	0.30 (0.03, 2.64)	0.93 (0.57, 1.53)	1.95 (1.33, 2.86)	1.60 (0.28, 9.24)	1.93 (1.32, 2.82)
Vitamin C*	0.96 (0.57, 1.62)	0.31 (0.04, 2.76)	0.91 (0.55, 1.50)	0.88 (0.60, 1.31)	1.30 (0.21, 7.88)	0.90 (0.61, 1.32)
	Genetic Risk Group = 1 risk allele from CFH or ARMS2					
Risk Factors	**Early AMD (n=181)**	**Late AMD (n=31)**	**Any AMD (n=212)**	**Early AMD (n=248)**	**Late AMD (n=43)**	**Any AMD (n=291)**
Age (per 10yrs)	2.46 (1.95, 3.10)	12.39 (6.13, 25.01)	2.59 (2.10, 3.20)	1.83 (1.48, 2.27)	5.61 (3.39, 9.29)	2.08 (1.70, 2.54)
Female	1.21 (0.85, 1.71)	2.74 (1.12, 6.69)	1.24 (0.89, 1.72)	1.26 (0.96, 1.67)	1.31 (0.68, 2.52)	1.25 (0.97, 1.63)
Current smoking	1.41 (0.84, 2.37)	2.28 (0.67, 7.78)	1.38 (0.85, 2.23)	1.19 (0.85, 1.66)	2.14 (1.02, 4.50)	1.26 (0.93, 1.72)
Fish (≥1 serving/week)†	0.94 (0.66, 1.34)	0.90 (0.39, 2.06)	0.96 (0.68, 1.34)	0.84 (0.63, 1.11)	1.14 (0.60, 2.16)	0..87 (0.67, 1.13)
Lutein/zeaxanthin*	0.85 (0.60, 1.21)	1.34 (0.55, 3.23)	0.90 (0.64, 1.27)	0.94 (0.71, 1.24)	0.90 (0.47, 1.73)	0.94 (0.72, 1.22)
Vitamin C*	0.91 (0.64, 1.31)	1.82 (0.78, 4.24)	0.98 (0.70, 1.38)	0.90 (0.67, 1.20)	0.87 (0.44, 1.72)	0.99 (0.78, 1.29)
	Genetic Risk Group =2+ risk alleles from CFH and ARMS2					
Risk Factors	**Early AMD (n=161)**	**Late AMD (n=47)**	**Any AMD (n=208)**	**Early AMD (n=269)**	**Late AMD (n=66)**	**Any AMD (n=335)**
Age (per 10yrs)	2.15 (1.69, 2.72)	5.94 (3.59, 9.83)	2.45 (1.96, 3.06)	2.00 (1.60, 2.51)	6.37 (4.07, 9.97)	2.48 (2.01, 3.06)
Female	0.99 (0.69, 1.42)	2.22 (1.09, 4.52)	1.06 (0.76, 1.48)	0.87 (0.66, 1.14)	1.18 (0.68, 2.05)	0.89 (0.69, 1.15)
Current smoking	1.02 (0.57, 1.83)	3.43 (2.50, 7.84)	1.20 (0.74, 1.94)	1.03 (0.74, 1.44)	2.03 (1.11, 3.74)	1.16 (0.86, 1.57)
Fish (≥1 serving/week)†	0.71 (0.49, 1.03)	0.18 (0.08, 0.38)	0.62 (0.44, 0.87)	0.99 (0.76, 1.30)	1.05 (0.61, 1.81)	1.01 (0.78, 1.30)
Lutein/zeaxanthin*	0.76 (0.51, 1.13)	0.58 (0.28, 1.20)	0.72 (0.50, 1.04)	0.78 (0.59, 1.05)	0.70 (0.38, 1.29)	0.77 (0.59, 1.01)
Vitamin C*	0.66 (0.44, 0.98)	0.42 (0.20, 0.87)	0.67 (0.47, 0.96)	1.02 (0.77, 1.35)	0.73 (0.41, 1.29)	0.97 (0.75, 1.25)

Abbreviations: AMD =age-related macular degeneration, ARMS2 = age-related maculopathy susceptibility 2, CFH = complement factor H, LZ = lutein/zeaxanthin.
Data are shown as age- and sex-adjusted odds ratios (95% confidence interval)
* Population-specific tertiles with the highest versus others two (middle+lowest) tertiles. -
† Compared to persons with fish consumption <1 serving/week.

TABLE 9 - Associations of Dietary Antioxidants with Incidence of Early and Late Age-related Macular Degeneration by subgroups of population with none, one, or two or more risk alleles of the *CFH* and *ARMS2* genes

	Genetic Risk Group = 0 risk allele from *CFH* or *ARMS2*		
Dietary Factors	**Early AMD (n=215)**	**Late AMD (n=14)**	**Any AMD (n=229)**
Fish (≥1 serving/week)†	1.17 (0.87, 1.57)	0.91 (0.29, 2.84)	1.14 (0.85, 1.51)
Lutein/zeaxanthin*	1.47 (1.09, 1.97)	0.65 (0.17, 2.43)	1.40 (1.05, 1.87)
Vitamin C*	0.91 (0.67, 1.24)	0.78 (0.20, 2.95)	0.91 (0.67, 1.23)
	Genetic Risk Group = 1 risk allele from *CFH* or *ARMS2*		
Dietary Factors	**Early AMD (n=429)**	**Late AMD (n=74)**	**Any AMD (n=503)**
Fish (≥1 serving/week)†	0.87 (0.70, 1.08)	0.98 (0.59, 1.63)	0.90 (0.73, 1.10)
Lutein/zeaxanthin*	0.91 (0.73, 1.13)	1.06 (0.63, 1.79)	0.92 (0.75, 1.13)
Vitamin C*	0.94 (0.75, 1.17)	1.33 (0.79, 2.24)	0.96 (0.78, 1.19)
	Genetic Risk Group =2+ risk alleles from *CFH* and *ARMS2*		
Dietary Factors	**Early AMD (n=430)**	**Late AMD (n=112)**	**Any AMD (n=542)**
Fish (≥1 serving/week)†	0.89 (0.71, 1.10)	0.54 (0.35, 0.85)	0.84 (0.69, 1.03)
Lutein/zeaxanthin*	0.78 (0.62, 0.99)	0.64 (0.40, 1.03)	0.75 (0.60, 0.93)
Vitamin C*	0.87 (0.70, 1.10)	0.67 (0.43, 1.06)	0.86 (0.70, 1.06)

Pooled data of BMES & RS, adjusting for age, sex, smoking and study site indicator
Abbreviations: AMD = age-related macular degeneration, *ARMS2* = age-related maculopathy susceptibility 2, BMES = Blue Mountains Eye Study, *CFH* = complement factor H, LZ = lutein/zeaxanthin, RS = Rotterdam Study
*Population-specific tertiles with the highest versus the other 2 (middle and lowest) tertiles, adjusted for age, sex, smoking, energy intake, and study site.
† Compared to persons with fish consumption <1 serving/week.

RESULTS

Comparisons of characteristics between the 1854 included and the 1800 excluded BMES participants, and between the 2778 included and the 3641 excluded RS participants, showed that those excluded were older, and more likely to have a history of diabetes or cardiovascular conditions, and early AMD. After adjusting for age, the significant differences in early AMD and AMD lesion distributions between the two groups disappeared (Table 2, available at http://aao.journal.org). In the BMES, excluded participants had lower mean intake levels of dietary antioxidants and ω-3 PUFA, compared to those who were included. In the RS, excluded and included participants had similar mean intake levels of dietary oxidants and ω-3 PUFA (Table 2, available at http://aao.journal.org). Of the 1800 BMES excluded participants, 32% died before the 5-year follow-up examination. Of the 3641 RS excluded participants, 22% died before the 6-year follow-up examination.

Of the 1854 BMES participants, 723 had no AMD at all visits, 467 had incident early AMD and 88 had incident late AMD. Of the 2778 RS participants, the corresponding numbers were 2006 without AMD, 657 with incident early AMD and 115 with incident late AMD.

As expected, the mean ages of the incident AMD groups were higher compared to controls. In addition, crude data comparisons confirmed that in both the incident early and late AMD groups, the proportions of participants homozygous for the risk genotypes of *CFH* and *ARMS2* were higher compared to controls, as were the proportions of current smokers in the late AMD groups (Table 3).

Comparison of Australian[25;26] and Dutch[27] national food consumption survey data (Table 4, available at http://aao.journal.org) showed that Australian men and women aged 45-64 years consumed more fruits, seafood and meat/poultry products than Dutch men and women aged 51-69 years. Although vegetable consumption levels were similar between Australian and Dutch men and women of similar age groups, Australians consumed less leafy vegetables than the Dutch, and Australian men consumed more carrots/root vegetables than Dutch men. Consumption levels of energy and other dietary items/food groups, macronutrients and micronutrients were similar, except for iron intake, which was substantially lower among Dutch men and women. We could not find data on LZ consumption levels from the national surveyreports.[25-27]

Energy and main macronutrient intake levels were similar between the BMES and RS populations (Table 5, available at http://aao.journal.org). While the mean intakes of ω-3 PUFA and zinc were similar between the two populations, the mean intakes of LZ (higher in the RS) and β carotene (higher in the BMES) differed substantially (Table 6). A consistent pattern was evident in both study samples that relatively lower proportions of participants in the incident late AMD group were in the highest intake tertile of LZ and ω-3 PUFA (Table 6).

Significant interaction between AMD genetic risk status and LZ intake with respect to risk of early or any AMD was evident in the RS but not the BMES. In pooled data analyses of the two cohorts, we found a significant interaction between AMD genetic risk status and LZ intake with respect to risk of early AMD ($p=0.002$) or any AMD ($p=0.0009$), and a marginally non-significant interaction between AMD genetic risk status and weekly consumption of fish ($p=0.05$) (Table 7, available at http://aao.journal.org). These interaction p values are smaller than the corresponding significance levels required after Bonferroni correction for three interaction tests for the three dietary factors ($p<0.05/3=0.017$). There was no significant interaction found between vitamin C, β-carotene or zinc intake and AMD genetic risk status on AMD risk.

We next stratified the study samples according to their genetic risk levels and investigated the associations between baseline weekly consumption of fish, dietary intakes of LZ or vitamin C and incidence of early, late or any AMD in each study (Table 8), and then in pooled data of the two studies (Table 9). In the subgroup with ≥2 risk alleles of *CFH* and/or *ARMS2*, weekly fish consumption and the highest tertile intake of vitamin C were associated with reduced risk of any AMD in the BMES but not the RS (Table 8). There was a marginally non-significant association between the highest tertile intake of LZ and reduced risk of any AMD in both studies, where the association magnitude was 28% and 23% risk reduction in the BMES and RS, respectively (Table 8).

In pooled data analysis of the two cohorts, among participants with ≥2 risk alleles of the *CFH* and/or *ARMS2*, a significant association between weekly consumption of fish and a 46% reduction in late AMD risk was evident. Similarly, significant associations were evident between the highest tertile intake of LZ and 22%-25% risk reduction in early and any AMD. The highest tertile intakes of LZ and vitamin C were non-significantly associated with an approximately 35% risk reduction in late AMD (Table 9). In the other two subgroups with no or one risk allele, no similar associations were evident (Tables 8 and 9).

DISCUSSION

By using data from two population-based cohorts we showed consistent evidence that participants with ≥2 risk alleles of either or both the *CFH* (rs1061170) and/or *ARMS2* (rs10490924) had a significantly reduced risk of early or any AMD, if they frequently consumed food items rich in LZ.

Findings from the BMES and RS individually were less consistent for the effect modification of weekly consumption of fish or high dietary vitamin C intake with AMD genetic risk level on AMD risk (Table 8, bottom panel). Pooling data from the two cohorts and incorporating three follow-up visits with similar time intervals, we demonstrated a significant association of regular fish consumption with a 46% reduction in late AMD risk, and a marginally non-significant association between high intake of vitamin C and reduced risk of late or any AMD, among participants with high genetic risk of AMD (Table 9, botom panel). These two effect modifications, however, appear to be driven by findings from the BMES. In contrast, the association between the highest tertile of LZ intake and reduced risk of early or any AMD in those with high genetic risk was driven by the findings from the RS, but the direction of the protective association was relatively consistent across the two cohorts, with risk estimates around 0.7-0.8, although not reaching statistical significance (Table 8, bottom panel). This effect modification became significant when data were pooled, with a 22% risk reduction in early AMD in participants with high genetic risk (Table 9, bottom panel).

A recent report from the AREDS2 documented a protective effect of LZ supplement use over 5 years compared to no LZ use on AMD progression in persons in the lowest quintile of dietary LZ intake.[27] The relatively short follow-up duration and lack of stratified analyses conducted in genetic risk subgroups may explain the non-significant findings in primary analyses.[28]

A beneficial effect of ω-3 PUFA and fish consumption on AMD [29-31] has been reported previously, where the anti-inflammatory property of ω-3 PUFA[32] is considered to be one of the underlying mechanisms. In addition, there is increasing recognition that a lipid metabolism pathway may be a key element in the course of AMD development.[33] The outer segments of photoreceptors, subjected to high photo-oxidative stress, have high concentrations of PUFAs and high oxygen tension, and PUFAs are susceptible to oxidation in the presence of oxygen or oxygen-derived radical species.[34] It is possible that lipid oxidation/ peroxidation products activate or amplify local inflammatory processes via the complement system,[33;35;36] and that ω-3 PUFA and antioxidants may counteract these processes. Evidence on possible mechanisms is emerging.[37]

Lutein and zeaxanthin are components of macular xanthophylls and dihydroxy-carotenoids. The light filtering capability is a passive antioxidant function of LZ, and thus potentially prevents bluelight from generating reactive oxygen species.[38] LZ may also have an anti-inflammatory property.[39] Our findings are in keeping with these known functions of ω-3 PUFA and LZ. The effect modification of LZ on participants with high AMD genetic risk suggests the possibility that susceptibility to activation and amplification of the complement pathways can be compensated by these antioxidants. An analogous observation is the significant association between blue light exposure and neovascular AMD in persons with low levels of plasma LZ, and vitamins C and E.[40]

The reliability of dietary data collected in nutritional epidemiology studies may be a concern. We compared dietary consumption survey data between two countries[25-27] (Table 4, available at http://aaojournal.org) and between two study samples (Table 5, available at http://aaojournal.org), that showed similar intake levels of energy, most main food groups and macronutrients between the two countries and the two study populations. The concordance of most dietary intakes between the

two populations suggests that the FFQs used and the resulting dietary data are likely to be robust, regardless of which specific FFQ was used. Nevertheless, we noticed differences in the intake levels of some food items between the two countries. The relatively high intake level of leafy vegetables by the Dutch and the relatively high intake level of carrots/root vegetables by Australian men, together with higher proportions of Australians eating these vegetables, may partly explain the differences in population mean intake levels of LZ and β carotene (Table 4, available at http://aaojournal.org). Different LZ intake estimation methods used by the two studies may also contribute to the difference in mean intake estimates of this nutrient.

Strengths of this study include long-term follow-up of population-based samples, photographic documentation of AMD status with incident late AMD cases cross-validated, and reasonable inter-grader reproducibility on early AMD detection between study graders. A major limitation of this study is a degree of heterogeneity in dietary intake patterns and consumption levels of some micronutrients between the two populations. We have used relative measures for dietary intakes and adjusted for different study sites in the statistical models. Findings for relative measures are directly applicable to populations of specific geographic locations regardless of absolute intake levels. Other limitations include survival bias to which our cohorts are subject, non-availability of serum or plasma nutrient levels and lack of specific data for oily fish consumption. Misclassification or reduced power from these limitations will tend to bias the associations towards the null. There was no evidence supporting associations between mortality and the two genotypes or the dietary antioxidants under investigation, so survival bias should have only minimal effect on the associations. Even with pooled data from two cohorts, we had a limited numbers of late AMD cases and therefore insufficient power to detect a significant association between LZ intake and late AMD incidence in the high genetic risk subgroup (Table 9).

Caution is needed in interpreting these findings. Nutrients do not work alone but interact with genes and the internal environment of the host, which may be influenced by many factors such as lifestyle, intestinal microorganisms and the uptake ability of the host, all of which may lead to differences in bioavailability of specific nutrients on disease pathways.

In conclusion, we showed that dietary intake of LZ is associated with an approximate 20% reduction in risk of developing early AMD among persons with high genetic risk of AMD. The relatively consistent pattern of the effect modifications between LZ intake and AMD-related genetic risk levels in our two cohorts may have clinical implications in the management of AMD patients. Future studies are warranted to confirm this effect modification of major AMD-related genes and dietary intake of antioxidants on the risk of AMD. Our findings also highlight the importance of incorporating information from both genetic and environmental exposures to capture the complexity of disease pathways and pathogeneses of conditions such as AMD.

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Chapter 6.1

Prediction of age-related macular degeneration in the general population: The Three Continent AMD Consortium

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ABSTRACT

Purpose Prediction models for age-related macular degeneration (AMD) based on case-control studies have a tendency to over-estimate risks. The aim of this study is to develop a prediction model for late AMD based on data from population-based studies.

Design Three population-based studies: the Rotterdam Study (RS), the Beaver Dam Eye Study (BDES), and the Blue Mountains Eye Study (BMES) from the Three Continent AMD Consortium (3CC).

Participants Persons (n=10,106) with gradable fundus photographs, genotype data, and follow up data without late AMD at baseline.

Methods AMD features were graded on fundus photographs using the 3CC AMD severity scale. Associations with known genetic and environmental AMD risk factors were tested using Cox proportional hazard analysis. In RS, the prediction of AMD was estimated for multivariate models by area under receiver operating characteristic curves (AUC). The best model was validated in BDES and BMES, and associations of variables were re-estimated in the pooled data set. Betas were used to construct a risk score, and risk of incident late AMD was calculated using Cox proportional hazard analysis. Cumulative incident risks were estimated using Kaplan-Meier product-limit analysis.

Main Outcome Measure Incident late AMD determined per visit during a median follow up period of 11.1 years with a total of 4-5 visits.

Results Overall, 363 participants developed incident late AMD, 3378 early AMD, and 6365 remained free of any AMD. The highest AUC was achieved with a model including age, sex, 26 single nucleotide polymorphisms in AMD risk genes, smoking, BMI, and baseline AMD phenotype. The AUC of this model was 0.88 in RS; 0.85 in BDES and BMES at validation; and 0.87 in the pooled analysis. Individuals with low risk scores had a hazard ratio (HR) 0.02 (95% confidence interval [CI] 0.01-0.04) to develop late AMD; those with high risk scores had HR 22.0 (95%CI 15.2-31.8). Cumulative risk of incident late AMD ranged from virtually 0 to over 65% for those with the highest risk scores.

Conclusion Our prediction model is robust and distinguishes well between those who will develop late AMD and those who will not. Estimated risks were lower in these population-based studies than in previous case-control studies.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly of industrialized countries[1,2]. Approximately 21 million elderly individuals are affected worldwide, and this number is expected to rise dramatically with the aging population[3,4]. AMD can be divided in several stages; early AMD, characterized by subcellular deposits (drusen) and pigmentary changes, and late AMD, subdivided into atrophy of the retinal pigment epithelium (dry AMD) and choroidal neovascularization (wet AMD). Despite improved treatment options, late AMD can cause irreversible blindness, while severe stages of early AMD are mostly asymptomatic they signal a high risk of progression to late AMD[5].

Age, early AMD phenotype, and genetic and environmental factors play important roles in the pathogenesis of late AMD[6-11]. These factors may be used to predict this end stage and to identify high risk individuals. Reasons for assessing predictive values may be risk-dependent (personalized) patient care and surveillance strategies for therapy. Future intervention research such as randomized controlled clinical trials can use prediction models to select individuals with a high risk of outcome events.

Previously reported prediction models were based on selections of cases and non-affected controls[12-28]. Most studies compared only the extreme ends of disease, excluding the majority of the population with an intermediate disease risk. This has inherent methodological concerns, because the disease risk is overestimated by design. Population-based studies include a whole spectrum of risk levels, and therefore findings from these studies would be more generalizable[29] and better suited for clinical implementation.

In this study, we present a prediction model for late AMD based on population-based cohort studies from three continents. We optimized a prediction model in one of the cohorts, and subsequently validated this in the other two cohorts. We included established genetic, environmental, and clinical risk factors in the model, assessed relative as well as cumulative risks, and provided a risk score which can be used to estimate the risk of AMD in individuals.

METHODS

For this paper we followed the guidelines for genetic risk prediction studies (GRIPS)[30].

Study Populations

The Three Continent AMD Consortium (3CC) consists of four population-based studies: the European Rotterdam Study (RS), the American Beaver Dam Eye Study (BDES) and the Los Angeles Latino Eye Study (LALES), and the Australian Blue Mountains Eye Study (BMES). For the purposes of this study, LALES was excluded due to absence of genotype and follow up data.

The RS is a prospective population-based cohort study investigating chronic diseases in the elderly. All inhabitants aged 55 years and older living in a suburb of Rotterdam, the Netherlands, were invited to participate in the study[31,32]. Of the initial cohort of 10,275 eligible individuals, 7,983 (78% of those eligible) participated in the overall study (98% Caucasian). The ophthalmologic part began later, and included 6780 participants (78% of those eligible) participated. Baseline examinations took place from 1990-1993, and four follow-up examinations were performed in 1993-1995, 1997-1999,

2002-2004 and 2009-2011, respectively. The Erasmus Medical Center Ethics Committee approved the study, which complies with the Declaration of Helsinki. All participants gave written informed consent for participation in the study.

The BDES is a prospective cohort study investigating eye diseases in the population of Beaver Dam, Wisconsin (USA)[33]. To identify all residents in the city or township of Beaver Dam who were 43 to 84 years of age, a private census was performed from 1987-1988. Of the 5,924 eligible individuals, 4,926 (83% of those eligible) participated in the baseline examination between 1988-1990 (99% Caucasian). There were follow-up examinations every five years; 1993-1995, 1998-2000, 2003-2005, 2008-2010. BDES was approved by the Institutional review board from the University of Wisconsin-Madison and adhered to the tenants of the Declaration of Helsinki. All participants provided signed, informed consent for participation in the study.

The BMES is a prospective cohort study of eye diseases and other health outcomes in an urban population[34]. All residents aged 49 years or older, living in two postcode areas of the Blue Mountains region in West Sydney, Australia, were invited to participate in the study. In 1992-1994 baseline examinations were performed in 3,654 participants (82.4% of those eligible). Re-examinations were performed after five, ten and fifteen years (in 1997-1999, 2002-2004 and 2007-2009, respectively). All BMES examinations were approved by the Human Research Ethics Committees of the Western Sydney Area Health Service and the University of Sydney, and complied the Declaration of Helsinki. All participants provided written informed consent for participation of the study.

Participants were eligible for the current analysis when genotype data, as well as gradable fundus photographs at baseline, and at least one follow-up eye-examination were available (Figure 1, available at http://aaojournal.org). Persons with late AMD at baseline were excluded. This resulted in 4753 (RS), 3542 (BDES) and 1811 (BMES) participants available for analysis, with a median follow-up of 10.7 (Interquartile range [IQR] 12.8; RS), 15.6 (IQR 10.4; BDES), 11.8 (IQR 5.6; BMES) years. In total, 10,106 participants with a median follow-up of 11.1 (IQR 11) years were included in the analysis. To investigate possible selection bias, we analyzed whether persons excluded from this study differed in baseline level of AMD from those who were included. The two groups did not differ in early AMD levels (10-40) after adjustment for age and sex (P=0.95).

Diagnosis of AMD

All participants underwent fundus photography after pharmacologic mydriasis. Fundus transparencies of all studies were graded according to the Wisconsin Age-Related Maculopathy Grading[35,36] by trained graders under the supervision of senior retinal specialists or senior researchers (RS: PTVMdJ, JRV, CCWK; BDES: RK, BEK; BMES: JJW and PM). The graded fundus photographs were classified using a classification common to all studies: the 3CC AMD severity scale[37] (Table 1, available at http://aaojournal.org). All prevalent and incident late AMD cases from each of these three studies were cross-checked by investigators of the other two studies, with consensus obtained via discussion over multiple teleconferences. The eyes of each participant were graded and classified separately; and the eye with the more severe grade was used to classify the person.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes. All eligible study participants in the RS were genotyped with the Illumina Infinium II HumanHap550 array, or Taqman assays (Applied Biosystems, Foster City, California, USA). HapMap CEU data (release #22) was used for imputation.

DNA from BDES participants was extracted from the buffy coats of blood obtained at baseline examinations or subsequent exams that have been stored frozen at -80ºC. DNA samples arrayed in 96-well plates were submitted for genotyping via an Illumina iSelect Custom Genotyping Panel (Illumina Inc, CA, USA) at the Genomics Core Facility at Case Western Reserve University, or via the KASP Assay at LCG Genomics (Teddington, Middlesex, UK). The data collected was analyzed using Illumina's Genome Studio, or via the KASP SNP Genotyping System. The assays were controlled for quality by examining cluster separation values, call frequency, ABR mean values and ABT mean values. Untyped SNPs were imputed using HapMap CEU (release #22) as reference.

In the BMES, all participants with DNA available were genotyped using Illumina Human670-Quad v1 custom array at the Wellcome Trust Centre for Human Genetics, Sanger Institute, Cambridge as part of the Wellcome Trust Case Control Consortium 2 (WTCCC2). A smaller subset of participants (N = 1,356) was also independently genotyped using the Illumina 610-Quad genotyping array at the Hunter Medical Research Institute, Newcastle, Australia. Following quality control, the genotyped data were imputed from the 1000 Genomes (Version 1) reference using IMPUTE software.

Selection of Single Nucleotide Polymorphisms (SNPs)

For selection of AMD genes, we reviewed publications on AMD genetics [38,39] and prediction of AMD [12-28]. From these, we selected 41 tag SNPs which were available in all three cohorts, and which were not in linkage disequilibrium (r^2 <0.60). Genotypes of SNPs were coded as 0 for carriers of two major alleles; 1 for the heterozygous genotype; and 2 for carriers of two minor alleles. When none of the cases were carriers of two minor alleles, genotypes of the SNPs were coded as 0 for carriers of two major alleles; and 1 for all carriers of at least one minor allele. As a first step, each SNP was tested for association with late AMD in all three cohorts.

For each locus with multiple SNPs, we performed a backwards Cox proportional hazard analysis to determine the best predictive SNPs for incident late AMD within each locus with data from the RS.

Assessment of non-genetic variables

All non-genetic variables used in the analyses were assessed using baseline data. Information on cigarette smoking was obtained in an interview at baseline and categorized as never, former, and current for smoking. Height, weight and blood pressure were measured at the beginning of baseline examination. Body mass index (BMI) was calculated by dividing weight (kg) by the height squared (m^2). The BMI variable was categorized as not overweight or obese (BMI=< 25) and overweight or obese (BMI> 25). Age (yrs) at baseline was categorized in three categories; <65, 65-75, >75 years. Baseline AMD grading was entered into the analysis as categorized variables with levels 10-40.

Statistical analyses

Throughout the entire study, incident late AMD was used as the outcome variable; non-incident late AMD, including those persons remaining at an early AMD stage, were used as the reference group. All analyses were performed using SPSS version 20.0; SPSS INC, Chicago, Illinois.

Variables were analyzed for association with incident late AMD in the three cohorts using Cox proportional hazard analysis, adjusting for age and gender. We constructed five different models based on a minimal and a maximal selection of clinical, genetic, and environmental factors. Model 1 was a minimal model including only age and sex; model 2 was a non-genetic model including age, sex, environmental and ocular factors; model 3 was a minimal genetic model including age, sex, and major genetic AMD risk variants (*CFH* Y402H, *ARMS2* A69S, *C3* R102G, *C2* L9H, *CB* R32Q); model

4 was a maximal genetic model including age, sex, and 26 genetic AMD risk variants (see section SNP selection above), and model 5 was a maximal gene-environmental model including age, sex, environmental, ocular factors, and the 26 genetic AMD risk variants. For each model, we calculated the area under the curve (AUC) of incident late AMD in the RS, and validated the best model in BDES/BMES.

Subsequently, we estimated the association of all variables in the best model using multivariate Cox proportional hazard analysis in the pooled dataset of all three cohorts and calculated the AUC. Calibration of the model was tested using the Hosmer-Lemeshow test. This goodness of fit test shows how well predicted risks match the observed risks. To test whether non-major genetic AMD risk variants could be discarded from this model without jeopardizing the AUC, backwards regression (eliminating SNPs with $P > 0.05$) using Cox proportional hazard analysis was carried out and the AUC of the new model was calculated within the pooled dataset. In this dataset, we estimated the beta of each variable from the best model using multivariate Cox proportional hazard analysis. The estimated beta of a variable was the individual risk score of that variable. Next, we created a summary risk score based on the sum of the betas from the multivariate Cox proportional hazard analysis. Risk scores were rounded off, and frequencies of the risk scores were plotted stratified for incident late AMD and no AMD. We calculated risk of incident late AMD with the middle risk score (3) as the reference using Cox proportional hazard analysis. Risk scores at the extreme ends were pooled to increase sample size due to limited numbers.

We calculated the cumulative risk of incident late AMD per risk score. We assigned the age of onset for incident late AMD as the median between the examination at which late AMD was first observed and the previous examination. For participants who did not develop late AMD, we used age at last examination for censoring. All participants aged 90+ years were censored at age 90 to maintain unbiased estimates. Risks were calculated using Kaplan-Meier product-limit analysis. Participants who died or were lost to follow-up were censored at the time of the last examination. Cumulative risks stratified for the risk score were compared with the overall cumulative risk based on incidence of late AMD (prior risk) using log-rank tests of equality (Mantel-Cox).

Missing data were encountered in the analysis of each model. Only participants with data on all variables in the model entered the analysis.

RESULTS

In total, 363 persons developed incident late AMD during a median follow-up (fup) time of 11.1 years (IQR 11), of which 132 cases in RS (fup 10.7; IQR 12.8), 153 cases in BDES (fup 15.6; IQR 10.4), and 78 cases in BMES (fup 11.8; IQR 5.6). Incidence rates for the three studies were 2.89/1000 person-years (PY), 2.96/1000 PY, and 3.66/1000 PY for RS, BDES and BMES respectively. The distribution of demographic characteristics and environmental risk factors differed slightly among the three cohorts (Table 2). Since the inclusion criteria for age were higher than for the other studies, participants in the RS were older. Early AMD (level 20-40) at baseline was more frequent in RS participants; BMI was higher in BDES participants; and current smokers were less frequent in BMES participants. The frequency of genetic risk alleles was not significantly different among the three studies, although there were slight differences in genotype distributions. Visual inspection of a principle component analysis of all genetic data against HapMap CEU data (NCBI build 36, release 22) as reference showed similar plots for the three cohorts (data not shown).

TABLE 2 - General characteristics of participants included in the analysis

Age inclusion	*RS (N=4753) aged 55+*	*BDES (N=3542) aged 45+*	*BMES (N=1811) aged 49+*	Age inclusion	*RS (N=4753) aged 55+*	BDES (N=3542) *aged 45+*	BMES (N=1811) *aged 49+*
Variables				**Variables**			
Age (yrs); Mean (SD)	67.6 (7.8)	60.2 (10.4)	64.0 (8.3)	*MYRIP rs2679798*			
Age categorized				AA	30.3	34.5	29.9
=< 65	42.5	67.3	56.8	AG	49.7	47.5	48.9
65-75	39.1	24.0	33.1	GG	20.0	18.1	21.1
75+	18.4	8.7	10.1	*SKIV2L* rs429608			
Gender; % Males	42.4	42.5	42.8	GG	74.4	74.7	71.4
AMD baseline grade				GA	23.6	23.1	26.7
Level 10	83.8	80.2	87.4	AA	2.0	2.2	1.9
Level 20	9.1	14.0	8.8	*ABAC1* rs1883025			
Level 30	6.0	5.2	3.4	CC	54.4	56.3	55.5
Level 40	1.1	0.6	0.3	CT	39.3	38.3	37.8
Smoking				TT	6.3	5.4	6.7
Never	33.9	45.1	51.4	*CETP* rs3764261			
Past	43.5	34.9	37.1	CC	46.4	44.5	45.3
Current	22.6	20.0	11.5	CA	43.1	44.8	45.6
BMI (kg/m^2); Mean (SD)	26.3 (3.6)	28.8 (5.4)	26.2 (4.2)	AA	10.5	10.7	9.1
BMI categorized				*TIMP3* rs5749482			
=<25	38.3	24.9	41.7	GG	78.7	78.0	75.8
25+	61.7	75.1	58.3	CG/CC	21.3	22.0	24.2
CFH (Y402H) rs1061170				*VEGFA* rs943080			
TT	41.8	39.7	42.5	CC	24.9	26.6	26.6
TC	45.0	47.0	46.5	TC	49.8	48.9	49.7
CC	13.1	13.4	11.0	TT	25.3	24.4	23.8
CFH rs12144939				*COL8A1* rs13081855			
GG	64.3	64.2	65.1	GG	82.4	82.8	82.3
GT	31.8	31.3	30.8	GT	16.9	16.3	16.9
TT	3.9	4.5	4.1	TT	0.7	0.9	0.8
CFH rs800292				*TNFRSF10A* rs13278062			
GG	52.6	59.8	56.9	TT	28.0	26.3	27.0
GA	40.0	35.0	37.2	GT	49.8	51.7	49.9
AA	5.3	5.3	6.0	GG	22.2	21.9	23.1

TABLE 2 - (continued)

Age inclusion	*RS (N=4753) aged 55+*	*BDES (N=3542) aged 45+*	*BMES (N=1811) aged 49+*	Age inclusion	*RS (N=4753) aged 55+*	BDES (N=3542) *aged 45+*	BMES (N=1811) *aged 49+*
ARMS2 (A69S) rs10490924				*FRK/COL10A1* rs3812111			
GG	62.6	60.7	62.0	TT	42.9	38.6	39.1
GT	33.6	34.8	34.0	AT	44.8	47.3	46.9
TT	3.8	4.5	4.0	AA	12.3	14.1	14.0
C2/CFB (L9H) rs4151667				*SLC16A8* rs8135665			
TT	90.8	90.7	91.1	CC	63.1	65.0	61.8
TA/AA	9.2	9.3	8.9	CT	32.7	31.3	33.7
C2/CFB (R32Q) rs641153				TT	4.2	3.7	4.4
GG	84.4	83.7	81.9	*ADAMTS9* rs6795735			
GA/AA	15.6	16.3	18.1	CC	33.9	33.5	36.6
C3 (R102G) rs2230199				TC	50.1	49.2	46.2
CC	61.3	64.8	67.9	TT	15.9	17.3	17.2
CG	33.9	31.2	28.8	*TGFBR1* rs334353			
GG	4.8	4.0	3.3	TT	58.1	54.7	54.8
C3 rs433594				GT	36.1	39.0	38.8
GG	39.4	39.4	35.1	GG	5.9	6.3	6.5
GA	45.7	46.0	49.3	*RAD51B* rs8017304			
AA	14.9	14.6	15.6	AA	38.2	38.8	41.9
CFI rs10033900				AG	46.6	47.9	44.1
CC	27.4	25.6	27.6	GG	15.2	13.3	14.0
CT	48.6	50.7	50.1	*IER3/DDR1* rs3130783			
TT	24	23.7	22.4	AA	64.5	64.9	62.3
LPL rs256				AG	32.0	26.0	33.4
CC	73.1	72.6	72.3	GG	3.5	9.2	4.3
CT/TT	26.9	27.4	27.7	*B3GALTL* rs9542236			
LIPC rs12912415				TT	31.0	31.9	33.1
AA	71.5	70.8	72.6	CT	48.7	49.3	48.1
AG/GG	28.5	29.2	27.4	CC	20.3	18.7	18.8

Data are percentages unless otherwise indicated

Abbreviations: AMD, age-related macular degeneration; BDES, Beaver Dam Eye Study; BMES, Blue Mountains Eye Study; BMI, body mass index; RS, Rotterdam Study; SD, standard deviation; yrs, years

Risk factors were tested separately for association with incident late AMD in all cohorts, adjusting for age and sex. (Table 3, available at http://aaojournal.org). Most SNPs in the genes *CFH, ARMS2, CFHR5* were significant in all three cohorts ($P<0.05$). SNPs in the genes *LIPC, TIMP3, ADAMTS9, IER3/DDR1, TNFRSF10A, TGFBR1*, and *B3GALTL* were not significant in any single cohort. In all other genes, SNPs were significant in at least one of the cohorts. In all three studies, increasing severity levels of early AMD stages at baseline, based on the 3-CC AMD severity scale (Table 1, available at http://aaojournal.org) was associated with a highly significant risk of incident late AMD. Of the environmental risk factors, current smoking showed a significant association in the RS and BMES, BMI showed no significant association with incident late AMD in all three cohorts. To determine the best set of markers for each locus, all SNPs were analyzed per locus in a multivariate Cox proportional hazard analysis. A total of 26 SNPs were found to be suitable for further analyses.

Prediction models were built using various sets of risk factors, and tested in RS (Table 4). Model 1, a minimal model which included only age and sex, provided an AUC of 0.60 (95% confidence interval [CI] 0.55-0.65). Adding environmental and ocular factors improved the AUC to 0.78 (95% CI 0.74-0.82, model 2). Adding only major AMD genes to model 1 increased the AUC to 0.73 (95% CI 0.69-0.78, model 3). Next, a maximal genetic model was created which included all 26 SNPs. This increased the AUC to 0.82 (95% CI 0.79-0.86, model 4). Finally, we combined all variables from models 1-4 to assess the best possible prediction. This resulted in an AUC of 0.88 (95% CI 0.85-0.90, model 5). Validation of this model in the pooled dataset of BDES and BMES (Table 5) showed an AUC of 0.85 (95% CI 0.82-0.88). To further improve the prediction model, we pooled all three cohorts and re-estimated the risks of the variables included in model 5 (Table 6). The AUC in the three cohorts combined was 0.87 (95%CI 0.85-0.89) and the model had a good calibration ($P= 0.55$). We also investigated the possibility to minimalize this model. Using backwards regression, 13 SNPs could be excluded from the model and provided a somewhat lower AUC of 0.86 (95% CI 0.84-0.88) (Table 7, available at http://aaojournal.org). The model with the best AUC in the three cohorts combined dataset was used for further analyses.

We calculated a risk score (Table 6) based on the betas from the pooled analysis, which ranged from -3.99 to 7.56. We rounded off the estimates and plotted their distribution stratified for incident or no incident late AMD (Figure 2). The plot showed a bimodal distribution with a large frequency difference between the groups for scores lower than 2, and scores greater than 3. The frequencies of risk scores 2 and 3 showed relatively small differences between cases and non-cases. Of note, all persons (n=8) with risk score 8 had risk alleles in the *CFH*, *ARMS2*, and *C3* genes, and no protective alleles in *C2*/*CFB*. By contrast, all persons with risk score -3 (n=29) carried a protective variant in *C2/CFB*, and were free of variants in *CFH* and *ARMS2*, except one person who carried a heterozygous variant in *ARMS2*. Risk of incident late AMD for individuals with risk score 6 to 8 was HR 23.2 (95% CI 15.9-34.0); for those with risk score -3 to 0 HR 0.02 (95% CI 0.01-0.04).

Cumulative risk of incident late AMD was calculated for each risk score, and compared to the overall AMD cumulative risk of incident late AMD. Individuals (n=181) with risk score 6 to 8 had a cumulative risk of 65.6% (SE 0.057) to develop late AMD at age 90 years, while those (n=2751) with risk score -3 to 0 had virtually no risk of developing incident late AMD (0.5%; SE 0.002). The overall risk of AMD for our study population prior to testing was 17.4% (SE 0.013) at age 90 (Figure 3), which was significantly different from all strata apart from risk score 3 ($P= 0.71$).

TABLE 4 - Predictive values for the tested models in the Rotterdam Study

Model	Variables	AUC (95% CI)	SE	No late AMD/ Late AMD (N)
1	**Minimal model**	0.60 (0.55-0.65)	0.023	4621/132
	Age			
	Sex			
2	**Non genetic model**	0.78 (0.74-0.82)	0.022	4561/132
	Age			
	Sex			
	AMD baseline grade			
	Smoking			
	BMI			
3	**Minimal genetic model**	0.73 (0.69-0.78)	0.022	4226/121
	Age			
	Sex			
	ARMS2 rs10490924			
	CFH rs1061170			
	C2/CFB rs641153			
	C3 rs2230199			
	C2/CFB rs4151667			
4	**Maximal genetic model**	0.82 (0.79-0.86)	0.016	4226/121
	Age			
	Sex			
	ARMS2 rs10490924			
	CFH rs800292			
	CFH rs12144939			
	CETP rs3764261			
	C2/CFB rs641153			
	COL8A1 rs13081855			
	C2/CFB rs4151667			
	C3 rs433594			
	TGBR1 rs334353			
	SKIV2L rs429608			
	C3 rs2230199			
	VEGFA rs943080			
	ADAMTS9 rs6795735			
	CFH rs1061170			
	TIMP3 rs5749482			
	IER3/DDR1 rs3130783			
	LPL rs256			
	MYRIP rs2679798			
	SLC16A8 rs8135665			
	RAD51B rs8017304			
	CFI rs10033900			

TABLE 4 - (continued)

Model	Variables	AUC (95% CI)	SE	No late AMD/ Late AMD (N)
	FRK/COL10A1 rs3812111			
	ABCA1 rs1883025			
	B3GALTL rs9542236			
	LIPC rs12912415			
	TNFRSF10A rs13278062			
5	**Maximal gene-environment model**	0.88 (0.85-0.90)	0.015	4171/121
	Age			
	Sex			
	ARMS2 rs10490924			
	CFH rs12144939			
	CFH rs800292			
	C3 rs433594			
	C2/CFB rs641153			
	TGBR1 rs334353			
	SKIV2L rs429608			
	CETP rs3764261			
	C2/CFB rs4151667			
	IER3/DDR1 rs3130783			
	C3 rs2230199			
	ADAMTS9 rs6795735			
	LPL rs256			
	COL8A1 rs13081855			
	SLC16A8 rs8135665			
	FRK/COL10A1 rs3812111			
	CFH rs1061170			
	TIMP3 rs5749482			
	VEGFA rs943080			
	MYRIP rs2679798			
	CFI rs10033900			
	TNFRSF10A rs13278062			
	RAD51B rs8017304			
	B3GALTL rs9542236			
	ABCA1 rs1883025			
	LIPC rs12912415			
	AMD baseline grade			
	Smoking			
	BMI			

Abbreviations: AMD, age-related macular degeneration; AUC, area under the curve; BMI, body mass index; CI, confidence interval; SE, standard error

TABLE 5 - Model 5, the maximal gene-environment model, in the three cohorts

Study	AUC (95% CI)	SE	No late AMD/late AMD (N)
RS	0.88 (0.85-0.90)	0.015	4171/121
BDES + BMES pooled	0.85 (0.82-0.88)	0.015	3634/156
3CC*	0.87 (0.85-0.89)	0.010	7805/277

* based on risk estimates re-analyzed in the complete data set
Abbreviations: 3CC, 3-Continent AMD Consortium; AMD, age-related macular degeneration; AUC, area under the curve; BDES, Beaver Dam Eye Study; BMES, Blue Mountains Eye Study; CI, confidence interval, RS, Rotterdam Study; SE, standard error

TABLE 6 - Risk estimates from Cox proportional hazard analysis based on the Three Continent AMD Consortium

Variable	Code	Beta per code
Age	=<65=0 / 65-75=1 / 75+=2	0 / 1.558 / 2.433
Gender	M=0 / F=1	0 / 0.320
ARMS2 rs10490924	GG=0 / GT=1 / TT=2	0 / 0.779 / 1.720
CFH rs800292	GG=0 / GA=1 / AA=2	0 / -0.899 / -1.614
C2/CFB rs4151667	TT=0 / TA or AA=1	0 / -1.245
CFH rs12144939	GG=0 / GT=1 / TT=2	0 / -0.947 / -1.195
COL8A1 rs13081855	GG=0 / GT=1 / TT=2	0 / 0.223 / 0.890
C3 rs2230199	CC=0 / GC=1 / GG=2	0 / -0.033 / 0.755
SLC16A8 rs8135665	CC=0 / TC=1 / TT=2	0 / 0.313 / 0.648
C3 rs433594	GG=0 / GA=1 / AA=2	0 / -0.110 / -0.591
C2/CFB rs641153	GG=0 / GA or AA=1	0 / -0.592
SKIV2L rs429608	GG=0 / GA=1 / AA=2	0 / 0.027 / 0.590
CETP rs3764261	CC=0 / CA=1 / AA=2	0 / 0.215 / 0.478
ADAMTS9 rs6795735	CC=0 / TC=1 / TT=2	0 / 0.130 / 0.424
RAD51B rs8017304	AA=0 / AG=1 / GG=2	0 / -0.414 / -0.138
TIMP3 rs5749482	GG=0 / GC or CC=1	0 / -0.357
TGBR1 rs334353	TT=0 / TG=1 / GG=2	0 / 0.039 / -0.336
CFH rs1061170	TT=0 / TC=1 / CC=2	0 / 0.175 / 0.278
FRK/COL10A1 rs3812111	TT=0 / TA=1 / AA=2	0 / -0.278 / -0.118
CFI rs10033900	CC=0 / TC=1 / TT=2	0 / -0.070 / -0.223
TNFRSF10A rs13278062	TT=0 / TG=1 / GG=2	0 / 0.093 / 0.196
B3GALTL rs9542236	TT=0 / TC=1 / CC=2	0 / -0.231 / -0.169
IER3/DDR1 rs3130783	AA=0 / AG=1 / GG=2	0 / 0.029 / 0.166
MYRIP rs2679798	AA=0 / AG=1 / GG=2	0 / 0.059 / 0.156
VEGFA rs943080	CC=0 / TC=1 / TT=2	0 / 0 / 0.098
ABCA1 rs1883025	CC=0 / TC=1 / TT=2	0 / -0.046 / 0.076
LIPC rs12912415	AA=0 / AG or GG=1	0 / -0.098
LPL rs256	CC=0 / TC or TT=1	0 / -0.048
AMD baseline grade	Level 10=0 / Level 20=1 / Level 30=2 / Level 40=3	0 / 1.458 / 2.560 / 3.398
Smoking	Never=0 / Past=1 / Current=2	0 / 0.164 / 0.651
BMI	=<25=0 / 25+=1	0 / 0.007

Abbreviations: AMD, age-related macular degeneration; BMI, body mass index; F, female; M, male

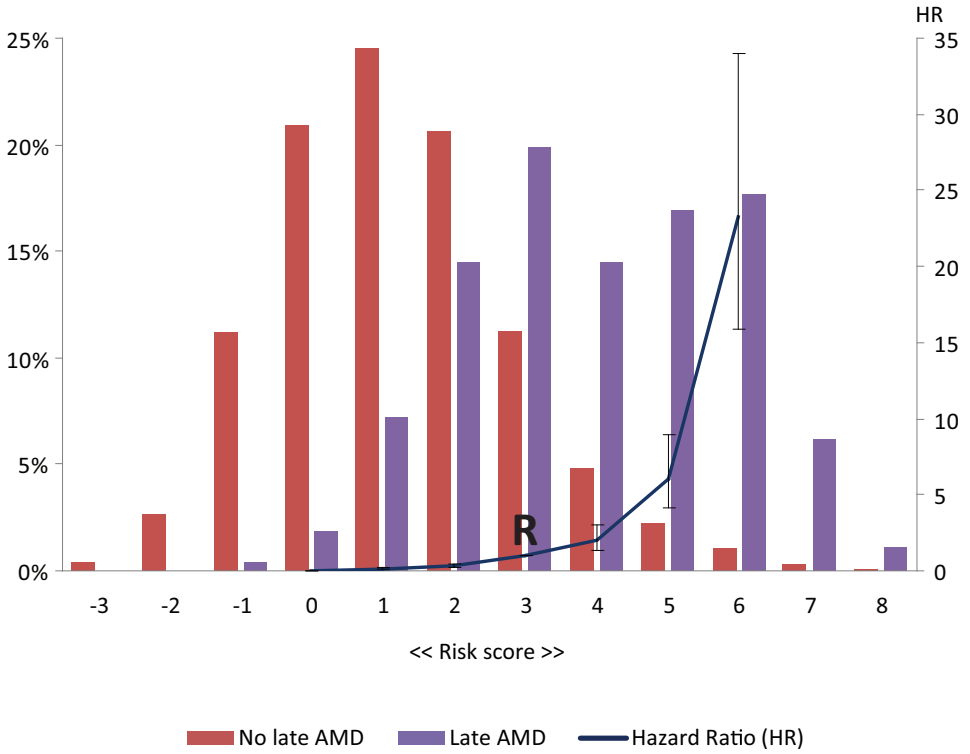

FIGURE 2 - Distribution of risk score in incident late AMD and no late AMD in 3CC, and hazard ratios of incident late AMD

The X-axis represents the risk score category, the left y-axis frequency as percentages, the right y-axis hazard ratio of incident late AMD. The red bars represent no late AMD; the purple bar incident late AMD. The dark blue line represents the hazard ratio (HR) of incident late AMD. Category 3 is the reference category (R) and has HR 1.00. Risk score -3 to 0, and risk scores 6 to 8 were combined for HR. Error bars indicate the 95% confidence interval (CI) of HR.

Abbreviations: 3CC, Three Continent AMD Consortium; AMD, age-related macular degeneration; R, reference

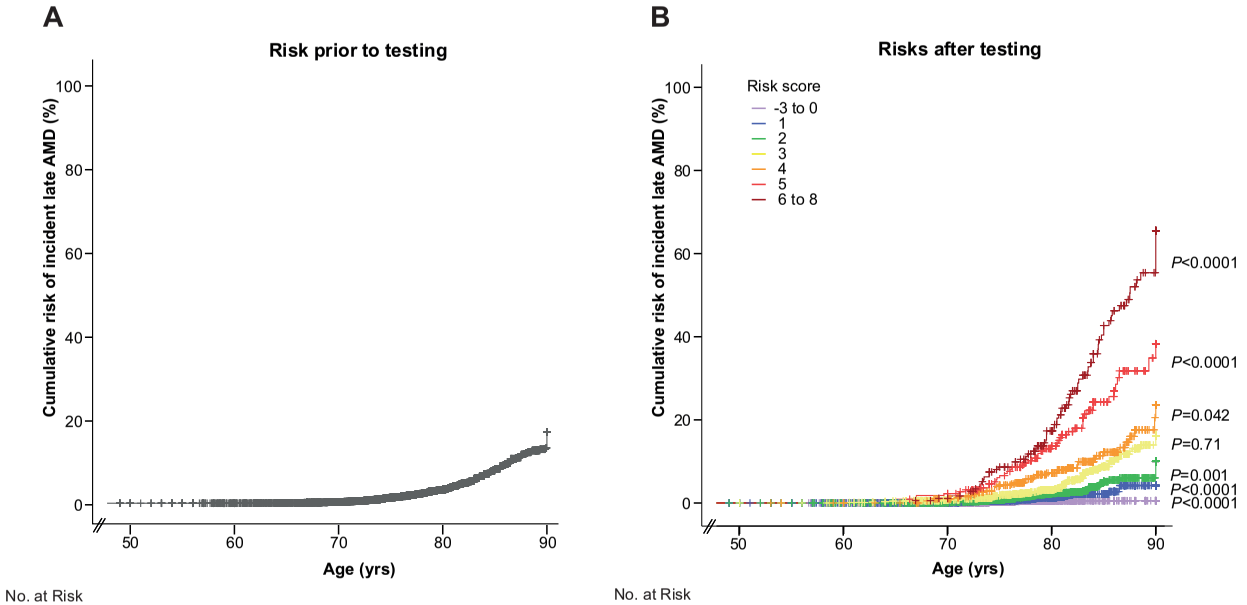

FIGURE 3 - Cumulative risk of incident late AMD prior and after testing of prediction model in 3CC

A – Overall cumulative risk of incident late AMD in 3CC; the x-axis represents the age of onset for incident late AMD cases and age at last examination for noncases. Y-axis represents the cumulative risk of incident late AMD. The number at risk for each decade is presented underneath the figure.

B – Cumulative risk of incident late AMD stratified for risk strata 0-6 in 3CC; X-axis represents the age of onset for incident late AMD cases and age at last examination for noncases. Y-axis represents the cumulative risk of incident late AMD. The risk scores are depicted by various colors. The number at risk for each decade and each risk score is presented underneath the figure.

Abbreviations: 3CC, Three Continent AMD Consortium; AMD, age-related macular degeneration; no., number; yrs, years.

DISCUSSION

In three independent population-based studies from three continents, we investigated all well-known genetic and non-genetic risk factors for AMD. We found that the best prediction for late AMD was based on age, sex, 26 genetic variants, two environmental variables, and early AMD phenotype. The accuracy of a prediction model including all these variables was 0.88 in the RS. As similar risk estimates were found in BDES and BMES, the model proved to be well generalizable to persons from Caucasian descent, living on other continents. Translation of the model to the individual level provided good discrimination between those at a high life-time risk of late AMD and those with virtually no risk , a risk difference of 65%.

A major strength of our study is the inclusion of a general population, unbiased by AMD risk factors. The study samples had included a wide spectrum of AMD lesion phenotypes but not only the extreme ends of disease, which is representative of real scenario in the population. Inclusion of wide spectrum of risk factors distributions and various levels of risk profiles of our population-based samples ensures a realistic, less biased prediction for all risk categories. Additional strengths are the use of longitudinal observational samples to predict incident cases and validation in two independent population-based cohorts with similar study designs. All the strengths facilitate our comprehensive analyses and calculation of cumulative risk of incident late AMD.

Limitations included the relatively low number of incident late AMD cases (n=363), hampering further risk estimation to AMD subtypes. In addition, we did not include several risk factors, such as dietary factors, biomarkers, or rare genetic variants[10,40-43]. Dietary factors and biomarkers are difficult variables to obtain, but their inclusion would have improved the sensitivity of the predictive value. Inclusion of genetic mutations is unlikely to contribute to population risk, due to their low frequencies. Finally, the three cohorts had subtle differences in methodology, which have been discussed by Klein et al.[37]

Most previously published AMD prediction models have been based on case-control studies (Table 8)[12-28]. Most of these models included demographic, genetic and environmental factors, and reached a good prediction for AMD (AUC 0.68-0.94). The study which reported the highest AUC (0.94) included complement activation[22]. However, measurement of activation fragments requires rather intense work-up and specific expertise, and is therefore unlikely to occur in a standard clinical setting. The other studies have some drawbacks as well. Gold et al. reported a sensitivity of 70% and a specificity of 50% for their model [14], making the prediction of low risk outcomes inaccurate. Hageman et al. showed a better specificity and sensitivity, but their model did not incorporate any non-genetic factors including age[16]. Furthermore, their model was based on prevalence data, which is less appropriate for estimation of prognosis[44]. Most other case control studies also lacked follow-up data. The reports with follow-up data were almost inclusively based on data from the AREDS study [20,23,24,27]. Although these investigated persons from the same source population, they differed substantially from each other in design and inclusion of risk factors, leading to great variation in prediction outcomes. Our study shows that the sensitivity of risk prediction depends on the number of variables included in the model, and highest sensitivity is achieved with a full model including the major genes, many of the recently discovered minor genetic variants, smoking, BMI, and existing AMD phenotypes.

TABLE 8 - Prediction models in the literature

Author	Year	Study population/ design	noncases/ cases	Follow-up	Prediction model variables				AUC	Sensitivity	Specificity	Validation in independent dataset?	Remarks
					Demographic	Environmental	Genetic	Ocular					
Gold et al[14]	2006	case-control	114/350				*CFH, C2/CB*			0.74	0.56	Yes	
Hughes et al[17]	2007	case-control	266/401			smoking	*CFH, ARMS2*					No	Riskscore
													Only CNV cases
Jakobsdottir et al[18]	2008	case control and family cohort	168/187; 1095/429		age, sex	smoking	*CFH, ARMS2, C2/FB*			0.70	0.74	No	
Jakobsdottir et al[19]	2009	case-control and family cohort	168/187; 1095/429				*CFH, ARMS2, C2/FB*		0.79			No	
Seddon et al[23]	2009	AREDS - RCT	1167/279	6.3 yrs	age, sex	education, smoking, BMI, antioxidant/zinc or both	*CFH, ARMS2, C2/FB, C3*	baseline grade	0.822	0.83	0.68	No	
Reynolds et al[22]	2009	case-control	60/110		age, sex	smoking, BMI, action fragments	*CFH, ARMS2, C2/FB, C3, CFI*		0.94			No	
Gibson et al[13]	2010	case-control	470/470		age, sex	smoking	*CFH, ARMS2, C3, SERPING1*		0.83	0.76	0.76	No	
McKay et al[21]	2010	case-control	436/437		age	smoking	*CFH, ARMS2, C2/FB, C3*		0.86			No	
Zanke et al[28]	2010	Risks from literature	NA			smoking	*CFH, ARMS2, C3, mtA4917G*					No	
Chen et al[12]	2011	AREDS (723) case-control (1121)	509/1335		age, sex	smoking, BMI	*CFH, ARMS2, C2/FB, C3*		0.82	0.755	0.747	No	
Hageman et al[16]	2011	case-control	467/482				*CFH, ARMS2, C2/FB, C3, CFHR4, CFHR5, F13B*		0.80	0.82	0.63	Yes	Only CNV cases

Klein et al[20]	2011	AREDS - RCT	2034/688	9.3 yrs	age	family history, smoking	*CFH, ARMS2*	baseline grade, drusen presence, advanced AMD in other eye	0.872^			Yes	
Seddon et al[24]	2011	AREDS - RCT	2118/819	9.2 yrs	age, sex	education, smoking, BMI, antioxidant	*CFH, ARMS2, C2/FB, C3*	drusen size, advanced AMD in other eye	0.908* 0.876^			No	
Spencer et al[25]	2011	case-control	216/349		age	smoking	*CFH, ARMS2, C2/FB, C3*		0.84	0.770	0.741	Yes	Outcome is early and late AMD
Spencer et al[25]	2011	nested case-control	148/85		age	smoking	*CFH, ARMS2, C2/FB, C3*			0.633	0.707	Yes	Outcome is early and late AMD
Ying et al[26]	2011	CAPT - trial	618/324	5 yrs	age	smoking, hypertension	…	Baseline grade, night vision score	0.68			No	Only GA cases
Grassmann et al[15]	2012	case-control	786/986				*CFH, ARMS2, C2/FB, LIPC, APOE, PLA2G12A, TIMP3*		0.813			No	
Yu et al[27]	2012	AREDS - RCT	1112/1448	10.3 yrs	age, sex	education, smoking, BMI, antioxidant	*CFH, ARMS2, C2/FB, C3,* *CFI, LIPC, TIMP3, CETP* *ABCA1, COL8A1, APOE*	Fellow eye status	0.895* 0.883^			No	Outcome is early and late AMD

* based on 10 year follow-up. ^ based on 5 year follow-up.

Abbreviations: AMD, age-related macular degeneration; AREDS, age-related eye disease study; AUC, area under the curve; BMI, body mass index; CAPT, complications of age-related macular degeneration prevention trial; CNV, choroidal neovascularization; GA, geographic atrophy; RCT, randomized controlled trial

What may be the benefits of prediction tests? Most current counseling provided to family members of late AMD cases is based on clinical parameters. A prediction test may improve the identification of true high-risk individuals. As the estimation of cumulative risk of incident AMD makes the risk very apparent, it may encourage an individuals to alter their lifestyle with the aim to decrease the risk of AMD. For instance, one can stop smoking, eat foods rich in antioxidants, and increase physical exercise to lower risk of progression to late AMD[9,45]. There may be benefits for patients with late AMD. Various studies have shown that persons with neovascular AMD who do not respond to anti-VEGF therapy are at higher genetic risk[46-49]. These patients may need more intensive treatment regimens. Lastly, current intervention trials select study participants mainly on the basis of phenotypes. Inclusion of high-risk individuals, identified by a prediction test, may improve homogeneity of the study population and prediction of AMD outcome events.

In summary, a risk score based on a large number of genetic risk variants for AMD, the environmental factors smoking and BMI, and early AMD phenotype provided a good prediction of incident late AMD cases in this study. A model incorporating non-genetic factors performed better than a model based on a minimal number of genetic factors, but after inclusion of many genes the model performed better than a model including only non-genetic factors. Inclusion of all risk factors provided the best prediction. As personalized medicine is the future, prediction tests will become more and more implemented as clinical tools. In such case, only comprehensive tests will be useful for AMD.

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Chapter 6.2

Direct-to-consumer personal genome testing for age-related macular degeneration

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ABSTRACT

Purpose Genetic testing may be the next step in clinical medicine for a more personalized approach in determining risk of disease. Direct-to-consumer (DTC) personal genome tests may fulfill this role. We explored the practicability and predictive value of DTC-tests from four companies (23andMe, deCODEme, Easy DNA, Genetic testing laboratories) for age-related macular degeneration (AMD).

Methods Body specimens of three individuals were collected and sent to four companies for DNA genotyping and disease risk estimation. In addition, DNA was also genotyped using Illumina HumanOmniExpress 12v1 array in the Rotterdam Study laboratory, and risk estimates of AMD were calculated using the validated prediction model from the population-based Three Continent AMD Consortium.

Results Genotyped results of the four DTC-tests matched genotyping performed by the Rotterdam Study laboratory. The estimated risks provided by the companies varied considerably in the tested individuals, from a 1.6-fold difference for overall relative risk to an up to 12-fold difference for lifetime risk. The lifetime risks for the individuals ranged from 1.4-16.1% in the DTC-tests, while they varied from 0.5-4.2% in the validated prediction model. Most important reasons for the differences in risks were the testing of only a limited set of genetic markers, the choice of the reference population, and the methodology applied for risk calculation.

Conclusion Direct-to-consumer personal genome tests are not suitable for clinical application as yet. More comprehensive genetic testing and inclusion of environmental risk factors may improve risk prediction of AMD.

INTRODUCTION

Genetic studies of age-related macular degeneration (AMD) have elucidated a major proportion of its genetic background. Currently, genome-wide studies (GWAS) have identified associations with >30 genetic loci for this disease, explaining a large part of the heritability of AMD[1,2]. Subsequently, these genomic findings have been incorporated into prediction models, many of which provide a >80% discriminative accuracy for late AMD[3-22]. This high predictive ability makes AMD particularly suitable for genetic testing, which may be the next step to a more personalized approach in clinical medicine.

Direct-to-consumer (DTC) personal genome tests had been made available for consumers and thousands have purchased these tests via the internet to determine a personal disease risk. Recently, methods of three DTC-tests have been examined and compared for several diseases[23]. AMD was the disease for which each test obtained the best predictive ability. Several companies offered genetic tests for AMD and implementation of these tests in the clinic could help identify individuals at risk of developing the disease to apply risk dependent patient care and surveillance strategies. Therefore, the accuracy of the risk estimates will be a great concern, and will determine whether such tests will be meaningful in the clinic.

In this study, we evaluated the results of AMD prediction tests provided by four major companies. We sent bio-samples from three individuals to these companies to test proof of principle, and reviewed the sampling process, the type of analysis, the genotyping, and the risk information. In addition, we compared results to a validated prediction model based on population studies.

METHODS

Experimental design

Evaluation of test methodology

Study participants

Three investigators (GB, JV, CK) agreed to voluntarily participate in the study, and signed informed consent.

DTC-tests for AMD

We searched for internet-based DTC-tests for AMD using a web search engine and the word groups "genetic testing for age-related macular degeneration", "genetic prediction of age-related macular degeneration", and "genetic tests for age-related macular degeneration". Only companies available for European citizens and testing more than one single nucleotide polymorphism (SNP) were eligible, and of these, four companies were selected; i.e., 23andMe, deCODEme, Easy-DNA, The Genetic Testing Laboratories, Inc.

23andMe

https://www.23andme.com/

This privately-held American company was founded in 2006 with the intention to empower individuals in accessing their own genetic information and to stimulate a way into more personalized medicine. One can order a single 'spit' kit for $99 (shipping costs $14.95 - $118.95) from the website on internet, and a sample collection kit will be sent by mail with instructions how to provide a saliva sample and

details for returning the sample. An assisted collection kit for persons having trouble to spit can be ordered together with the DTC-kit for an additional $25, requiring only half the amount of saliva. The returned saliva sample will arrive at the contracted LabCorp's Clinical Laboratory Improvement Amendments (CLIA) certified laboratory, where DNA will be isolated from cells in the saliva and processed on an Illumina® HumanOmniExpress array customized by 23andMe (>1 million SNPs, call rate above 98%). These SNPs provide information about traits, carrier status, and risks for over hundred diseases, including AMD. The risk for developing AMD is estimated based on the risk in the reference population and an overall relative risk (RR) representing risks of five SNPs: *CFH* rs1061147; *C2* rs547154; *LOC387715/ARMS2* rs3750847; *C3* rs2230199; *TIMP3* rs9621532 [11,24-36]. AMD risk in the reference population differed for males and females and was 6.5 and 7% respectively. Methods of risk calculation have been described in a white paper[37], accessible after login to the 23andMe website. No health reports including risk prediction and carrier status are currently provided for new customers.

DeCODE
http://www.decodeme.com/

DeCODE was founded in 1996 and the headquarters are located in Reykjavik, Iceland. This company developed the deCODEme test, which provide results for 47 conditions and traits. Unfortunately, new tests are no longer offered by the company. Costs were $1100 per test, with no extra costs for shipping. After purchasing the test from the internet, a buccal swab kit will was sent in the mail with instructions how to collect and return the sample. The samples were processed at a CLIA certified lab, the deCODE laboratory in Reykjavik, for DNA isolation. Genotyping was performed on an Illumina Human 1M Beadchip (Illumina, Inc., San Diego, California, USA) which determines >1 million SNPs. Validation occurred by bi-directional Sanger sequencing and independent SNP genotyping platforms.

A overall RR for developing AMD was calculated based on six risk variants: *ARMS2/HTRA1* rs3750847, *C2/FB* rs9332739 and rs547154, *C3* rs230199 and, *CFH* rs1061147 and rs1329428 [27,38]. Subsequently, for the tested individual a lifetime risk was calculated based on the overall relative risk and the AMD risk in the reference population, which was set at 8%. A white paper[39] describing the risk calculation is available after login to the deCODEme website.

Easy-DNA
http://www.easy-dna.com / http://www.easydna.co.uk / http://www.easydna.eu

Easy-DNA is an international company which provides a genetic DNA predisposition test on 25 conditions and diseases. This test can be purchased from the internet for €299/$299/£299 including shipping costs. A kit will be sent by mail for collection of a blood sample, and includes submission forms, instructions for collecting the blood sample from a punctured finger, the sample collection kit and a self-addressed envelope. This company does not provide information on the genotyping method, but states that results are provided for *CFH* rs1061170 and *C2* rs800292 [40,41]. Risk estimates are presented as lifetime and overall RR of AMD. Risk of AMD in the reference population was set at 8%. Methods for risk calculation was not provided by the company.

The Genetic Testing Laboratories, Inc (GTL)
http://www.gtldna.com/predisposition.html

This company provides a DNA predisposition test which will reveal the genetic and environmental predisposition for 25 diseases and conditions including AMD. The DNA predisposition test costs $285 with additional costs of $45 for shipping outside the Contiguous United States. After purchasing the kit from the internet, it will be sent to your own physician or a professional collector agency appointed by GTL to collect the sample, which can be a bucal or a blood sample. The sample will be processed by a CLIA accredited laboratory. As for Easy-DNA, this company also is unclear on genotyping method, but states that results are provided for *CFH* rs1061170 and *C2* rs800292 [40,41]. Lifetime and overall RR are provided for each tested person. Risk of AMD in the reference population was set at 8%.The risk calculation method of this company was not available for consumers or professionals.

We followed each company's instructions for the collection of bio-samples used for DNA extraction. We sent the samples to the various laboratories associated with the companies, and awaited the results.

Genotyping in Rotterdam

Genotyping for the three individuals was also performed at the Rotterdam Study Laboratory: Genetic Laboratory of Internal Medicine at the Erasmus Medical Center in Rotterdam, the Netherlands. Genomic DNA was extracted from peripheral leukocytes and all participants were genotyped using the Illumina HumanOmniExpress 12v1_J microarray (Illumina, Inc., San Diego, California, USA). Call rate for the genotyping was >97.5%.We imputed genotype data to Hapmap 3 release 2 and 1000 genomes phase I V3.

Assessment of covariates

The covariates age, length, weight, smoking status, and family history regarding AMD were obtained by interview. Body mass index (BMI) was calculated by dividing weight (kg) by the height squared (m^2). AMD phenotype was evaluated by standard ophthalmologic examination including fundus photography (Topcon TRC-50EX fundus camera, Topcon Optical Co, Tokyo, Japan and Sony DXC-950P digital camera, Sony Corporation, Tokyo, Japan) after pharmacological mydriasis. Images were graded according to the Wisconsin Age-Related Maculopathy Grading[42] and the modified international classification system[43] by graders from the Rotterdam Study.

Risk score Three Continent AMD Consortium prediction model and DTC-tests

The Three Continent AMD Consortium (3CC) developed a validated prediction model including a total risk score based on 31 variables; 26 genetic variants associated with AMD, age, sex, smoking, BMI, and AMD phenotype. The prediction model had 87% discriminative accuracy for incident late AMD [22]. For each individual in this study this summary risk score was calculated. Based on the risk score, lifetime risks could be assessed for each individual.

Ancestry assessment

Ancestry of the three individuals was determined using multi-dimensional scaling (MDS) protocol from ENIGMA [44] using Hapmap 3 release 2 as the reference.

Statistical analysis

Test results included predicted risks for several diseases from four companies. For the purpose of this study, we only evaluated the predicted risks for AMD. 23andMe provided odds ratios (OR) and the other companies relative risks (RR) per SNP per genotype, but all were adjusted for the average risk of the SNP in the population, and will be referred to as OR and RR, respectively. Genotype frequency, risks per genotype, overall RR, lifetime population risk and lifetime risk of the tested individual were obtained from the test results.

Minor allele frequencies were not provided by the companies, but calculated using the formula:

$$p+q = 1$$

With p representing the major allele and q the minor allele. For the different genotypes, frequencies could be calculated after applying this information; homozygous for major alleles = p^2, heterozygous = $2pq$ and homozygous for minor alleles = q^2.

All analyses were performed using SPSS version 20.0 (SPSS INC, Chicago, Illinois) except for the MDS-analysis which was performed using R software (R-project, Institute for Statistics and Mathematics, R core team (2013), Vienna, Austria, version 3.0.2).

RESULTS

Demographic characteristics of the three study subjects are provided in Table 1. All three were younger than the average age of AMD onset, and none had any features of AMD, as determined by grading of fundus photographs. One had a history of smoking, and one had a positive family history for late AMD. All three were Caucasian and had northern/western European ancestry (Supplementary Figure 1).

TABLE 1 - Discriptives of the participants

Variable	Individual 1	Individual 2	Individual 3
Age (yrs)	45	29	51
Sexe	Female	Female	Male
Ethnicity	Caucasian	Caucasian	Caucasian
Ancestry	Northern/western European	Northern/western European	Northern/western European
BMI (kg/m^2)	22.7	20.2	24.3
Smoking	never	never	past
AMD phenotype	none	none	none
Family history of AMD	grandmother	none	none

Abbreviations: AMD = age-related macular degeneration, BMI = body mass index, yrs = years

DTC-tests

Details of the DTC-tests are given in Table 2. Tests differed considerably in price, the most costly being 11x more expensive than the cheapest test. Sampling methods varied from saliva, buccal swap to blood from a finger prick. One participant particularly had difficulty to deliver the saliva specimen of 2.5 ml for 23andMe, which required ~1 hour of sampling time. Genetic Testing Laboratories (GTL) required for all participants and Easy-DNA only for US-residents a physician or another health professional assigned by the company to collect the blood sample and only the collectors obtained the test results. However, the forms for requesting the test from GTL were open access. Delivery time for test results ranged from 2-4 weeks for most tests; results from one Easy-DNA test were delayed up to 8 weeks without notice or explanation.

TABLE 2 - Overview genetic testing companies

Company name	Website	Costs per kit	DNA source	Easy to collect?	Additional notes
23andMe	https://www.23andme.com	$99 / € 74	saliva	Difficult in one participant	Street address is needed to deliver DTC-test
deCODEme*	https://www.decodeme.com	$1100 / € 821	bucal	yes	-
Easy-DNA	http://www.easygenetictest.com	$299 / € 299	blood	yes	For US residents: Sample needs to be collected by physician or professional collector
The Genetic Testing Laboratories	http://www.gtldna.com/	$285 / € 213	blood	yes	Sample needs to be collected by physician or professional collector

* deCODEme do not offer any new testing possibilities

In contrast to the statement of Easy-DNA and GTL, the SNP rs800292 is located in the *CFH* gene, not in *C2* (Table 3). Thus, these two companies only tested risk variants in *CFH*. DeCODEme and 23andMe covered 4 and 5 AMD loci, respectively. The tested SNPs varied among tests, however, there was considerable overlap. Individual genotypes at these SNP locations are shown in Table 3. Risk-increasing as well as risk-decreasing variants were present in all three individuals. The effect estimates of these variants showed the largest range in individual 2, in particular for the risks predicted by 23andMe and deCODEme. The lifetime AMD population risk used by the companies varied from 6.5-8%, and varied for gender in the 23andMe calculations. For 23andMe and deCODEme the ancestry of the reference populations was European, for GTL and Easy-DNA this was European Tuscan. Only for individual 1 the Easy-DNA test listed European ancestry as the reference population. Genotypes identified by the DTC-tests were identical to those determined at the Rotterdam Study laboratory in all three individuals.

The inter-test variability of the overall relative and life-time risks was large in all three individuals , but most profoundly in individual 3 (Table 3). For this person, these risks were lower and higher than the population risk, depending on the test. Lifetime risks between lowest and highest estimate differed by factor 1.7, 1.6, and 11.5 for individuals 1, 2, and 3, respectively.

TABLE 3 - Risks of the tested variants, overall risk and lifetime risk per company for each individual

		Individual 1								Individual 2								Individual 3							
		23andMe		deCODEme		Easy-DNA*		GTL		23andMe		deCODEme		Easy-DNA		GTL		23andMe		deCODEme		Easy-DNA		GTL	
AMD Gene	SNP number	genotype	OR	genotype	RR	genotype	RR	genotype	RR	genotype	OR	genotype	RR	genotype	RR	genotype	RR	genotype	OR	genotype	RR	genotype	RR	genotype	RR
CFH	rs1061147	AC	0,97	AC	1.56||					CC	0,34	CC	0.21||				AC	0,97	AC	1.56||				
CFH	rs1329428			GG								AA								GG					
CFH	rs1061170					CT	1,26	CT	1,60					TT	0,64	TT	0,64					CT	1,60	CT	1,60
CFH†	rs800292					CC	0,67	CC	0,63					CT	1,26	CT	1,26					CT	1,26	CT	1,26
C2	rs547154	GG	1,07	CC	1,10					GG	1,07	CC	1,10					GT	0,57	AC	0,58				
C2	rs9332739			GG	1,06							GG	1,06							GG	1,06				
LOC387715/ ARMS2	rs3750847	CC	0,47	GG	0,46					CT	1,63	AG	1,59					CC	0,47	GG	0,46				
C3	rs2230199	CG	1,37	CG	1,29					CG	1,37	CG	1,29					GG	0,79	CC	0,76				
TIMP3	rs9621532	AA	1,02							AA	1,02							AA	1,02						
		23andMe		deCODEme		Easy-DNA*		GTL		23andMe		deCODEme		Easy-DNA		GTL		23andMe		deCODEme		Easy-DNA		GTL	
Overall RR‡		0,70		1,01		0,85		1,00		0,70		0,50		0,81		0,81		0,22		0,34		2,01		2,01	
Lifetime population risk (%)		7,0		8,0		8,0		8,0		7,0		8,0		8,0		8,0		6,5		8,0		8,0		8,0	
Lifetime risk§ (%)		4,9		8,6		6,8		8,1		5,9		4,0		6,5		6,5		1,4		2,7		16,1		16,1	

abbreviations: AMD = age-related macular degeneration, GTL = The Genetic Testing Laboratories, OR = Odds Ratio, RR = Relative Risk

* Reference population for individual 1 was set to European and differed from individual 2 and 3 for the Easy-DNA test which was set to European (Tuscans)

† Easy-DNA and GTL referred to this SNP as though it was located within the C2 Gene

‡ The overall RR provided by each company is based on all the tested genetic variants.

§ The lifetime risk is calculated multiplying the overall RR with the population risk

|| RR based on haplotype rs1061147 and rs1329428 in the CFH gene

TABLE 4 - Risk estimates from the Three Continent AMD Consortium prediction model

Variable	Code	Risk per code	Individual 1	Individual 2	Individual 3
ARMS2 rs10490924	GG=0 / GT=1 / TT=2	0 / 0.779 / 1.720	0	0.779	0
ADAMTS9 rs6795735	CC=0 / TC=1 / TT=2	0 / 0.130 / 0.424	0	0.424	0.424
SLC16A8 rs8135665	CC=0 / TC=1 / TT=2	0 / 0.313 / 0.648	0.313	0	0.313
Sexe	M=0 / F=1	0 / 0.320	0.320	0.320	0
CETP rs3764261	CC=0 / CA=1 / AA=2	0 / 0.215 / 0.478	0.215	0	0
CFH rs1061170	TT=0 / TC=1 / CC=2	0 / 0.175 / 0.278	0.175	0	0.175
Smoking	Never=0 / Past=1 / Current=2	0 / 0.164 / 0.651	0	0	0.164
MYRIP rs2679798	AA=0 / AG=1 / GG=2	0 / 0.059 / 0.156	0.059	0.156	0
VEGFA rs943080	CC=0 / TC=1 / TT=2	0 / 0 / 0.098	0	0	0.098
TNFRSF10A rs13278062	TT=0 / TG=1 / GG=2	0 / 0.093 / 0.196	0.093	0	0
TGBR1 rs334353	TT=0 / TG=1 / GG=2	0 / 0.039 / -0.336	0.039	0.039	0
IER3/DDR1 rs3130783	AA=0 / AG=1 / GG=2	0 / 0.029 / 0.166	0	0.029	0.029
SKIV2L rs429608	GG=0 / GA=1 / AA=2	0 / 0.027 / 0.590	0	0	0.027
Age (yrs)	=<65=0 / 65-75=1 / 75+=2	0 / 1.558 / 2.433	0	0	0
AMD baseline grade	Level 10=0 / Level 20=1 / Level 30=2 / Level 40=3	0 / 1.458 / 2.560 / 3.398	0	0	0
BMI (kg/m^2)	=<25=0 / 25+=1	0 / 0.007	0	0	0
C2/CFB rs4151667	TT=0 / TA or AA=1	0 / -1.245	0	0	0
B3GALTL rs9542236	TT=0 / TC=1 / CC=2	0 / -0.231 / -0.169	0	0	0
LIPC rs12912415	AA=0 / AG or GG=1	0 / -0.098	0	0	0
COL8A1 rs13081855	GG=0 / GT=1 / TT=2	0 / 0.223 / 0.890	0	0	0
TIMP3 rs5749482	GG=0 / GC or CC=1	0 / -0.357	0	0	0
C3 rs2230199	CC=0 / GC=1 / GG=2	0 / -0.033 / 0.755	-0.033	-0.033	0
ABCA1 rs1883025	CC=0 / TC=1 / TT=2	0 / -0.046 / 0.076	-0.046	-0.046	0
LPL rs256	CC=0 / TC or TT=1	0 / -0.048	0	-0.048	-0.048
CFI rs10033900	CC=0 / TC=1 / TT=2	0 / -0.070 / -0.223	0	-0.070	-0.070
C3 rs433594	GG=0 / GA=1 / AA=2	0 / -0.110 / -0.591	-0.110	-0.110	0
FRK/COL10A1 rs3812111	TT=0 / TA=1 / AA=2	0 / -0.278 / -0.118	0	0	-0.118
RAD51B rs8017304	AA=0 / AG=1 / GG=2	0 / -0.414 / -0.138	0	0	-0.414
C2/CFB rs641153	GG=0 / GA or AA=1	0 / -0.592	0	0	-0.592
CFH rs800292	GG=0 / GA=1 / AA=2	0 / -0.899 / -1.614	0	-0.899	-0.899
CFH rs12144939	GG=0 / GT=1 / TT=2	0 / -0.947 / -1.195	0	-0.947	0
Total risk score			**1.025**	**-0.406**	**-0.911**
Lifetime risk (%)			**4.2**	**0.5**	**0.5**

Abbreviations: AMD = age-related macular degeneration; BMI = body mass index; F = female; M= male; yrs = years

Risk prediction based on Three Continent AMD Consortium

The prediction model developed by the population-based Three Continent AMD Consortium (3CC) consists of 31 variables which were represented in a total risk score indicating the risk of developing late AMD[22]. For each individual the total risk score was calculated (Table 4) and used to assess lifetime risks. Lifetime population risk for developing late AMD was 17.4% at life expectancy of 90 years in the 3CC cohort. Lifetime risks for all three individuals were also calculated using the 3CC risk score, and were 4.2%, 0.5%, and 0.5% respectively (Table 4). Although the population risk in the 3CC cohort was much higher than for the DTC-tests, lifetime risks for the three individuals were considerably lower than the lifetime risks provided by the companies (4.9-8.6; 4.0-6.5; 1.4-16.1, Table 3).

DISCUSSION

Until recently, anyone could order a DTC-test and get a personal risk estimate for common diseases. Interpretation of the test results and evaluation of their validity has been difficult, even for professionals. Our study shows that predicted risks of AMD vary considerably among DTC-tests, and none may represent the true disease risk.

We examined four DTC-tests in three individuals, and compared test results to predicted risks from a validated model developed in the large population-based Three Continent AMD Consortium (3CC)[22]. Predicted risks varied widely within each individual, and differences between highest and lowest estimates for lifetime risk were up to 12-fold. Within the same person, overall relative risks could be increased as well as decreased, depending on which test was used. All tests provided higher estimates for lifetime risk than the 3CC model. Several key points explain these differences.

First, the DTC-tests genotyped only 2-6 SNPs to calculate the risk of AMD. These risks were often based on case-control studies instead of population-based studies which often comprise lower risks[22]. Recent reports show that >30 loci have been associated by GWAS studies[1,2]. Not testing a comprehensive set of SNPs may lead to imbalance of harmful and protective SNPs, and provide a very different overall risk estimate. For example, individual 2 had several important risk-increasing as well as risk-decreasing variants (Table 4), and not testing these hampered accurate risk profiling (Table 3). This was also acknowledged for the population at large; inclusion of an extended set of variants increased risk prediction in three population-based studies[22]. We expect that even more common and rare variants will be identified for AMD in the near future, and inclusion of these variants will further refine personalized risk prediction.

Second, the lifetime population risk and reference population differed among the DTC-tests. The lifetime population risk used by 23andMe was lower than that used by the other companies, and differed for men and women. Which population had been used as reference for the calculation of the lifetime AMD population risk was not specified by any of the companies. They were all lower than the lifetime population risk estimate in 3CC (6.5-8% versus 17.4%, respectively). Lifetime population risks were based on life expectancy of 79 years for 23andMe and 90 years for 3CC. No information was provided on life expectancy by the other companies. The average life expectancy is currently above 80 years in western Europe and 79 years in the United States [45]. Life expectancy increases once a certain age has been reached: for instance, persons who reached the age of 80 years during 2008-2010 in France still had an average life expectancy of 8.3 years for men and 10.6 years for women [46]. In these persons, a life expectancy of 90 years is not unrealistic. Ancestry also influences the risk estimates. All companies asked the applicant for their ethnicity and used questionnaire data for analysis. However, calculation of ancestry is more accurate using multi-dimensional scaling

(MDS) analysis with genotype data. In GTL and Easy DNA, all results were based on European Tuscan ancestry, although European ethnicity was stated by the individuals at application. MDS analysis with genotype data from all three individuals confirmed their northern/western European ancestry comparable with their appearance (Supplementary Figure 1). Why a Tuscan ancestry was chosen for these individuals is unclear and incorrect. The choice of two different ancestries (European Tuscan and European) in one individual (Table 3) in these tests is presumably an unintended error.

The conversion to a different ancestry can lead to an alteration of the risk, since the frequency of genotypes may differ among ethnicities. The minor allele frequency (MAF) for the *CFH* rs1061170 variant in the Easy-DNA and GTL tests was set at 17% for those with Tuscan ancestry. MAF for this variant varies among ethnicities: ~36% in Europeans and Africans, ~17% in Latinos/Hispanics and ~10-15% in Asians[47]. Tuscans cluster more closely with northern/western Europeans than with Latinos/Hispanics (Supplementary Figure 1), and literature indicates that the actual MAF of the *CFH* rs1061170 variant in an Italian population is also 36%[48]. Therefore, these companies should have used a MAF of 36% rather than 17% for European Tuscans. Not using the correct MAF resulted in higher risks since all risks per SNP have been adjusted for the average risk of the SNP in the population, which can be calculated using the risk per genotype and genotype frequency. This effect is particularly visible in the risks for individual 1 (Table 3); risks provided by Easy-DNA used the European ancestry as reference population and a MAF of 36% resulting in an RR of 1.26, while GTL used the European Tuscan ancestry with a MAF of 17% resulting in a higher RR of 1.60. For carriers of the *CFH* rs1061170 CC-genotype this difference in risk will be even more extreme. In summary, an incorrect reference population was assigned to the three individuals and to this reference population (Tuscans) an incorrect MAF for the *CFH* rs1061170 SNP was assigned. In this particular case the largest effect on risk prediction of AMD was the incorrect assigned MAF. This most likely influenced the risk prediction for the other diseases predicted by the companies as well.

Third, there were mistakes in assignment of an AMD risk variant. Easy-DNA and GTL stated that the tested SNP rs800292 was located in the *C2* gene, when in fact this particular rs-number is located in the *CFH* gene[49]. Apart from the incorrect gene, the direction of the risk for this variant was opposite of that reported in 3CC[22]; in the tests from Easy-DNA and GTL the T allele was set as the risk variant, increasing the risk of AMD, while in 3CC this allele decreased the risk of AMD.

Fourth, the DTC-tests lacked inclusion of non-genetic risk factors. Only 23andMe took age and gender into account in their risk calculation. Age is the most important non-genetic factor associated with AMD known to date, and it is therefore prudent to incorporate this factor in risk predictions of AMD [50]. None of the companies included environmental factors in their risk prediction. We recommend inclusion of smoking since this factor is an important environmental risk factor for AMD[51], which also shows interaction with genetic risk variants[40]. Inclusion of non-genetic risk factors can improve the predictive ability of the test[22].

Lastly, the companies applied different methods for their risk calculation. A recent study examined and compared the methods from three DTC-tests (23andMe, deCODEme and Navigenics) for several diseases including AMD[23]. The authors showed that the formulas used by deCODEme can lead to a predicted risk exceeding 100% in high risk cases. The formulas used by 23andMe followed the Bayes' theorem preventing risks to exceed 100%, leading to more realistic risk estimates. Unfortunately, methods for risk calculation were not provided by Easy-DNA or GTL, and could therefore not be evaluated.

Recently, many companies stopped offering DTC-tests. Several issues played a role. First, the Food and Drug Administration (FDA) questioned the evidence of the safety and efficacy of these prediction tests[52]. Second, it was unclear what actions the individual will take when made aware of his/her genetic profile. Third, health care professionals lacked guidelines for counselling and patient management after genetic profiling. Do these issues apply to DTC-tests for AMD? Our study encountered no genotyping errors. Nevertheless, predictions were inaccurate based on methodology. It is indeed unclear what an individual should do when diagnosed with a high genetic risk of AMD, and what a clinician should advise such patients. Cessation of smoking and lowering BMI is advice which applies to all persons. However, it is likely that individuals who have been made aware of a high genetic risk after testing will be more motivated to make drastic life style changes than persons who are ignorant.

Although genetic testing for prediction of disease risk is the next step to personalized medicine, the current state of the art is that most DTC-tests are accurate at genotyping, but not at risk prediction. Improvement can be achieved by incorporation of a more comprehensive set of genetic markers with population-based risks. Inclusion of non-genetic risk factors, a more adequate choice of the reference population, and implementation of valid methodology for risk calculation will further improve these tests. Only then will these genetic tests become suitable for clinical practice.

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Chapter 7.1

General discussion

The aim of this thesis was to expand our genetic and epidemiological knowledge of age-related macular degeneration (AMD), in order to gain more comprehension of the pathogenesis of AMD. This general discussion will summarize and review the most important findings described in this thesis and their clinical relevance, discuss methodological considerations, and provide suggestions for future research.

MAIN FINDINGS AND CLINICAL RELEVANCE

Burden of AMD

Although the prevalence of AMD in the Western World has been studied extensively in large studies,[1,2] estimates in Europe were based on single studies and a clear overview for Europe was lacking.[3] Thus far, only a few small studies have described the prevalence of AMD in Europe and its' relation to visual decline.[4-6] We therefore investigated the prevalence of AMD over two decades in Europe in **Chapter 2.1** and observed a decreasing prevalence of AMD after the year 2006. This was also seen in other studies[7,8] and is most likely due to increase of awareness[9] and improved lifestyle in the elderly.[10,11] However, the projections we made for the year 2040 indicate that, although age-specific prevalences may be decreasing, that the actual number of affected persons with AMD will increase due to the aging population, resulting in more affected elderly in Europe than residents currently living in the Netherlands.

In **Chapter 2.2** we have shown that AMD is the major cause of visual impairment and blindness in an elderly population. Visually impaired persons, due to AMD, experience a significantly reduced quality of life.[12] For society, both visual impairment and blindness cause a considerable economic burden.[13] Annual expenses are inversely correlated with visual acuity; for blind patients costs are almost two-fold the costs for non-blind patients. Costs are even expected to increase due to the aging population. Even though, improved diagnostics and the introduction of anti-vascular endothelial growth factor (VEGF) therapy have most likely resulted in fewer visually impaired eyes,[14] studies indicated that long term visual prognosis after anti-VEGF treatment often show substantial visual decline.[15,16] However, all improvement in visual outcome, even for a short period, will decrease demands on healthcare and costs. Therefore, anti-VEGF therapy should still be continued, since it improves visual outcome on the short term.[15,16] Our data indicates that AMD is still a major health problem and a more profound solution is needed, since long lasting effects of the current therapy is insufficient.

Environmental and phenotypical risk factors of AMD

Risk factors have been investigated to identify those at risk, predict disease outcome and help elucidate the complex pathogenesis of AMD. These factors can also be used for screening and modifiable factors may even alter disease onset and outcome. As stated in **Chapter 1.1** many risk factors for AMD were known before the onset of this thesis. Yet, we were able to identify a new risk factor for AMD: thyroid hormone. Previous studies identified a positive relationship of thyroid medication use[17,18] or self-reported hypothyroidism[19] associated with a higher risk of AMD. However all of these studies were lacking laboratory assessment of thyroid function, and were therefore unable to investigate the true relationship. In this thesis we have shown that higher values of free thyroxine, even within the normal range, were associated with an increased risk of AMD and retinal pigment alterations (**Chapter 4.3**). Higher levels of free thyroxine can increase metabolism leading to oxidative stress, which plays an important role in the pathogenesis of AMD (Figure 1). [20,21] Our findings were partly confirmed by the Blue Mountains Eye study. They found a significant positive association of overt hyperthyroidism and 10-year incidence of AMD. No association was found for

hypothyroidism, but use of thyroxine medication was significantly associated with an increased risk of AMD. Both findings seem to be driven by higher free thyroxine levels. Unfortunately, no association was found for free thyroxine levels and incident AMD. However, the number of cases in their study was small, since data was only available for 5-year incidence of AMD.[22] More studies are necessary to understand the exact role of thyroid hormone in the pathogenesis of AMD and maybe in the future thyroid hormone can be used as a biomarker for AMD.

An important modifiable risk factor is dietary intake. Several studies focusing on dietary intake and supplementation of nutrients have shown that in particular carotenoids, zinc, and omega-3 fatty acids were associated with a lower risk of AMD. These nutrients are mainly present in dark-leafy vegetables, yellow-orange vegetables and fruit, fortified cereal, meats and fatty-fish. (**Chapter 4.1**) Unclear was what the recommended minimum intake of these foods should be to obtain this protective effect. In this thesis we have shown that a diet including at least 200 grams of vegetables a day, 2 large pieces of fruit a day and 2 times fish per week is associated with a reduced risk of AMD. No significant association was found for the intake of grains or meats with AMD (**Chapter 4.2**). The beneficial effect from vegetables, fruit and fish intake on AMD is most likely due to carotenoids, in vegetables and fruit, and omega-3-fatty acids in fish. Higher intake of these nutrients could also reduce the overall risk of AMD in those with a high genetic risk (**Chapter 5.3**). Carotenoids, in specific lutein and zeaxanthin, are important for macular pigment. The macular pigment is highly concentrated with these carotenoids and offer protection to the retina by absorbing hazardous ionizing blue and ultraviolet light. Furthermore, it has antioxidant properties and has anti-inflammatory effects.[23] Polyunsaturated fatty acids, like omega-3-fatty acids, are one of the main components of the retina. They are highly present in the photoreceptor outer segments and improve fluidity of the photoreceptor membranes, resulting in faster response to stimulation. Additionally, omega-3-fatty acids may also protect against ischemia, and have antioxidant and anti-inflammatory properties.[24] Both oxidative stress and inflammation are part of the pathogenesis of AMD (Figure 1).[20,21] Other modifiable risk factors associated with AMD are smoking and body mass index. Cardiovascular risk factors may be associated with AMD, although findings have been inconclusive.[21,25]

Regarding the modifiable risk factors, clinicians should advice patients at risk of AMD, for example those with a positive family history, to adopt a healthy life-style. This should include physical exercise, a diet with recommended intake of vegetables, fruit and fish, have a normal body mass-index and quit smoking, the latter if applicable.

Reticular pseudodrusen (RPD), also known as subretinal drusenoid deposits, have been identified as an important clinical risk factor for progression to late AMD. Those with RPD have a higher risk compared to other drusen types.[26-28] Presence of RPD is often bilateral and the highest prevalence is found in late AMD cases.[29] Identification of persons at high risk for late AMD can be important for patient management. **Chapter 3.1** and **3.2** provide evidence that near-infrared imaging was the most sensitive for detection of RPD of the tested imaging modalities. Detection improved even further when multi-modal imaging was applied. We also found that RPD was associated with a different risk profile compared to soft indistinct drusen, another drusen type which coincide often with RPD. These findings indicate that RPD are distinct AMD entities.

In neovascular AMD, antiplatelet and anticoagulant (AP/AC) use may potentially worsen clinical outcomes, leading to more severe hemorrhages and increase fibrovascular scarring.[30] A question often asked in the clinic is whether AP/AC therapy should be discontinued in patients with neovascular AMD. In **Chapter 4.4** we show that the use of AP/AC therapy in patients with active

choroidal neovascularization is not associated with visual impairment, nor the presence of retinal hemorrhages. We could not find an association of AP/AC use with choroidal neovascularization (CNV) lesion size. For retinal foveal thickness a borderline significant association was found for aspirin use only. After the start of anti-VEGF medication no differences were observed between users and nonusers. In contrast, those using AP/AC medication did not have an increased risk of visual impairment and had a lower risk of retinal hemorrhages. Aspirin seemed to be the major driver of the beneficial effect of AP/AC in our study. Beside the well-known effect on platelet aggregation, aspirin has anti-oxidant properties, reduces inflammation and angiogenesis, and stimulates apoptosis.[31] The anti-oxidant, anti-inflammatory and anti-angiogenic properties may explain the found protective effect. Our findings imply that patients with neovascular AMD can continue their prescribed use of AP/AC medication without negative effects on AMD outcome.

Genetic risk factors

Even before the start of genome-wide association studies (GWAS), it was known for many years that heritability was an important factor in AMD.[32] Only after the start of GWAS identification of the disease-associated risk variants became possible. Several risk variants in thirteen genes have been associated with AMD, by single or small collaborations between studies (**Chapter 5.1**). The associated genes were part of the complement pathway (*CFH, CFB/C2, C3, CFI*), lipid related genes (*APOE, LIPC, CETP*), collagen related genes (*COL8A1, COL10A1*) and other genes (*ARMS2, VEGFA, TIMP3, TNFRSF10A*). The AMD gene consortium conducted a GWAS including ~17,100 advanced AMD cases and ~60,000 controls, and confirmed the previous associations and identified seven new loci (*COL8A1-FILIP1L, IER3-DDR1, SLC16A8, TGFBR1, RAD51B, ADAMTS9, B3GALTL*) (**Chapter 5.2**). All nineteen risk variants were common and involved in complement activity, lipid metabolism, extracellular matrix remodeling and angiogenesis.

More recently, The AMD Gene Consortium published their results using exome chip data from ~16,100 cases with intermediate or late AMD and ~17,800 controls.[33] In total 52 independently associated variants in 34 loci were identified, of which 17 loci were replicated from the previously mentioned GWAS. The associated variants were mostly common, seven were rare variants. Four genes had a significant disease burden: *CFH, CFI, TIMP3* and *SLC16A8*, indicating a causal role for these genes. Pathway analyses identified complement, collagen, lipid and extracellular matrix pathways to play a role in the pathogenesis of AMD. The heritability of AMD has been determined to be between 65-70%.[34,35] All identified risk variants to date explain approximately 27.2% of disease variability, of which 1.4% is contributed by rare variants. This means that approximately 40% of the heritability is not explained by the identified genes. The missing heritability might be explained by additional genetic variation[36], gene-gene or gene-environment interactions.

Identification of risk factors has helped to understand more of the pathogenesis of AMD. This information can be used to develop future therapies, but also to identify those at high risk. In Figure 1, risk factors for AMD discussed in this this thesis have been incorporated to provide an overview of the pathogenesis of AMD.

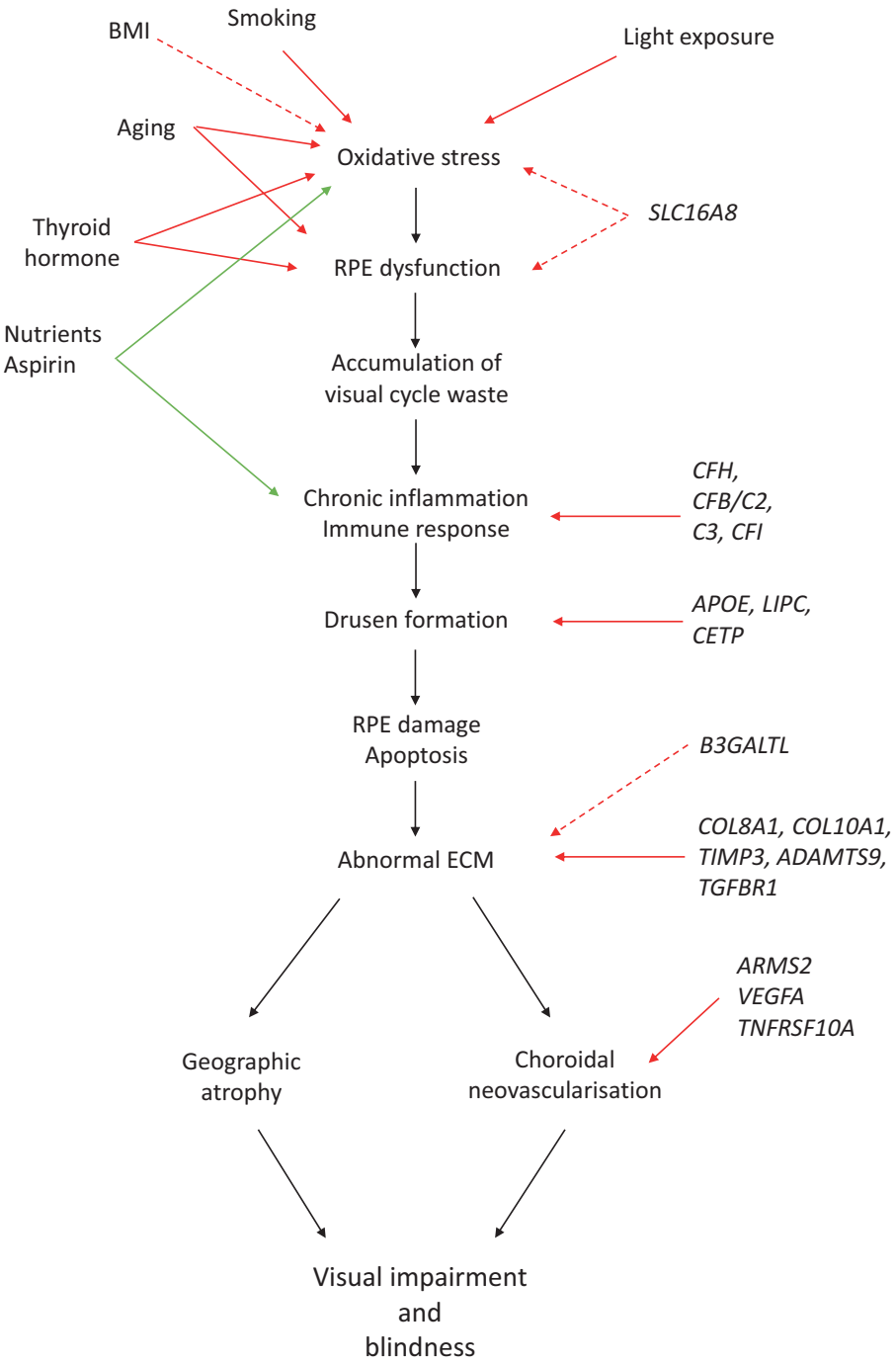

FIGURE 1 - Pathogenesis of AMD.
Simplified scheme of the pathogenesis of AMD. Black arrows are the steps in the pathogenesis, red arrow displays risk increasing factors, green arrow displays risk reducing factors. Dotted arrows indicate a potential relationship.

Prediction of AMD

In **Chapter 6.1** we have provided an overview of previous reported prediction models for late AMD. All of these models were based on a case-control study design, in which the extreme ends of disease are compared, while excluding the majority of the population with signs of early AMD. We developed a prediction model in the population-based Rotterdam Study and validated it in the Three Continent AMD Consortium. The full model included the variables: age, sex, smoking, body mass index, baseline AMD phenotype, and 26 common risk variants in genes associated with AMD. This model could distinguish those who will develop late AMD between those who will not, with an accuracy of 87%. Exclusion of variables led to a lower predictive value. Subsequently we showed that the cumulative risk of developing late AMD is approximately 17% at the age of 90 years. Using our prediction model, the cumulative risk for seven risk categories, ranged from zero to almost 66% at the age of 90 years. This indicates that a prediction test can help to identify those at high risk of late AMD.

Although published prediction models led to several web-based risk calculators for prediction of late AMD (http://caseyamdcalc.ohsu.edu[37]; http://www.myvisiontest.com/riskcalc2.php[38]; http://www.sightrisk.com) and progression of AMD (www.seddonamdriskscore.org[39]), implementation in the clinic will be difficult, since most of these risk calculators need genetic data beside information on lifestyle and phenotypical factors. Currently, there is no easy accessible, inexpensive genetic test available for AMD, which tests all known associated genetic variants. There are, however, companies offering genetic tests for AMD, which can be purchased on the internet. We have tested the direct-to-consumer personal genome tests for genotyping quality and accuracy of risk prediction of AMD in **Chapter 6.2**. The genotyping was of high quality, but the risk prediction was not accurate. Inclusion of more genetic and environmental risk factors for AMD will improve future tests. Interestingly, from the tested companies, one stopped offering genetic tests shortly after we had received our results, one offers raw genotype data only, and two companies are currently not offering any tests for AMD.

The costs of sequencing has dropped in a rapid pace over the past years. Sequencing of an entire genome, was approximately $10 million in 2007, and currently available for less than $1000.[40,41] Although lower costs for genetic testing will make it more available for clinical practice, cost-effectiveness[42], the possibility of secondary findings[43], psychological and decision making risks, all need to be addressed before it can be applied for routine genetic testing in AMD as long as no cure is available.[44]

METHODOLOGICAL CONSIDERATIONS

Within this thesis, relevant methodological issues have been addressed in the discussion sections of each chapter. Here, we will discuss some general methods and issues we encountered.

Phenotyping issues

The studies described in this thesis have different outcomes, mostly: 1) late AMD, 2) early AMD, 3) any AMD, both late and early AMD combined. Late AMD is the visual threatening end-stage of the disease, due to a low prevalence in the general population, the power to investigate some associations has been challenging. Early AMD is mostly asymptomatic, but more frequently present in the general population compared to late AMD. However, several characteristics describe early AMD, leading to a large variation of phenotypes. A possibility to increase power is to collaborate with other studies, however phenotypical issues could reduce the power. For the collaboration with the Three Continent AMD Consortium we harmonized our classification to minimize differences in AMD definitions, which

was predominantly the case for early AMD.[45] This resulted in a new 5-step AMD severity scale, which was used in **Chapter 5.3,** and **6.1**. To increase power for analyses restricted to the Rotterdam Study, we mainly used the outcome any AMD.

Another phenotypical issue is improved detection of AMD lesions with multimodal imaging compared to color fundus photographs (CFP) only. Differences in detection may lead to changes in prevalence and incidence of AMD. For instance, in **Chapter 3.1** and **3.2** we showed that detection of RPD was better using near-infrared imaging, and the best results were obtained using multi modal imaging. This also accounts for other AMD lesions.[46-49] Nevertheless, CFP have been the golden standard for AMD grading in epidemiological studies, including the Rotterdam Study.[50-52] Many new techniques have been developed and since 2007, when high resolution imaging was available,[53] imaging techniques like optical coherence tomography (OCT), near-infrared and auto fluorescence imaging, have been incorporated in the Rotterdam Study beside CFP. Unfortunately, a consensus for grading and classification of these pathologies using imaging other than CFP is lacking.

Genetics

Since the start of the GWAS era, thousands of genetic variants associated with complex diseases and traits have been identified successfully.[54] However, these types of analyses have limitations: large sample sizes are mostly needed to increase the power to find associations. This requires collaborations between different studies and could introduce phenotypical and genetical heterogeneity, limiting the possibility to find associations. Furthermore, the found association is in most cases a marker in high linkage disequilibrium with the causal variant. Identification of the causal variant can be difficult due to many or no genes near the associated marker. Identification of the causal variants is necessary, since it helps to identify the true relationship with the disease and may provide a drug target. In **Chapter 5.2** we identified 19 common variants associated with AMD using GWAS data from 33 different studies. The heterogeneity was high for some associations, and most likely due to differences in methodology and ancestry of study populations (Asian and European descent). Of the previously identified loci, 17 were replicated in the most recent study from the AMD gene consortium using genomic data from 26 different studies.[33] Exome chip data was enriched for the previously identified loci and imputed with 1000 genomes data, to increase the chance of finding causal risk variants in protein-coding regions of the DNA, also known as the exome. All study samples were genotyped at the same facility, which led to a reduction of heterogeneity. The study using exome chip data was executed with a larger amount of variants, but with a lower number of cases and controls in comparison to the GWAS. Many new risk variants, including rare variants, were identified in the recent study, and explained more of the phenotypical variance. However, these newly identified variants were involved in largely the same pathways which were also identified in the GWAS and there is still missing heritability.

FUTURE DIRECTIONS

The studies described in this thesis have provided new insights in the pathogenesis of AMD. However, there are still many questions that can be answered by future studies.

As a start, identifying the missing heritability of AMD could help to understand the complete genetic architecture of AMD. Many genes involved in several pathways have been identified, but we are not there yet. For instance, it is unknown what the exact function of the *ARMS2* gene is and which role it plays in the pathogenesis of AMD, although the risk variant in this gene has the most significant association of all common variants associated with AMD.[33] Unidentified risk variants, gene-gene

and gene-environment interaction could help to understand the role of identified genes and identify the missing heritability of AMD. For identification of novel risk variants it is difficult to state which approach would be the most successful, since several strategies have been explored: Exome sequencing of families with AMD[55,56] or patients with specific phenotypes,[57] sequencing of previously associated genes,[58-62] and the GWAS approach using large case-control samples for the outcomes early[63] as well as late AMD.[33] Enlargement of the study sample and whole genome sequencing might help to identify new risk variants for AMD.

Gene-gene or gene-environment interactions can modulate disease risk. In this thesis we have shown that intake of nutrients reduced the overall risk of individuals at high genetic risk based on gene-environment interactions (**Chapter 5.3**). In order to study multifactorial relationships between genes and environmental factors, collaboration between epidemiological studies is needed, since single studies are quickly outnumbered because of the many different strata in the analyses. Gene-gene and gene-environment interactions can also be investigated in a genome-wide setting, so called genome-wide association gene-gene interaction and genome-wide environmental interaction studies. This, however, needs to be done in a large study setting with good quality of phenotyping and environmental data, since studies can be quickly underpowered due to countless possibilities of interaction combinations and misclassification may introduce bias.[64,65]

Newer fields of interest in AMD research have been epigenomics, transcriptomics, proteomics, and metabolomics. Like GWAS, omic studies are hypothesis-free, enabling association analyses at once in a large set of markers. Epigenomics focusses on heritable regulatory mechanisms of gene expression without changes in the DNA sequence. Transcriptomics conveys gene expression and noncoding RNAs like microRNAs, which are small RNA molecules that regulate gene expression after transcription. Proteomics focusses on peptides or proteins and in metabolomics derivatives of metabolism are measured and analyzed. Several small studies using these new techniques have published interesting results, however findings need to be replicated in independent studies.[21,66] The most interesting of these new techniques are proteomics and metabolomics. These techniques may help to understand what is actually happening in a patient with AMD. Beside data acquired from plasma or serum, which is much easier to obtain from the patient, ocular tissue and fluids of patients with AMD in various stages of the disease are also necessary, since these are closer to the location of the disease. Proteomics and metabolomics may help to identify potential biomarkers, find new pathways and link current pathways together. Epigenomics and transcriptomics are also interesting, but might be of more interest in a later stage, when more of the pathogenesis of AMD is understood. These techniques focus on regulatory mechanisms and may help to understand for example the influence of environmental factors on gene expression.

Many different imaging techniques of the posterior pole are available and improvement of current and development of newer techniques is ongoing. For example in OCT imaging many different methods have been developed, and the latest is OCT angiography.[67] This provides a non-invasive technique for visualization of functional blood vessels in the eye and is potentially interesting for neovascular AMD. However, as stated earlier, a consensus for grading and classification of imaging other than CFP is lacking. Currently, we are working on a consensus for OCT grading of macular diseases within the E3 consortium.

Manual grading of images is time-consuming, which is one of the reasons that automatic grading with machine learning algorithms have been developed for different image modalities.[68-71] This field is still under development and the predictive power of the machine learning algorithms

depend on the size and quality of the dataset.[69] Consensus of grading and classification, together with collaboration of studies with imaging, may result in large high quality datasets, leading to improvement of the algorithms. Automatic grading is especially interesting for screening purposes, which has successfully been used for detection of diabetic retinopathy.[70] Early detection of AMD in persons without any symptoms, may identify those at risk of late AMD, increase awareness and lead to positive life-style changes. Another opportunity for machine learning is development of prediction models. Currently there models available for prediction of drusen regression[72], which is associated with development of late AMD, and prediction of anti-VEGF treatment needs[73], which may help to determine individual treatment intervals. When these prediction models will become available for clinical application, this can lead to a substantial improvement of AMD management.

Systems biology can help to link all the pieces together. It is a powerful computational and mathematical method of modeling complex diseases to comprehend complex interactions within biological systems. For example, in systems biology data on phenome, genome, epigenome, transcriptome, proteome, interactome, and metabolome can be integrated to assemble models and pathways, which can help explain the pathogenesis of AMD.[74]

The ultimate goal is to identify patients at an early stage, obtain insights in individual risk profiles and how to modulate these, monitor progression of the disease, and measure treatment response, all with a simple test. However, before this test will have added value, there is need for better treatment options. Understanding the pathogenesis of AMD will help to provide drug targets and understand how to prevent progression to visual threatening late AMD.

Currently, many ongoing trials are investigating different therapeutic options.[75-78] For neovascular AMD the main focus is to decrease treatment burden. Implants providing constant release of anti-VEGF are being tested. New anti-VEGF agents have been developed, but also anti-platelet derived growth factor and anti-angiopoietin agents are interesting therapies, because of their anti-angiogenic effect. These new agents are mostly tested in combination with anti-VEGF therapy. Gene therapy might be another option to reduce treatment burden. A viral vector containing the code for the VEGF receptor is administered via subretinal injections and then expressed by the host retinal cells. This reduces the effect of VEGF in the eye with a longer duration compared to current treatment with anti-VEGF.

Since no treatment options have been available for geographic atrophy (GA) or early AMD, many trials have been focusing on this type of AMD with the main goal of preservation of vision. Ongoing trials can be divided in five categories: 1) neuroprotective agents, 2) anti-inflammatory agents, 3) lipofuscin and visual cycle inhibitors, 4) choroidal blood flow restoration agents, 5) stem cell therapy. Most of these trials have completed phase I or II and a few started phase III. The first results of most of these trials have been promising, in particular for stem cell therapy. Human embryonic stem cells were injected in the subretinal area, which improved visual acuity in the injected eyes of patients with geographic atrophy.[79,80] However, larger studies with follow-up measurements are needed before some of these therapeutic options will be used as a treatment for AMD.

The latest results of the phase III trial investigating the safety and efficacy of lampalizumab, which is an antibody selectively inhibiting complement factor D, was less promising.[81] The first results did not show a difference in mean GA lesion area in comparison to the sham injection. The target, complement factor D, was chosen carefully: genetic studies have indicated that the complement system, in particular the alternative pathway in which factor D plays a role, is involved in the pathogenesis of AMD. It could be that once geographic atrophy starts to develop, there is no way

back. A recent study observed that before GA is visible, small disruptions of the retinal anatomy occur, which are best visualized on OCT.[49] These disruptions include subsidence of the outer plexiform and inner nuclear layers, while the retinal pigment epithelium is still intact. This stage is named nascent GA and eventually will result in loss of photoreceptor and retinal pigment epithelial cells, which are characteristics of GA. Thus, treatments targeting the complement system might have more success when tested in patients with early signs of AMD, since the complement system plays a major role in drusen formation.[82] Nevertheless, patients with early AMD are mostly asymptomatic, but at risk of visual threatening end stage disease. Whether these patients should be treated or not is a difficult discussion. As a start, future studies focusing on treatment of early AMD could start with those at high risk of developing late AMD.

CONCLUDING REMARKS

AMD is a chronic complex disease and still the leading cause of blindness in the Western World. In this thesis we have identified genetic variants associated with AMD through an international collaboration of studies, which have helped to elucidate a large part of the genetic architecture of AMD. Furthermore, we have investigated environmental risk factors and gene-environment interactions, using large well-designed longitudinal studies for which we harmonized our methodology and grading protocols. Combining these risk factors in a prediction model offered a good method to distinguish between those who will develop late AMD and those who will not. These findings can help future studies to further unravel the pathogenesis of AMD and ultimately develop preventative measures for this blinding disease.

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Chapter 7.2

Summary

SUMMARY

Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in elderly in the Western world. AMD is characterized by drusen, pigmentary changes, geographic atrophy and choroidal neovascularization, which affect the normal anatomy of the macula. Early AMD is characterized by the first two features, and late AMD by the latter two. Although several demographic, genetic, and environmental risk factors have been identified, the etiology of AMD is still largely unknown.

The main objectives described in this theses were: 1) assess frequency and impact of AMD, 2) evaluate the merit of new imaging techniques for diagnosis and risk assessment, 3) identify new environmental risk factors for AMD, 4) investigate genetic associations and gene-environmental interactions, 5) assess predictive value of risk factors associated with AMD.

Our study population included the population-based Rotterdam Study, population-based studies from the Three Continent AMD Consortium and from the European Eye Epidemiology (E3) Consortium, double blind randomized-controlled multicenter trial BRAMD study, and population-based and case-control studies from the AMD Gene Consortium.

Chapter 1 provides a general introduction to AMD and describes the main aims of this thesis. **Chapter 2** discusses the frequency and impact of AMD. In **Chapter 2.1** we investigated the prevalence of AMD in Europe over the past two decades using 14 population-based studies from the E3 Consortium. The frequency of AMD increases with age; overall prevalence of early AMD and late AMD was 13.2% and 3.0%, respectively, in those aged 70 years and older. We observed a decreasing age-specific prevalence of late AMD. We also observed a decreasing number of visually impaired eyes and persons due to choroidal neovascularization after 2006, most likely due to improved diagnostic procedures and introduction of anti-vascular endothelial growth factor therapy. We also made projections for the year 2040, and predicted that the actual numbers of affected persons will still increase. Europe will reside more affected elderly persons, than residents currently living in the Netherlands. In **Chapter 2.2** we studied the causes of blindness and low vision in relation to refractive error using the Rotterdam Study data. We found that the major cause of visual impairment in all refractive categories, except for high myopia (spherical equivalent ≤ -6D), was AMD.

In **Chapter 3**, we explored diagnostic properties for detection of reticular pseudodrusen (RPD) in AMD using different imaging techniques and investigated the risk profiles of RPD in a population-based setting. In **Chapter 3.1**, we describe the epidemiology of RPD in the Rotterdam Study. We showed that detection of RPD was better on near-infrared imaging than on color fundus photographs. Subsequently, we assessed demographic, environmental, and genetic risk factors for RPD versus soft indistinct drusen, since these drusen types coincide frequently. Several demographic and several genetic (*ARMS2, C3, VEGFA*) risk factors were more associated with RPD than soft indistinct drusen. Indicating that these features are distinct AMD entities. **Chapter 3.2** proposes an automatic system for RPD quantification, which showed similar performance of RPD detection as human graders. The study also indicated that multimodal imaging improves detection of RPD for both automatic as human grading.

In **Chapter 4**, we explored risk factors for AMD. We provided an overview of investigated nutrients and their associations with AMD in **Chapter 4.1**. In particular carotenoids, zinc and omega-3 fatty acids were associated with a reduced risk of AMD. Diet recommendations from the Dutch food center were studied in **Chapter 4.2**, using data from the Rotterdam Study. Recommended intake of at least

200 grams of vegetables a day, 2 large pieces of fruit a day and 2 times fish per week, was associated with a reduced the risk of AMD of 42%. However, only 3.5% of the studied population met these recommendations. **Chapter 4.3** shows that higher values of free thyroxine, even within the normal range, are associated with an increased risk of AMD and retinal pigmentary alterations in the macula, suggesting a role for thyroid hormone in the pathway of AMD. **Chapter 4.4** shows that in the double blind randomized-controlled multicenter trial BRAMD study, usage of antiplatelet or anticoagulant drugs in active choroidal neovascular AMD did not negatively affect visual outcome, nor presence of retinal/subretinal hemorrhages, lesions size, or retinal thickness. From our study, we concluded that continuation of antiplatelet or anticoagulant therapy in patients with active choroidal neovascular AMD is not contraindicated.

Chapter 5 focusses on genetic risk factors and gene-environment interactions in AMD. **Chapter 5.1** discusses the knowledge about the genetic background of AMD. Most of the genetic risk of AMD can be explained by two genes, *CFH* and *ARMS2*. In **Chapter 5.2**, seven new loci for AMD were identified, using a large-scale genome wide association study meta-analysis, which was established in the AMD Gene consortium. These loci were located near the genes: *COL8A1, IER3-DDR1, SLC16A8, TGFBR1, RAD51B, ADAMTS9* and *B3GALTL*, which are involved in complement activity, lipid metabolism, extracellular matrix remodeling and angiogenesis. **Chapter 5.3** shows that higher intake of lutein and zeaxanthin, and weekly consumption of fish, could reduce the overall risk of AMD in individuals with a high genetic risk, based on two or more risk variants in major AMD genes (*CFH, ARMS2*). This was observed in two population based studies from the Three Continent AMD Consortium. These findings can enable personalized preventive interventions.

In **Chapter 6**, we discuss prediction and personal genome testing. In **Chapter 6.1** we studied the predictive value of various prediction models based on demographic, genetic and environmental data in the Rotterdam study, and validated this in the Three Continent AMD Consortium. We found that the full model, including age, sex, 26 single nucleotide polymorphisms in AMD risk genes, smoking, body mass index and baseline AMD phenotype had the best predictive value. The prediction model could distinguish between those who will develop late AMD and those who will not with an accuracy of 87%. In **Chapter 6.2** we tested direct-to-consumer personal genome tests available on the internet. Although the genotyping was of a high quality, these tests appeared not yet suitable for clinical application, as risk prediction was not accurate. Inclusion of more genetic and environmental risk factors for AMD will improve future tests.

Lastly, **Chapter 7** provides a general interpretation and implication of these main findings. This chapter also addresses methodological considerations, clinical implications, and suggestions for future research.

To conclude, the studies described in this thesis have investigated several aspects of AMD. We have identified new genetic variants and risk factors, which may lead to new insights in the complex pathogenesis of AMD, and eventually to new directions for treatment and/or preventive measures.

Chapter 7.3

Samenvatting

SAMENVATTING

Leeftijdsgebonden maculadegeneratie (LMD) is de meest voorkomende oorzaak van slechtziendheid bij ouderen in de Westerse Wereld. LMD wordt gekenmerkt door drusen, pigment veranderingen, verdunning van het netvlies (geografische atrofie) en vorming van nieuwe bloedvaten (choroïdale neovascularisaties) waardoor de normale anatomie van de macula verloren gaat. De eerste twee kenmerken zijn de karakteristieken voor vroege LMD en de laatste twee voor late LMD. Hoewel er meerdere demografische, genetische en omgevingsfactoren gevonden zijn die een rol spelen in het ontstaan van LMD, is de etiologie van LMD nog steeds grotendeels onbekend.

De belangrijkste vragen die dit proefschrift beoogt te beantwoorden waren: 1) Hoe vaak komt LMD voor en wat zijn de gevolgen hiervan? 2) Welke nieuwe beeldvormende technieken zijn nuttig voor de diagnostiek van LMD? 3) Welke nog onbekende omgevingsfactoren spelen een rol in het ontstaan van LMD? 4) Welke genetisch en gen-omgevingsfactoren veroorzaken LMD? 5) Hoe goed kunnen reeds bekende risicofactoren LMD voorspellen?

Onze studiepopulatie bestond uit het Erasmus Rotterdam Gezondheid Onderzoek (ERGO, ook wel Rotterdam Studie genoemd), bevolkingsonderzoeken van het Three Continent AMD Consortium en van het European Eye Epidemiology (E3) Consortium, de BRAMD studie, een dubbelblinde gerandomiseerde gecontroleerde trial, en bevolkingsonderzoeken, patiënt-controle en patiënt studies van het AMD Gene Consortium.

Hoofdstuk 1 geeft een algemene introductie over LMD. **Hoofdstuk 2** geeft weer hoe vaak LMD voorkomt en wat de gevolgen hiervan zijn. In **Hoofdstuk 2.1** hebben we onderzocht hoe vaak LMD voorkomt in Europa in de laatste twee decennia. Hiervoor hebben we data van 14 bevolkingsonderzoeken van het E3 Consortium gebruikt. De frequentie van LMD neemt toe met de leeftijd; in deelnemers van 70 jaar en ouder bleek 13.2% vroege LMD en 3.0% late LMD te hebben. Verder vonden wij dat de leeftijdsspecifieke frequentie van late LMD afnam na het jaar 2006. Ook bleek het aantal blinde en slechtziende ogen door choroïdale neovascularisaties af genomen te zijn, waarschijnlijk door verbeterde diagnostische technieken en introductie van anti-vasculaire endotheliale groeifactor, een behandeling tegen bloedvat nieuwvormingen in het netvlies. Ondanks deze dalende frequentie laten de projecties voor het jaar 2040 zien dat het werkelijke aantal aangedane personen zal toenemen. Dit betekent dat het totale aantal aangedane ouderen door LMD in Europa, het aantal inwoners van Nederland zal overschrijden. In **Hoofdstuk 2.2** hebben we de oorzaken van blindheid en slechtziendheid in relatie tot refractieafwijkingen bestudeerd binnen het ERGO onderzoek. We vonden dat de belangrijkste oorzaak van blindheid en slechtziendheid LMD was. Dit was onafhankelijk van refractieafwijkingen, met uitzondering van hoge myopie (sferisch equivalent van -6 D of meer) waar myope maculadegeneratie de belangrijkste oorzaak was.

In **Hoofdstuk 3** hebben we naar verschillende aspecten van beeldvormende technieken voor het diagnosticeren van reticulaire pseudodrusen (RPD) gekeken en onderzochten de risicoprofielen van RPD in kader van LMD. In **Hoofdstuk 3.1** beschrijven we de epidemiologie van RPD in het ERGO onderzoek. We hebben aangetoond dat detectie van RPD beter was op nabij-infraroodbeelden dan op kleurenfundusfoto's. Vervolgens hebben we demografische, genetische en omgevingsfactoren voor RPD versus zachte drusen geanalyseerd, aangezien deze soorten drusen vaak samen voorkomen. Demografische factoren (leeftijd en geslacht) en verschillende genetische factoren (*ARMS2, C3, VEGFA*) waren meer geassocieerd met RPD dan met zachte drusen. Deze bevindingen suggereren dat RPD een op zichzelf staande entiteit is binnen LMD. **Hoofdstuk 3.2** beschrijft automatische

kwantificatie van RPD, die evengoed als humane gradeerders de aanwezigheid van RPD vast stelt. Verder komt uit deze studie voort, dat multimodale beeldvorming de detectie van RPD, voor zowel automatische als humane gradering verbetert.

In **Hoofdstuk 4** hebben we risicofactoren voor LMD onderzocht. We hebben een overzicht gegeven van de verschillende voedingsstoffen die bestudeerd zijn in relatie tot LMD in **Hoofdstuk 4.1**. Met name carotenoïden, zink en omega-3 vetzuren waren geassocieerd met een lager risico van LMD. Dieetaanbevelingen van het voedingscentrum zijn onderzocht in **Hoofdstuk 4.2**, met behulp van het ERGO onderzoek. Er werd aangetoond dat de aanbevolen inname van tenminste 200 gram groenten per dag, 2 grote stukken fruit per dag en 2 keer per week vis, het risico op LMD met 42% kan verminderen. Echter, slechts 3,5% van de ouderen behaalde de geadviseerde minimale inname. **Hoofdstuk 4.3** laat zien dat hogere waarden, die nog binnen de normale spreiding vallen, van vrije thyroxine geassocieerd zijn met een verhoogd risico op LMD en pigment veranderingen in de macula. Deze bevindingen suggereren een rol voor schildklierhormoon in de pathogenese van LMD. **Hoofdstuk 4.4** toont aan dat in de BRAMD studie het gebruik van trombocytenaggregatieremmers of anticoagulantia bij actieve choroïdale neovascularisaties, in het kader van LMD, geen negatieve invloed heeft op de visus, noch op retinale / subretinale bloedingen, laesiegrootte of retinale dikte. Uit deze studie kunnen we concluderen dat het doorgebruiken van deze medicatie bij patiënten met actieve choroïdale neovascularisaties in het kader van LMD, niet gecontra-indiceerd is.

Hoofdstuk 5 richt zich op genetische risicofactoren en gen-omgevingsinteracties geassocieerd met LMD. **Hoofdstuk 5.1** geeft een samenvatting van de genetische achtergrond van LMD. Het grootste deel van het genetische risico van AMD kan verklaard worden door slechts twee genen, namelijk *CFH* en *ARMS2*. In **Hoofdstuk 5.2** werden zeven nieuwe genetische varianten geïdentificeerd voor LMD met behulp van een grootschalige meta-analyse van genoomwijde associatie studies binnen het AMD Gene consortium. Deze varianten bevinden zich nabij de genen: *COL8A1*, *IER3-DDR1*, *SLC16A8*, *TGFBR1*, *RAD51B*, *ADAMTS9* en *B3GALTL*. Deze genen zijn betrokken bij diverse processen, waaronder de complement cascade, lipide metabolisme, weefselstructuren en het vormen van nieuwe bloedvaten. **Hoofdstuk 5.3** laat zien dat een hogere inname van luteïne en zeaxanthine en wekelijkse consumptie van vis het totale risico op LMD verlaagt voor personen met een hoog genetisch risico op basis van twee of meer risicovarianten in de belangrijke LMD genen (*CFH, ARMS2*). Dit onderzoek werd verricht in twee bevolkingsonderzoeken van het Three Continent AMD Consortium. Deze bevindingen zijn interessant voor counseling van individuele LMD patiënten.

In **Hoofdstuk 6** bespreken we predictie en persoonlijke DNA testen. **Hoofdstuk 6.1** onderzoekt de voorspellende waarde van verschillende predictiemodellen op basis van alle bekende risicofactoren. Dit is onderzocht in het ERGO onderzoek en gevalideerd in het Three Continent AMD Consortium. We vonden dat het volledige model, inclusief leeftijd, geslacht, 26 genetische risicovarianten, roken, 'body mass index' en het LMD fenotype de beste voorspellende waarde had. Het predictiemodel kon met 87% zekerheid onderscheid maken tussen diegenen die wel een eindstadium LMD zullen ontwikkelen en diegenen die dat niet doen. In **Hoofdstuk 6.2** hebben we persoonlijke DNA testen getest die beschikbaar waren op het internet. Hoewel de genotypering van hoge kwaliteit was, bleek dat deze testen niet geschikt waren voor klinische toepassingen, aangezien de risicovoorspellingen niet nauwkeurig genoeg waren. Daarnaast ontbraken omgevingsfactoren in de predictie modellen van de onderzochte testen; incorporatie hiervan zal de voorspelling ook verbeteren.

Ten slotte geeft **Hoofdstuk 7** een algemene interpretatie van deze belangrijkste bevindingen. Dit hoofdstuk beschrijft ook methodologische overwegingen, klinische implicaties en suggesties voor toekomstig onderzoek.

Concluderend, de beschreven studies in dit proefschrift hebben verschillende aspecten van LMD onderzocht. Wij hebben nieuwe genetische varianten en risico factoren geïdentificeerd die kunnen zorgen voor nieuwe inzichten in de complexe pathogenese van LMD en zullen leiden tot nieuwe richtlijnen voor behandelingen en/of maatregelen ter voorkoming van deze ziekte.

Chapter 8.1

Acknowledgements - Dankwoord

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Chapter 8.2

PhD portfolio

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Gabriëlle H.S. Buitendijk
Departments: Ophthalmology / Epidemiology
Research School: Netherlands Insititute for Health Sciences
PhD-period: 2010-2017
Supervisors: Prof. dr. C.C.W. Klaver and Prof. dr. J.R. Vingerling

PhD Training	Year	Workload (ECTS*)
Courses		
Master of Health Sciences, Genetic Epidemiology (NIHES)	2010-2012	70
Workshop on Photoshop and Illustrator CS5 (MolMed)	2011	0.3
Workshop on InDesign CS5 (MolMed)	2011	0.2
Biomedical English Writing and Communication (David Alexander)	2011	4.0
Research Integrity (prof dr. S. van de Vathorst)	2015	0.3
Seminars, symposia and workshops		
Research seminars, department of Epidemiology, Erasmus MC	2010-2014	4.5
International Course Genetics in Retinal Disease, Rotterdam	2010	0.3
Myopia workshop for opticians and optometrists, Rotterdam	2011	0.3
AMD workshop for general practitioners, Rotterdam	2011	0.3
Brainstorm session Nanotechnology, Rotterdam	2011	0.3
1st European Eye Epidemiology Workshop, Bordeaux, France (oral presentation)	2011	1.0
2nd annual meeting International AMD Genetics Consortium, Bethesda, USA (oral presentation)	2011	1.0
2nd ErasmusAge & SIGN-E Workshop Basic Principles of nutritional Epidemiology, Rotterdam	2012	0.3
ARVO-NED, Utrecht (oral presentation)	2011	1.0
2nd European Eye Epidemiology Workshop, Bordeaux, France (oral presentation)	2012	1.0
CHARGE investigators meeting 2013, Rotterdam	2013	0.3
International Course Genetics in Retinal Disease, Ghent, Belgium	2013	0.3
3rd European Eye Epidemiology Workshop, Bordeaux, France (oral presentation)	2013	1.0
Patient day Juvenile Macular degeneration, Nijmegen (oral presentation)	2013	1.0
Exome chip meeting International AMD Genetics Consortium, Miami, USA	2013	0.3
ARVO-NED, Utrecht	2013	0.3
Masterclass for clinical PhD students on Therapies for Inherited Retinal Dystrophies, Nijmegen	2014	0.3
Symposium on Novel Therapies for Inherited Retinal Dystrophies, Nijmegen	2014	0.3
2020 meeting of the department of Epidemiology, Erasmus MC, Rotterdam (oral presentation)	2014	1.0
4th European Eye Epidemiology Workshop, Rome, Italy (oral presentation)	2014	1.0
Exome chip meeting International AMD Genetics Consortium, Regensburg, Germany (oral presentation)	2014	1.0
Fundus autofluorescence workshop, Rotterdam	2014	0.3
5th European Eye Epidemiology Workshop, London, UK	2015	0.3

PhD Training	Year	Workload (ECTS*)
National conferences		
15th Molecular Medicine Day, Rotterdam	2010	0.3
NOG Annual Meeting 2011, Maastricht (oral presentation)	2011	1.0
NOG Annual Meeting 2012, Groningen (oral presentation)	2012	1.0
NOG Annual Meeting 2013, Groningen (oral presentation)	2013	1.0
2nd Dutch Ophthalmology PhD Day, Nijmegen (oral presentation)	2013	1.0
NOG Annual Meeting 2014, Maastricht (oral presentation)	2014	1.0
3rd Dutch Ophthalmology PhD Day, Nijmegen (oral presentation)	2014	1.0
NOG Annual Meeting 2015, Groningen (oral presentation)	2015	1.0
NOG Annual Meeting 2016, Maastricht (oral presentation)	2016	1.0
International conferences		
ARVO Annual Meeting 2011, Fort Lauderdale, USA (poster presentation)	2011	1.0
V. International Symposium of the German Ophthalmology Society (DOG), Baden-Baden, Germany (oral presentation)	2011	1.0
Macula of Paris, 6th international conference on AMD, Paris, France (oral presentation)	2012	1.0
ARVO Annual Meeting 2012, Fort Lauderdale, USA (poster presentation)	2012	1.0
ARVO Annual Meeting 2013, Seattle, USA (poster presentation)	2013	1.0
ARVO Annual Meeting 2014, Orlando, USA (oral presentation)	2014	1.0
14th Euretina Meeting 2014, London, UK (oral presentation)	2014	1.0
European University Professors of Ophthalmology (EUPO), Nice, France (oral presentation)	2014	1.0
ARVO Annual Meeting 2015, Denver, USA (oral presentation)	2015	1.0
6th Euretina Winter Meeting, Rotterdam	2016	0.3
ARVO Annual Meeting 2016, Seattle, USA (oral presentation)	2016	1.0
ARVO Annual Meeting 2017, Baltimore, USA (poster presentation)	2017	1.0
Teaching		
Supervising research internship, Ashley Wills	2011	0.4
Supervising master's thesis, Sheila Backus	2014-2017	3.0
Other		
Founder and co-organizer of the 1st Dutch Ophthalmology PhD Conference, Nijmegen	2012	3.0
Chair of AMD session, ARVO Annual Meeting 2014, Orlando, USA	2015	0.1
Chair of AMD session, 6th Euretina Winter Meeting, Rotterdam	2016	0.1
Moderator AMD poster session, ARVO Annual Meeting 2016, Seattle, USA	2016	0.1

* 1 ECTS (European Credit Transfer System) equals a workload of 28 hours.

Chapter 8.3

Biblography

PUBLICATIONS AND MANUSCRIPTS ON WHICH THIS THESIS IS BASED

1. **Buitendijk GHS***, Springelkamp S*, Ho L, Klaver CC (2013) Age-related macular degeneration and primary open-angle glaucoma: genetics and gene-environment interaction. In: Scholl HP, Massof RW, West S (Eds), Ophthalmology and the ageing society, (pp57-82). Berlin Heidelberg: Springer [Chapter 5.1]
2. Fritsche LG*, Chen W*, Schu M*, Yaspan BL*, Yu Y*, Thorleifsson G, Zack DJ, Arakawa S, Cipriani V, Ripke S, Igo RP Jr, **Buitendijk GHS**, Sim X, Weeks DE, Guymer RH, Merriam JE, Francis PJ, Hannum G, Agarwal A, Armbrecht AM, Audo I, Aung T, Barile GR, Benchaboune M, Bird AC, Bishop PN, Branham KE, Brooks M, Brucker AJ, Cade WH, Cain MS, Campochiaro PA, Chan CC, Cheng CY, Chew EY, Chin KA, Chowers I, Clayton DG, Cojocaru R, Conley YP, Cornes BK, Daly MJ, Dhillon B, Edwards AO, Evangelou E, Fagerness J, Ferreyra HA, Friedman JS, Geirsdottir A, George RJ, Gieger C, Gupta N, Hagstrom SA, Harding SP, aritoglou C, Heckenlively JR, Holz FG, Hughes G, Ioannidis JP, Ishibashi T, Joseph P, Jun G, Kamatani Y, Katsanis N, N Keilhauer C, Khan JC, Kim IK, Kiyohara Y, Klein BE, Klein R, Kovach JL, Kozak I, Lee CJ, Lee KE, Lichtner P, Lotery AJ, Meitinger T, Mitchell P, Mohand-Saïd S, Moore AT, Morgan DJ, Morrison MA, Myers CE, Naj AC, Nakamura Y, Okada Y, Orlin A, Ortube MC, Othman MI, Pappas C, Park KH, Pauer GJ, Peachey NS, Poch O, Priya RR, Reynolds R, Richardson AJ, Ripp R, Rudolph G, Ryu E, Sahel JA, Schaumberg DA, Scholl HP, Schwartz SG, Scott WK, Shahid H, Sigurdsson H, Silvestri G, Sivakumaran TA, Smith RT, Sobrin L, Souied EH, Stambolian DE, Stefansson H, Sturgill-Short GM, Takahashi A, Tosakulwong N, Truitt BJ, Tsironi EE, Uitterlinden AG, van Duijn CM, Vijaya L, Vingerling JR, Vithana EN, Webster AR, Wichmann HE, Winkler TW, Wong TY, Wright AF, Zelenika D, Zhang M, Zhao L, Zhang K, Klein ML, Hageman GS, Lathrop GM, Stefansson K, Allikmets R, Baird PN, Gorin MB, Wang JJ, Klaver CC, Seddon JM, Pericak-Vance MA, Iyengar SK, Yates JR, Swaroop A, Weber BH, Kubo M, Deangelis MM, Léveillard T, Thorsteinsdottir U, Haines JL, Farrer LA, Heid IM, Abecasis GR; AMD Gene Consortium. Seven new loci associated with age-related macular degeneration. Nat Genet. 2013 Apr;45(4):433-439. [Chapter 5.2]
3. **Buitendijk GHS**, Rochtchina E, Myers C, van Duijn CM, Lee KE, Klein BE, Meuer SM, de Jong PT, Holliday EG, Tan AG, Uitterlinden AG, Sivakumaran TA, Attia J, Hofman A, Mitchell P, Vingerling JR, Iyengar SK, Janssens AC, Wang JJ, Klein R, Klaver CC. Prediction of age-related macular degeneration in the general population: the Three Continent AMD Consortium. Ophthalmology. 2013 Dec;120(12):2644-2655. [Chapter 6.1]
4. **Buitendijk GHS**, Klaver CC (2014) Trace elements, Vitamins, and Lipids and Age-related Macular Degeneration: An overview of the current concepts on nutrients and AMD. In: Preedy VR (Ed.), Handbook of nutrition, diet, and the eye, (pp55-63). Waltham, MA: Elsevier [Chapter 4.1]
5. Wang JJ, **Buitendijk GHS**, Rochtchina E, Lee KE, Klein BE, van Duijn CM, Flood VM, Meuer SM, Attia J, Myers C, Holliday EG, Tan AG, Smith WT, Iyengar SK, de Jong PT, Hofman A, Vingerling JR, Mitchell P, Klein R, Klaver CC. Genetic susceptibility, dietary antioxidants, and long-term incidence of age-related macular degeneration in two populations. Ophthalmology. 2014 Mar;121(3):667-675. [Chapter 5.3]
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9. Chaker L*, **Buitendijk GHS***, Dehghan A, Medici M, Hofman A, Vingerling JR, Franco OH, Klaver CC, Peeters RP. Thyroid function and age-related macular degeneration: a prospective population-based cohort study--the Rotterdam Study. BMC Med. 2015 Apr 23;13:94. [Chapter 4.3]
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Chapter 8.4

About the author

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