Environmental and Genetic Risk Factors for Aging Macula Disorder

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Externe en genetische risicofactoren van ouderdoms macula aandoening

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Chapter 1

Introduction

INTRODUCTION

What's in a name? Since the first description of age-related macular degeneration in 1874 as "Symmetrical central choroido-retinal disease occurring in senile persons",[1] over 20 different names have been given to this disorder, often reflecting the pathophysiological thinking at a certain time. Because there was need for an internationally accepted classification, the International ARM Epidemiological Study Group named all signs of age-related macular changes age-related maculopathy (ARM), and its end stages, age-related macular degeneration (AMD) following the convention at that time.[2] Both the laymen and the medical public found these two names confusing, so gradually all ARM was called AMD, to be divided in early and late AMD. Recently it was proposed to use AMD as an acronym for "Aging Macula Disorder", essentially the same disease as age-related macular degeneration, for the following reasons.[3] Age-related does not differentiate between juvenile macular disease and that associated with old age. Aging better describes the process of becoming older. Although AMD is a complex disorder with a large genetic component, the lifelong changes in the retinal pigment epithelium (RPE) and surrounding tissues in the macula seem a key component in its pathogenesis. Patients do not like to hear associations with senility anymore, nor with degeneration. Finally, it is not clear if and when early or late AMD becomes a disease, therefore we prefer calling it a disorder. Thus we opted for using aging macula disorder as the basis for AMD in this thesis.

Aging macula disorder (AMD) is a progressive disorder affecting the central part of the retina, the macula lutea. The macula is the specialized region of the retina responsible for the ability to see fine details. Deterioration of the macular area, and essentially its central point, the fovea, impairs contrast sensitivity and thus the ability of patients to conduct a wide range of activities, including recognizing familiar faces, reading, watching television, driving, and cycling. Most of these activities only can be performed with a visual acuity above 0.2 or 0.3. Because the visual acuity drops fast, the more eccentric to the fovea one fixates, we call this acuity range central visual acuity, in contrast to the lower visual acuity in the periphery of and outside the macula. AMD patients have impaired central vision, and because peripheral vision usually remains intact, AMD does not cause total blindness when defined as loss of all light perception. This can make it difficult for family and friends of patients to understand why patients can navigate through a room effortlessly, even though they cannot read or drive. The impact of this visual handicap can be huge, especially in case of rapid visual acuity loss that may occur in the wet or neovascular type of late AMD. Patients and their partners need to deal with the emotional shock that rapid loss of vision will probably bring, and reorganize their way of living.

The diagnosis of AMD usually is made by funduscopy, looking at the inner posterior part of the eye. Based on fundus signs AMD can be divided into early and late AMD. Early AMD is characterized by the appearance of white subretinal deposits, so-called drusen, and pigmentary abnormalities in the RPE. Most patients with early AMD are asymptomatic, although some psychophysical tests, for example recovery time after a strong light stimulus, are abnormal.[4] Late AMD can be subdivided in two distinct end stages, dry AMD and wet AMD, and is associated with severe central visual loss. Dry AMD is characterized by degeneration of the RPE, leading to a loss of photoreceptors. In cases of wet AMD new blood vessels form under the retina, leading to RPE detachment or sub retinal hemorrhage, resulting in fibrovascular disciform scarring in the macular area. Severe vision loss in dry AMD usually progresses over the course of years, while patients with wet AMD may lose central vision overnight. Based on our data, we estimated that of the more than 4 million persons over 55 years living in the Netherlands,[5] approximately 400.000 (9.2%) persons have early AMD and more than 70.000 (1.7%) have late AMD. It has been predicted that in our rapidly aging population, by 2020 the number of blind persons due to AMD will double unless better prevention and therapy will be found.[6] The fast growing burden to public health makes it important to identify strategies for this disorder. Although AMD is now the leading cause of incurable blindness in the Western world,[7-9] there is a low public awareness of AMD. It is essential to inform the population about primary prevention and symptoms of especially beginning wet AMD because for the latter new therapeutic strategies became available.[10] AMD is considered to be a multifactorial disease, which means that both environmental and genetic factors play a role in its development. The pathogenesis of AMD is not yet elucidated, however several epidemiological studies have tried to shed light on various factors associated with AMD. As indicated by the name, age is a strong risk factor and the prevalence and incidence of AMD rise steeply with increasing age. The most consistent modifiable risk factor found for AMD is smoking.[11] Other risk factors include atherosclerosis, a diet low in antioxidants, zinc and fatty fish, and potentially hypertension, high cholesterol levels, and sunlight exposure.[12] Over the years, twin studies, research into familial aggregation and segregation analyses have provided compelling evidence for a strong role of genetics in AMD.[13] Recently, a major risk variant was identified in the complement factor H gene. This Y402H single nucleotide polymorphism (SNP) in the complement factor H gene is thought to explain between 24 and 54% of all AMD cases.[3] It has been reported that blacks have a lower prevalence of AMD than whites.[14, 15] However, *CFH* genotype frequencies are similar in blacks and whites, suggesting that other, yet unidentified, genetic or environmental risk factors are important in the pathogenesis of AMD. The search for genetic risk factors for AMD is rapidly progressing, and recent studies have

suggested that SNPs in the *LOC387715*, *C2/FB* and *HTRA1* genes are also strongly associated with AMD.[16-19]

The aim of the epidemiological studies described in this thesis was to identify external and genetic factors that are involved in the pathogenesis of AMD. All studies were performed within the context of the Rotterdam Study, a large-scale population-based prospective cohort study among 7983 participants (response rate 78%) aged 55 years and older living in a suburb of Rotterdam. In epidemiologic studies we prefer not to speak of patients but of persons or participants. **Chapter 2** presents studies on environmental risk factors for AMD. Because recent evidence has shown a possible benefit of antioxidant and zinc intake in reducing loss of visual function due to AMD,[20] we investigated whether dietary intake of antioxidant micronutrients was associated with a decreased risk of AMD (**chapter 2.1**). It has been suggested that inflammation plays a role in the pathogenesis of AMD,[21] therefore we assessed the association between serum C-reactive protein levels and AMD (**chapter 2.2**). We also examined the association between alcohol consumption and the risk of AMD (**chapter 2.3**), a possible modifiable risk factor. **Chapter 3** contains studies on the association between genetic polymorphisms and AMD. Some studies proposed a protective effect of estrogen on AMD,[22] subsequently we studied a genetic variation in the estrogen receptor 1 gene (**chapter 3.1**). Growth factors have been implicated in the pathogenesis of AMD and especially in wet AMD.[23, 24] For this reason we examined genetic variations in the insulin-like growth factor gene 1 (**chapter 3.2**) and in the vascular endothelial growth factor gene (**chapter 3.3**). Finally in **chapter 4**, we reflect on our main findings, discuss relevant methodological aspects, and provide suggestions for further research.

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Chapter 2

Environmental risk factors

2.1

Dietary intake of antioxidants and risk of aging macula disorder

ABSTRACT

Context: Aging macula disorder (AMD) is the most prevalent cause of irreversible blindness in the developed countries. Recently, high-dose supplementation with beta carotene, vitamins C and E, and zinc was shown to slow the progression of AMD.

Objective: To investigate whether regular dietary intake of antioxidants is associated with a lower risk of AMD.

Design: Dietary intake was assessed at baseline in the Rotterdam Study (1990-1993) using a semiquantitative food frequency questionnaire. Incident AMD until final follow-up in 2004 was determined by grading fundus color transparencies in a masked way according to the International Classification and Grading System.

Setting: Population-based cohort study of all inhabitants aged 55 years or older in a middle-class suburb of Rotterdam, the Netherlands.

Participants: Of 5836 persons at risk of AMD at baseline, 4765 had reliable dietary data, and 4170 participated in the follow-up.

Main outcome measure: Incident AMD, defined as soft distinct drusen with pigment alterations, indistinct or reticular drusen, geographic atrophy, or choroidal neovascularization.

Results: Incident AMD occurred in 560 participants after a mean follow-up of 8.0 years (range, 0.3-13.9 years). Dietary intake of both vitamin E and zinc was inversely associated with incident AMD. The hazard ratio (HR) per standard deviation increase of intake for vitamin E was 0.92 (95% confidence interval (CI), 0.84 - 1.00) and for zinc was 0.91 (95% CI, 0.83- 0.98). An above-median intake of all 4 nutrients, beta carotene, vitamin C, vitamin E, and zinc, was associated with a 35% reduced risk (HR, 0.65; 95% CI, 0.46 - 0.92) of AMD. Exclusion of supplement users did not affect the results.

Conclusions: In this study, a high dietary intake of beta carotene, vitamins C and E, and zinc was associated with a substantially reduced risk of AMD in elderly persons.

INTRODUCTION

Aging macula disorder (AMD) is a degenerative disorder of the macula, the central part of the retina. Late AMD results in an inability to read, recognize faces, drive, or move freely. Early AMD is the subclinical stage of the disease and can be diagnosed by funduscopy. The prevalence of late AMD steeply increases with age, affecting 11.5% of white persons older than 80 years.[1] In the absence of effective treatment for AMD, the number of patients severely disabled by late AMD is expected to increase in the next 20 years by more than 50% to 3 million in the United States alone.[1]
The pathophysiology of AMD is still poorly understood, and AMD may in fact be a constellation of diseases with different causes. As in other age-related disorders, oxidative stress has been implicated in the etiology of AMD.[2] The retina seems particularly susceptible to oxidative stress because of its high concentration of oxygen, polyunsaturated fatty acids, and photosensitizers, in combination with an intense exposure to light.[3] Epidemiological studies evaluating both dietary intake and serum levels of antioxidant vitamins and AMD have provided conflicting results.[4-7] In the randomized, placebo-controlled Age-Related Eye Disease Study (AREDS), supplements containing 5 to 13 times the recommended daily allowance (RDA) of beta carotene, vitamins C and E, and zinc given to participants from retinal clinics with early or monocular late AMD resulted in a 25% reduction in the 5-year progression to late AMD.[8] We sought to investigate whether antioxidants, as present in normal daily foods, may play a role in the primary prevention of AMD.

METHODS

Study population

The Rotterdam Study is a population-based, prospective cohort study of the frequency and determinants of common cardiovascular, locomotor, neurologic, and ophthalmologic diseases.[9, 10] The eligible population comprised all 10,275 inhabitants aged 55 years or older of a middle-class suburb of Rotterdam, the Netherlands, of whom 7,983 (78%) participated. Because the ophthalmologic part of the study became operational after the pilot phase of the study had started, 6,780 (66%) took part in the ophthalmic examinations. A baseline home interview and examinations at the study center were performed from 1990 to mid 1993, followed by a first follow-up examination from 1993 to 1994, a second from mid 1997 to the end of 1999, and a third examination from 2000 to the end of 2004. Written informed consent was obtained from all participants. The medical ethics committee of Erasmus University approved the study protocol.

Diagnosis of AMD

The eye examination included 35° fundus photography (Topcon TRV-50VT fundus camera, Topcon Optical Co, Tokyo, Japan) after pharmacological mydriasis.[10] Transparencies were graded with 12.5x magnification according to the International Classification and Grading System.[11] Two experienced graders, masked to dietary intake, graded the follow-up transparencies and afterward compared these with the baseline ones. The grading procedures, definitions, and graders were identical at baseline and follow-up. Consensus sessions and between-grader comparisons were performed regularly. Weighted kappa values were 0.72 for soft distinct drusen, 0.80 for hyperpigmentation, and 0.58 for hypopigmentation.

Early AMD was defined as the presence of either large (≥ 63 µm), soft, distinct drusen with pigment irregularities or indistinct (≥ 125 µm) or reticular drusen with or without pigment irregularities. Drusen are white deposits in the retina that are considered to be the hallmark of early AMD and are important predictors of late AMD.[10, 12] Late AMD, mostly leading to blindness, was defined as dry (geographic atrophy) and wet (neovascular) AMD, or a combination of both.[11]

Dietary assessment

At baseline, participants completed a checklist at home that queried foods and drinks they had consumed at least twice a month during the preceding year as well as dietary habits, use of supplements, and prescribed diets. Next, during their visit to the research center, they underwent a standardized interview by a dietitian based on the checklist, using a 170-item semi-quantitative food frequency questionnaire.[13, 14] A validation study comparing this questionnaire with a 2-week food diary demonstrated reproducible and valid estimates.[13, 14] These dietary data were converted to total energy intake and nutrient intake per day with the computerized Dutch Food Composition Table.[15] For the current study, we selected the carotenoids alpha and beta carotene, beta cryptoxanthin, lutein/zeaxanthin, lycopene, vitamins A (retinol equivalents), C and E, and iron and zinc as cofactors for antioxidant enzymes. Persons, who reported taking supplements containing carotenoids, vitamins A, C, or E, iron, or zinc, as well as multivitamins or multiminerals, were classified as supplement users.

Assessment of confounders

Information on potential confounders was collected at baseline. Smoking status was categorized as current, former or never, and number of pack-years was calculated. Serum total cholesterol level was measured in nonfasting blood samples with an automated enzymatic procedure. Blood pressure was defined as the mean of 2 measurements in sitting position at the right brachial artery with a random zero

sphygmomanometer. The ankle-arm index was calculated by taking the ratio of the systolic blood pressure at the ankle to the systolic blood pressure at the arm, using the lowest ratio of both legs. Carotid intima-media thickness and atherosclerotic plaques were assessed ultrasonographically and aortic calcifications on lateral radiographic films of the lumbar spine. A sub clinical atherosclerosis composite score (range, 1-4) was constructed by summing points for the population-based deciles of carotid wall thickness and ankle-arm index, with points added for the presence of carotid plaques and aortic calcifications.[16]

Study sample

The cohort at risk consisted of 5836 persons with no AMD in either eye at baseline; i.e. with no drusen or pigment irregularities, hard drusen only, or soft drusen without pigment irregularities. Incidence of AMD was defined as the presence of early or late AMD in at least 1 eye at 1 of the follow-up examinations. Persons with incident AMD (iAMD) were compared with those with no AMD at baseline and no AMD at any follow-up examinations.

Dietary intake was not assessed in 227 participants with decreased cognitive function (defined as a score < 80 on the Cambridge Examination of Mental Disorders in the Elderly),[17] because their dietary history was deemed unreliable. We also excluded 179 nursing home residents because their food was prepared by nursing home staff and would not reflect past dietary habits. Reliable dietary data were missing in 665 participants because of logical inconsistencies in dietary interviews, to missing the baseline dietitian visit when the food-frequency questionnaire was administered, or to various other logistical reasons. Baseline characteristics were similar in the 2 groups, although eligible respondents without dietary data were, on average, somewhat older compared with those with data and included fewer women.

Of this baseline cohort, 156 participants died, 419 refused any follow-up examination, and 20 were lost to follow up before the first follow-up examination. Nonparticipants tended to be older; included more women, nursing home residents, and smokers; and more often had systemic hypertension. They did not differ from participants in their dietary intake of antioxidants; e.g., vitamin E ($p = 0.75$) or zinc ($p = 0.69$). The study sample thus consisted of 4170 participants who had normal cognition, lived independently, had reliable dietary assessment and gradable fundus transparencies, and participated in at least 1 follow-up examination.

Data analysis

We adjusted the dietary intake of antioxidant nutrients for the total energy intake by means of the residual method described by Willett.[18] For each nutrient, linear regression analysis was performed with antioxidant intake as the dependent variable and

total energy intake as the independent variable. This regression equation was used to calculate the expected mean antioxidant intake of the study population for the mean total energy intake of the study population. Next, for each individual, the energy-adjusted intake was calculated by adding the expected mean antioxidant intake of the study population to the residual derived from the regression analysis.
We estimated the risk of AMD associated with the dietary intake of antioxidant nutrients at baseline with Cox proportional hazards regression analysis. Intake of each nutrient was entered into the model either as a linear term per standard deviation (SD) or as a dummy variable representing 1 of the 3 highest quartiles. Quartiles were analyzed both as a categorical and as a continuous variable to test for trend. Quartiles and SDs were based on the distribution within the study sample. We adjusted for age, sex, body mass index, smoking status, pack-years of smoking, systolic blood pressure, serum total cholesterol, composite atherosclerosis score, and alcohol intake in all analyses. We additionally adjusted for intake of polyunsaturated fat in the analysis of the fat-soluble vitamin E because of a reported association between this fat and AMD.[19] Missing values of categorical variables were represented in the model by a missing indicator. For continuous variables, missing values were replaced by the mean or median of the study sample, depending on the distribution. Only the atherosclerosis composite score had more than 1% of the data missing (12.7%). To distinguish between the effect of antioxidants from food and from supplements, all analyses were repeated after exclusion of supplement users at baseline and also after adding supplement users to the highest quartile of dietary intake. Also, analyses were repeated after stratification for smoking status.
One of our aims was to study the regular dietary intake of the combination of nutrients that had been administered at a high dose in the AREDS.[8] To secure large-enough groups with a relatively high or low intake of each of the 4 nutrients, we used the median intake per nutrient, based on the total sample, as the cutoff value. The high-intake group consisted of persons with an above median intake of each of the 4 nutrients. The low-intake group had a below-median intake of each nutrient, and all persons in between were considered the reference category.
Associations are presented as hazard ratios (HRs) with 95% confidence intervals (CIs). All analyses were performed using SPSS, release 11.0 (SPSS Inc, Chicago, Ill).

RESULTS

Mean follow-up of participants was 8.0 years, with a range of 0.3 to 13.9 years (median, 10.6 years). During this period, 560 persons (13.4%) were diagnosed as having iAMD, the majority of whom had early AMD. Persons with early iAMD had

either large, soft drusen with pigment irregularities (n = 317) or indistinct drusen without (n = 124) or with (n = 77) pigment irregularities. Of the 42 persons with late iAMD, 14 had dry and 28 wet AMD. Twelve of them had AMD at the second follow-up examination while 30 did so at the third follow-up. The incidence of AMD in the study sample was similar to the incidence in those with missing data on dietary intake who were not included in the sample ($p = 0.60$, adjusted for age and sex). Baseline characteristics of persons with iAMD as well as the remainder of the cohort are presented in Table 1. The mean age was 68.2 for the incident cases compared to 66.4 years for the remainder ($p < 0.001$). Persons with iAMD reported more pack-years of cigarette smoking ($p = 0.04$) and had a higher serum high-density lipoprotein cholesterol level ($p = 0.02$). Other baseline characteristics were not different in the 2 groups.

Table 2 shows the mean daily dietary intake of the antioxidant nutrients in the study sample, adjusted for total energy intake. In Table 3, the risk of AMD in relation to nutrient intake is presented. A significant inverse association was observed for intake of vitamin E, iron, and zinc. After adjustment, a 1 SD increase in intake was

Table 1. Baseline characteristics of the study sample (n = 4170)*

Characteristics	Incident AMD (n = 560)		No AMD (n = 3610)		p-value†
Age, y	68.2	(7.1)	66.4	(7.2)	< 0.001
Sex, female number (%)	321	(57.3)	2151	(59.6)	0.31
Body mass index‡	26.3	(3.5)	26.4	(3.6)	0.66
Smoking status number (%)					
Never	183	(32.8)	1207	(33.6)	
Former	248	(44.4)	1561	(43.4)	0.36
Current	127	(22.8)	825	(23.0)	0.55
Pack-years of smoking	18.5	(23.8)	16.4	(21.8)	0.04
Systolic blood pressure, mm Hg	138.9	(20.3)	137.1	(21.5)	0.76
Diastolic blood pressure, mm Hg	73.4	(10.7)	73.9	(11.0)	0.56
Total cholesterol, mmol/l	6.6	(1.2)	6.7	(1.2)	0.93
HDL cholesterol, mmol/l	1.4	(0.4)	1.4	(0.4)	0.02
Atherosclerosis composite score§	2.7	(1.1)	2.5	(1.1)	0.21
Alcohol intake, g/d	10.8	(15.3)	10.6	(15.3)	0.20
Number of antioxidant supplement users (%)	60	(10.7)	499	(13.8)	0.09

* Data are expressed as mean (SD) unless otherwise indicated

† Adjusted for age and sex

‡ Body mass index was calculated as weight in kilograms divided by the square of height in meters

§ Information on the atherosclerosis composite score is presented in the "Methods" section of the text

Table 2. Mean dietary intake within each quartile of intake in the total study sample (n = 4170).

	Quartiles							
	1		2		3		4	
	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)
Carotenoids								
Alpha carotene	≤ 0.7	0.5 (0.2)	> 0.7 - ≤ 1.0	0.9 (0.01)	> 1.0 - ≤ 1.4	1.2 (0.1)	> 1.4	2.0 (1.2)
Beta carotene	≤ 2.7	2.1 (0.6)	> 2.7 - ≤ 3.6	3.2 (0.2)	> 3.6 - ≤ 4.6	4.0 (0.3)	> 4.6	6.2 (0.3)
Beta cryptoxanthin	≤ 0.1	0.05 (0.04)	> 0.1 - ≤ 0.3	0.2 (0.04)	> 0.3 - ≤ 0.4	0.3 (0.04)	> 0.4	0.6 (0.2)
Lutein/Zeaxanthin	≤ 1.8	1.4 (0.3)	> 1.8 - ≤ 2.2	2.0 (0.1)	> 2.2 - ≤ 2.8	2.5 (0.2)	> 2.8	3.6 (1.3)
Lycopene	≤ 0.3	0.1 (0.07)	> 0.3 - ≤ 0.7	0.5 (0.1)	> 0.7 - ≤ 1.1	0.8 (0.1)	> 1.1	1.8 (0.8)
Vitamins								
Vitamin A (retinol equivalents)	≤ 0.6	0.5 (0.1)	> 0.6 - ≤ 0.8	0.7 (0.03)	> 0.8 - ≤ 0.9	0.8 (0.04)	> 0.9	1.2 (0.5)
Vitamin C	≤ 84.5	63.7 (15.5)	> 84.5 - ≤ 113.6	99.4 (8.2)	> 113.6 - ≤ 146.1	128.4 (9.2)	> 146.1	189.3 (46.6)
Vitamin E	≤ 9.9	7.6 (1.9)	> 9.9 - ≤ 12.8	11.4 (0.8)	> 12.8 - ≤ 16.2	14.4 (1.0)	> 16.2	20.2 (4.1)
Trace elements								
Iron	≤ 10.7	9.5 (1.0)	> 10.7 - ≤ 11.9	11.3 (0.4)	> 11.9 - ≤ 13.3	12.6 (0.4)	> 13.3	14.8 (1.6)
Zinc	≤ 8.3	7.3 (0.9)	> 8.3 - ≤ 9.6	9.0 (0.4)	> 9.6 - ≤ 10.9	10.2 (0.4)	> 10.9	12.3 (1.4)

Table 3. Risk of aging macula disorder per standard deviation (SD) increase in dietary intake of antioxidant nutrients.

Nutrients	Mean dietary intake (SD), mg/d	Adjusted Hazard Ratio per 1-SD Increase (95% confidence interval)*	
Carotenoids			
Alpha carotene	1.12 (0.84)	0.99	(0.94 - 1.06)
Beta carotene	3.84 (2.23)	1.00	(0.94 - 1.06)
Beta cryptoxanthin	0.29 (0.22)	1.01	(0.92 - 1.10)
Lutein/Zeaxanthin	2.37 (1.08)	1.01	(0.93 - 1.09)
Lycopene	0.80 (0.80)	1.01	(0.97 - 1.04)
Vitamins			
Vitamin A (retinol equivalents)	0.82 (0.35)	0.95	(0.86 - 1.05)
Vitamin C	120.20 (52.49)	1.02	(0.94 - 1.10)
Vitamin E	13.42 (5.19)	0.92	(0.84 - 1.00)
Trace elements			
Iron	12.04 (2.16)	0.95	(0.86 - 1.04)
Zinc	9.67 (2.01)	0.91	(0.83 - 0.98)

* Adjusted for age, sex, body-mass index, smoking status, pack-years of smoking, systolic blood pressure, atherosclerosis composite score, serum total cholesterol, and alcohol intake.

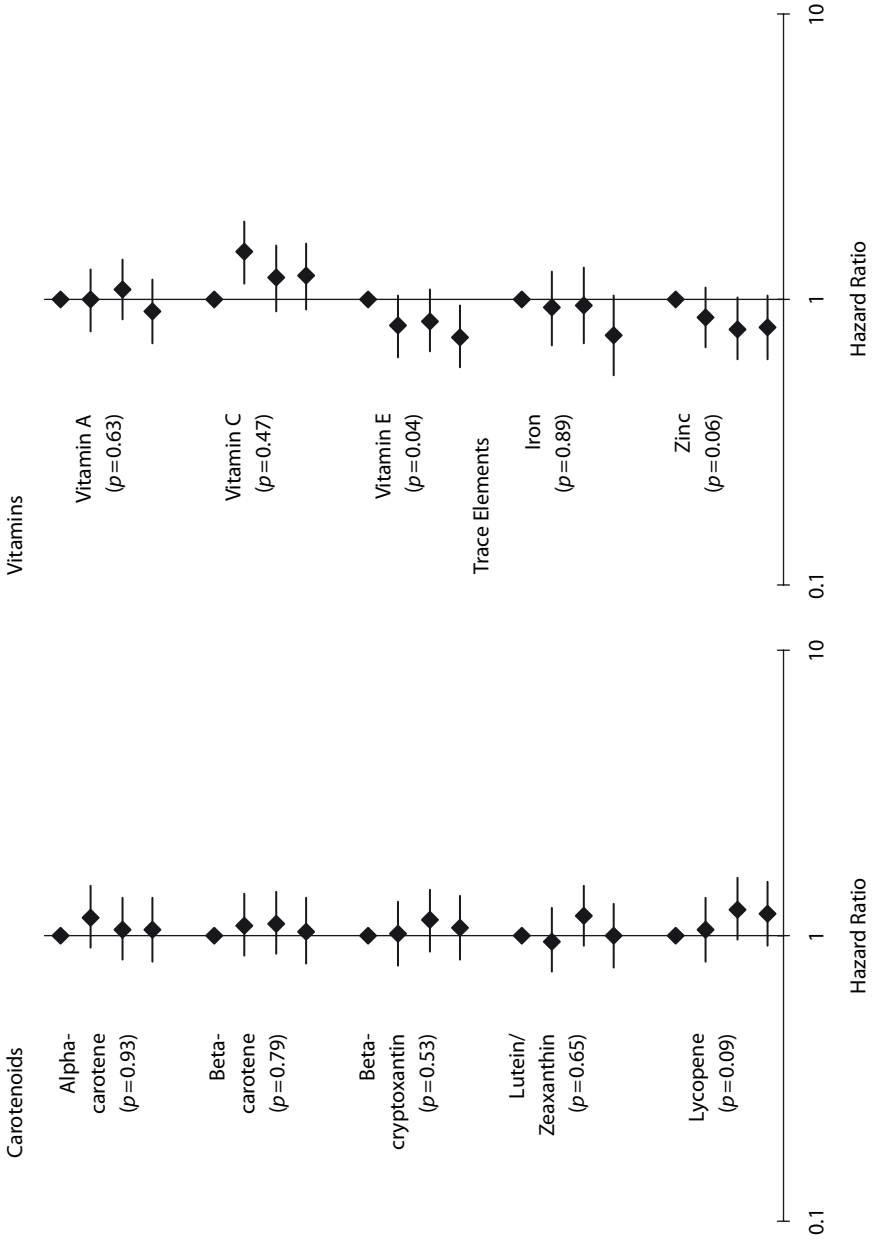
associated with a reduced risk of AMD of 8% (95% CI, 0%–16%) for vitamin E and 9% (95% CI, 2%-17%) for zinc.

The risk of AMD by quartiles of nutrient intake is presented in the Figure. The test for trend for both vitamin E ($p = 0.04$) and zinc ($p = 0.06$) intake indicated a dose-response relationship between vitamin E and zinc intake and reduced risk of AMD.

Table 4 presents the impact of the combined dietary intake of the 4 antioxidants that were studied in AREDS.[8] Intake of these nutrients in the present study was considerably lower than the high-dose supplements used in the AREDS. An above-median intake of beta carotene, vitamins C and E, and zinc, compared with a below-median intake of at least 1 of these nutrients, was associated with a reduced risk of AMD (HR, 0.65; 95% CI, 0.46-0.92) adjusted for all potential confounders. In persons with a below-median intake of all 4 nutrients, the risk of AMD was increased but not significantly so (HR 1.20; 95% CI, 0.92-1.56).

Exclusion of the 559 participants who used antioxidant supplements at baseline did not substantially alter the risk estimates (Table 5). In addition, adding supplement users to the highest quartile of dietary intake did not change the results (HR, adjusted for the same factors as in Table 5, 0.77; 95% CI, 0.61-0.98). Stratification for smoking status did not substantially change point estimates but widened the confidence intervals (Table 5).

Figure: Hazard ratios for incident aging macula disorder by quartile of energy-adjusted dietary intake of antioxidant carotenoids



Error bars indicate 95% confidence intervals. Adjusted for age, sex, body mass index, smoking status, pack-years of smoking, systolic blood pressure, atherosclerosis composite score, serum total cholesterol, and alcohol intake. The lowest quartile was considered the reference group. For each nutrient, the p-value of the test for trend is given.

Table 4. Risk of aging macula disorder by category of combined intake of 4 predefined antioxidant nutrients (vitamins C and E, beta carotene, and zinc).

	Category of dietary intake*		
	Low n = 466	Middle n = 3270	High n = 434
Cases (%)	76 (16.3)	442 (13.5)	42 (9.7)
Hazard ratio (95% confidence interval)			
Unadjusted	1.31 (1.03 - 1.67)	1.00	0.65 (0.48 - 0.89)
Age- and sex-adjusted	1.23 (0.97 - 1.58)	1.00	0.68 (0.49 - 0.93)
Fully adjusted†	1.20 (0.92 - 1.56)	1.00	0.65 (0.46 - 0.92)

* Categories were defined by using the median energy-adjusted intake per nutrient as a cutoff value and classifying above-median intake of all nutrients as high intake and below-median intake of all nutrients as low intake. Cutoff values were 114 mg for vitamin C, 13 mg for vitamin E, 3.6 mg for beta carotene, and 9.6 mg for zinc

† Adjusted for age, sex, body-mass index, smoking status, pack-years of smoking, systolic blood pressure, atherosclerosis composite score, serum total cholesterol, and alcohol intake

Table 5. Risk of aging macula disorder by category of combined intake of 4 predefined antioxidant nutrients (vitamins C and E, beta carotene, and zinc), excluding supplement users and stratified by smoking status.

	Categories of intake*		
	Low	Middle	High
Excluding supplement users	n = 419	n = 2816	n = 376
Cases (%)	67 (16.0)	396 (14.1)	37 (9.8
HR (95% CI)†	1.11 (0.84 - 1.46)	1.00	0.62 (0.43 - 0.90)
Smoking Status			
Never	n = 128	n = 1096	n = 160
Cases (%)	17 (13.3)	144 (13.3)	16 (10.0)
HR (95% CI)‡	0.85 (0.48 - 1.50)	1.00	0.74 (0.42 - 1.31)
Former	n = 182	n = 1434	n = 193
Cases (%)	31 (17.0)	199 (13.9)	18 (9.3)
HR (95% CI)‡	1.26 (0.84 - 1.99)	1.00	0.58 (0.34 - 0.98)
Current	n = 154	n = 727	n = 77
Cases (%)	28 (18.2)	97 (13.3)	8 (10.4)
HR (95% CI)‡	1.44 (0.93 - 2.22)	1.00	0.65 (0.30 - 1.40)

Abbreviations: CI indicates confidence interval; HR, hazard ratio

* Categories were defined by using the median energy-adjusted intake per nutrient as a cutoff value and classifying above-median intake of all nutrients as high intake and below-median intake of all nutrients as low intake. Cutoff values were 114 mg for vitamin C, 13 mg for vitamin E, 3.6 mg for beta carotene and 9.6 mg for zinc

† Adjusted for age, sex, body-mass index, smoking status, pack-years of smoking, systolic blood pressure, atherosclerosis composite score, serum total cholesterol, and alcohol intake

‡ Adjusted for all of the above except smoking status and pack-years of smoking

DISCUSSION

We found that high dietary intake of vitamin E and zinc was associated with a lower risk of iAMD. An above-median intake of the combination of vitamins C and E, beta carotene, and zinc was associated with a 35% lower risk of incident AMD.

The strengths of our study were the prospective design, the population-based cohort, the detailed and similar grading of AMD at baseline and follow-up, and the long follow-up. Potential weaknesses were, as in all observational studies, selection bias, information bias and confounding. Selective nonresponse was unlikely because non-participants did not differ from participants in the dietary intake of antioxidants. Bias in the diagnosis of AMD was minimized by the masked grading of photographs by persons unaware of the antioxidant nutrient status. Misclassification potentially could result from the use of only 1 food questionnaire at baseline, but such misclassification would be nondifferential and, therefore, more likely to underestimate the true associations. The questionnaire was not validated for all nutrients included in the current analysis; e.g., specific carotenoids and vitamin E. However, for other nutrients, the validity of the questionnaire was shown to be moderate to good. The adjusted Pearson correlation coefficient for vitamin A (including retinol and beta carotene) was 0.48; for vitamin C, 0.64; for iron, 0.42; and for zinc, 0.51.[13,14] For vegetables, the correlation was 0.39, and for fruit 0.60. Since alpha and beta carotene, and lutein intake are well correlated with total vegetable intake, we presumed equal validity. The same held for the correlation between beta cryptoxanthin and vitamin C. It is possible that other factors can explain the reported associations. Although we adjusted for known confounders, such as smoking and atherosclerosis, unknown factors associated with a healthy diet still may have played a role.

The median nutrient intake used as a cutoff value was at or above the RDA, so the majority of our population presumably consumed a healthy diet. A larger risk reduction was observed for dietary intake above the RDA for all 4 micronutrients than for individual micronutrients. To ensure that diet was the only source of antioxidant intake, we repeated the analysis excluding persons using antioxidant supplements at baseline (13.4%) and also investigated the combined effect of antioxidants from food and from supplements. This resulted in similar risk estimates. The independent association between antioxidant supplements and AMD could not be examined because of the relatively small number of antioxidant supplement users in our population and the lack of data on duration and dosage of use.

Recent data suggest that oxidative protein modifications may play a critical role in the formation of drusen.[20] This implies that antioxidants may have their strongest effect at the initiation of the disease. We studied a cohort that was free of clinical signs of early AMD at baseline, and our incident cases were primarily affected by

early AMD. Early AMD, however, is a strong predictor of late AMD.[10, 12] Exclusion of the 42 persons with late iAMD did not change the results. We therefore conclude that dietary antioxidants may delay the development of early and, possibly, of AMD in general.

Different antioxidants may act synergistically;[3] therefore, we studied the combined effect of nutrients and used the combination previously investigated in AREDS.[8] We observed a dose-response relationship with a mean intake of beta carotene, vitamins C and E, and zinc as reference. Persons with an above-median intake of these nutrients may be different in other aspects. This residual confounding is inherent to an observational study and can only be dealt with in a randomized trial. However, experimental studies with a randomized change in food consumption are difficult to perform.

Previous studies have shown variable degrees of protection against AMD by different antioxidants. Dietary intake of vitamin E was not associated with AMD in 1 case-control study in persons with wet late AMD[4] but showed an inverse association with large drusen in a population-based study.[7] A high intake of lutein and zeaxanthin was associated with a 40% lower risk of late AMD in 1 study[4] but history of intake of these 2 nutrients was not associated in another study.[7] A randomized controlled trial of vitamin E supplementation did not show an effect on the incidence of early AMD after 4 years of follow-up.[21] An inverse association between zinc intake and both prevalent and incident early AMD was reported in 1 population-based cohort study[22] but could not be confirmed in another similar study[23, 24] or in a pooled study in which late AMD and visual acuity data were obtained by self-report.[25] In contrast the aforementioned studies, our results were based on long-term follow-up of a large, population-based cohort with thorough baseline assessment of dietary intake.

Recently, a meta-analysis of 19 clinical trials including AREDS showed that high-dosage (≥ 400 IU/day) vitamin E supplementation may increase all-cause mortality.[26] This finding would challenge recommendations for supplement use.[8] It should be noted that most trials were performed in patients with chronic diseases, in contrast with the general population in our study sample. The mean amount of vitamin E consumed in diet in the highest quartile of our cohort (20.2 mg/d [30 IU/d]) was still considerably lower than high-dose supplementation, and the bioavailability of dietary antioxidants may be different from that in supplements. Dietary replacement also may be less expensive than supplement use.

This study suggests that the risk of AMD can be modified by diet; in particular, by dietary vitamin E and zinc. A higher intake of vitamin E can be achieved by consumption of whole grains, vegetable oil, eggs, and nuts. High concentrations of zinc can be found in meat, poultry, fish, whole grains, and dairy products. Carrots,

kale, and spinach are the main suppliers of beta carotene, while vitamin C is found in citrus fruits, and juices, green peppers, broccoli, and potatoes. Based on this study, foods high in these nutrients appear to be more important than nutritional supplements. Until more definitive data become available, this information may be useful to persons with signs of early AMD or to those with a strong family history of AMD.[27] Although in need of confirmation, our observational data suggest that a high intake of specific antioxidants from a regular diet may delay the development of AMD.

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2.2

C-reactive protein and risk of aging macula disorder

The Rotterdam Study

ABSTRACT

Context: Recently some clinic-based studies found an association between serum C-reactive Protein (CRP) levels and aging macula disorder (AMD).

Objective: To examine whether CRP levels are a risk factor for AMD in a general population.

Methods: We examined serum high sensitivity CRP (HsCRP) levels in 4914 participants of the population-based Rotterdam Study at risk for AMD. After a mean follow-up time of 7.7 years, 561 cases of early and 97 cases of late incident AMD (iAMD) were identified. We used Cox proportional hazards regression analysis to estimate hazard ratios and corresponding 95% confidence intervals (CIs).

Results: After adjustment for age and sex, hazard ratios for early iAMD were 1.11 (95% CI 1.02-1.21) per standard deviation increase of HsCRP and for late iAMD 1.28 (95% CI 1.02-1.60). Hazard ratios for early iAMD also increased per quartile increase of HsCRP level: 2nd quartile: 1.19 (95% CI 0.94-1.52), 3rd quartile: 1.29 (95% CI 1.01-1.64), 4th quartile: 1.33 (95% CI 1.05-1.70). The risk of late iAMD was also higher in all-upper quartiles of HsCRP.

Conclusion: Elevated baseline levels of HsCRP were associated with the development of early and late AMD in this large population-based cohort.

INTRODUCTION

Since its first description of age-related macular degeneration in senile persons[1], at least 20 different names have been given to this disease according to the views about its pathogenesis at the subsequent times. We now prefer the name "aging macula disorder" (AMD)[2] for the following reasons. Age-related does not differentiate between juvenile macular disease and that associated with old age, it is open for debate if and when early or late AMD becomes a disease, and patients do not want to be associated with senility or degeneration.

AMD is a condition affecting the center of the retina in the elderly. Late AMD is the main cause of incurable vision loss in the Western world[3-5] and its prevalence is estimated to double by 2020.[6] Its pathogenesis is still not clear although some modifiable risk factors as smoking and hypertension have been found.[7]

Local inflammatory and immune-mediated events play a role in the development of drusen, white subretinal extracellular deposits that are a hallmark of AMD.[8-10] Direct analysis by liquid chromatography and immunocytochemical analyses confirmed that drusen contain proteins associated with inflammation, such as complement components, C-reactive protein (CRP), fibrinogen and vitronectin.[11, 12] A number of these proteins appears to be locally produced by damaged RPE cells.[13] Also inflammatory cells such as multinucleated giant cells and leukocytes were described in the choroid of eyes with late AMD and in excised choroidal neovascular membranes.[14-17]

Chronic inflammation seems to be an etiological factor in the development of AMD. Several studies have investigated this possible association from different perspectives. A mouse model with defects in macrophage mobilization demonstrated many pathological features of AMD, suggesting that macrophage dysfunction plays a role in AMD.[18] Data from a case-control study found an association between antibodies against Chlamydia pneumoniae and wet (neovascular) late AMD.[19] In addition a modest association was found between pigmentary abnormalities and wet late AMD and emphysema, and gout was associated with the incidence of dry late AMD.[20]

Recently a strong association between the Y402H single nucleotide polymorphism (SNP) in the complement factor H (CFH) gene and AMD was found in three clinic-based case-control studies[21-23] and in a longitudinal population-based one.[24] CFH plays an essential role in the inhibition of the alternative complement pathway and abnormal regulation of this pathway leads to an increased inflammatory state.

CRP is a non-specific marker of systemic inflammation. It activates the classical route of complement activation directly and via cytokines through Fc-receptor binding by antibodies, which enhances the inflammatory respons.[12] Two clinic-based cross-sectional studies[25, 26] and a longitudinal clinical one by similar authors[27] reported an

association between CRP and AMD, supporting the inflammatory pathogenesis of AMD. We investigated whether baseline high sensitivity CRP (HsCRP) serum levels were also a risk factor for AMD in the general population.

METHODS

Population

The Rotterdam Study is a prospective, population-based cohort study investigating the incidence and determinants of chronic disabling diseases in the elderly. All inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands were invited.[28] Of the 10,275 eligible individuals, 7983 (78%) participated. The ophthalmologic part of the study started after screening of the participants had begun, leading to 6780 (also 78% response) ophthalmic participants. The tenets of the Declaration of Helsinki were followed and the appropriate medical ethics committees approved of the study. All participants signed an informed consent and gave permission to retrieve information from medical records. Baseline examinations, including a home interview and physical examinations at the research center took place from 1990 till 1993 and were followed by three examinations from 1993 to 1994 (response rate 88%), from 1997 to 1999 (response rate 80%) and from 2000 till the end of 2004 (response rate 74%).

Measurement of high sensitivity C-reactive protein

At baseline nonfasting blood was collected and processed by standard techniques, and stored at -20°C.[29] In 2003 and 2004, serum levels of HsCRP were determined by Rate Near Infrared Particle Immunoassay method (IMMAGE® high sensitive CRP, Beckman Coulter, USA). This system measures concentrations ranging from 0.2 to 1140 mg/L, with a within-run precision < 5.0%, a total precision < 7.5%, and a reliability coefficient of 0.995. In a random sample of the study (n = 29), we compared HsCRP measurements from baseline blood from the same participants stored at -20°C and -80°C. The correlation between these measurements was high (Spearman correlation 0.99; $p < 0.001$), although HsCRP levels were somewhat lower in blood stored at -20°C (mean difference, -0.5097; 95% CI, -1.637 to 0.618). This difference was not statistically significant. Because we used for all our analyses these -20°C stored samples, we do not expect that this affected our point estimates. The HsCRP distribution was skewed. Outliers (values > 3 standard deviations (SD) of the population distribution) of the logarithmically transformed HsCRP values were excluded from the analyses, since they may indicate the presence of an acute inflammatory disease.[29]

AMD definition

For the diagnosis of AMD, 35° color pictures were taken of the macular area of each eye (Topcon TRV-50VT fundus camera, Topcon Corporation, Tokyo, Japan) after dilatation of the pupils with tropicamide 0.5% and phenylephrine 5%. These transparencies, and in the last follow-up digitized images, were graded with 12.5x magnification according to the International Classification and Grading System[30] by the same two trained professionals grading AMD from baseline on, who were masked for all other determinants.[31, 32] We deviated from this system by changing the name age-related maculopathy in AMD. Also by categorizing the range of AMD fundus signs into five mutually exclusive stages 0 to 4 that had an increasing risk of late AMD.[32] No AMD was defined as stage 0, no signs of AMD at all or only hard drusen (< 63 μm); stage 1 included soft distinct drusen (≥ 63 μm), or pigmentary abnormalities. Because for our analyses we wanted to have a clear separation between participants with no or early AMD and quite some participants with only one or two soft distinct drusen were classified as stage 1, we considered stage 1 as no AMD in the present analyses. For these analyses we included as early AMD stage 2, soft indistinct drusen (≥ 125 μm) or reticular drusen only or soft distinct drusen (≥ 63 μm) with pigmentary abnormalities, and stage 3, soft indistinct drusen (≥ 125 μm) or reticular drusen with pigmentary abnormalities. Stage 4 was similar to late AMD, usually associated with severe visual loss.[32] Late AMD was subdivided in dry (geographic atrophy) and wet (neovascular) AMD. A person was classified according to the highest stage of AMD in either eye and in case both dry and wet AMD were present, as wet AMD. Early incident AMD (iAMD) was defined as any sign of early AMD in at least one eye during follow-up among participants with no AMD at baseline in either eye. Persons who were free of late AMD at baseline in both eyes and developed it in at least one eye during follow-up were classified as having late iAMD.

Population for analysis

At baseline, gradable fundus transparencies of 6418 participants were available, of whom 476 (7.4%) had early and 106 (1.7%) late AMD. This resulted in 5836 persons at risk for early or late AMD and 6312 persons at risk for late AMD only. Of the 6312 participants at risk for early and late iAMD, 4914 (77.9% of those at risk) participated in at least one follow-up examination. Our study population consisted of 4624 (73.3%) participants from these in whom we had baseline HsCRP measurements. HsCRP levels were missing from persons who did not visit the research center or refused blood sampling and from participants of whom no blood was available due to random logistic reasons. We excluded 20 (0.43%) persons at risk of any AMD with outliers in HsCRP levels, leaving 4604 participants as our population for analysis.

Assessment of confounders

Information on all potential confounders was collected at baseline. During a home interview, trained research assistants asked participants for their smoking habits. Smoking was categorized as current, past or never smoker. Anthropometric measurements were obtained at the research center. Body mass index was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Systolic and diastolic blood pressures were measured twice at the right brachial artery with a random zero sphygmomanometer in a sitting position. The average of these two measurements was used to determine blood pressure levels. Non-fasting blood samples were obtained from all participants. Serum total cholesterol and high-density lipoprotein (HDL) levels were measured by an automated enzymatic procedure. Diabetes mellitus was considered to be present when persons currently used blood-glucose lowering medication, or had a non-fasting or post-load glucose level above 11.0 mmol/L.

Data analysis

We investigated associations between HsCRP levels and early or late iAMD using a Cox proportional hazards model to compute hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). Follow-up time in years was used as time-axis of the model. Hazard ratios take time to event into account, and can be interpreted as relative risks. Linear trends were analyzed in which the regression coefficient was expressed per SD increase and quartiles were analyzed both continuously and as categorical variables. All analyses were initially adjusted for age and sex. To check whether associations could be attributed to confounding, analyses were repeated with possible confounders added to the model (smoking, body mass index, diastolic and systolic blood pressure, diabetes mellitus, total and HDL cholesterol). All analyses were carried out using SPSS 11.0.

RESULTS

Baseline characteristics of participants free of any AMD at baseline and follow-up, and persons at risk for early and late iAMD are shown in Table 1. Persons with missing HsCRP values were on average older, included more women, were more often resident in a nursing home and had lower HDL levels. Follow-up of participants was on average 7.7 years (median 10.4 years; range 0.3 to 13.9 years). During this period, 658 persons were diagnosed with any iAMD of which 561 persons developed early and 97 late iAMD. Among all participants with iAMD, HsCRP levels ranged from 0.20 to 33.60 (mean 2.67 mg/L, SD 3.22); in those with early iAMD these ranged from

Table 1: Baseline characteristics of the 4604 participants of our population for analysis.

Variable	No AMD n = 3946		Early AMD n = 561		Late AMD n = 97	
Age, years	66.7	(7.7)*	68.2	(7.6)	72.0	(6.5)
Sex, female (%)	2315	(58.7)	315	(56.1)	55	(56.7)
Smoking status (%)						
Never	1324	(33.8)	186	(33.6)	26	(27.7)
Former	1705	(43.6)	240	(43.4)	43	(45.7)
Current	886	(22.6)	127	(23.0)	25	(26.6)
Diabetes Mellitus (%)	362	(9.2)	39	(7.0)	8	(8.2)
Body Mass Index, kg/m^2	26.4	(3.7)	26.2	(3.5)	26.1	(3.3)
Systolic blood pressure, mm Hg	137.5	(21.7)	138.9	(20.7)	139.1	(19.5)
Diastolic blood pressure, mm Hg	74.0	(11.1)	73.4	(11.2)	71.8	(11.1)
Total cholesterol, mmol/L	6.7	(1.2)	6.6	(1.2)	6.6	(1.1)
HDL cholesterol, mmol/L	1.3	(0.4)	1.4	(0.4)	1.3	(0.4)

* Means and standard deviations between brackets, unless otherwise indicated

Table 2: Risk of early iAMD for quartiles of baseline HsCRP levels.

Quartiles (range)	n (cases)	Hazard ratio (95%CI)*	Hazard ratio (95%CI)**
1st (≤ 0.83)	1135 (123)	1.00 (reference)	1.00 (reference)
2nd (0.84 - 1.72)	1132 (146)	1.19 (0.94 - 1.52)	1.26 (0.99 - 1.61)
3rd (1.73 - 3.25)	1116 (147)	1.29 (1.01 - 1.64)	1.35 (1.05 - 1.74)
4th (≥ 3.26)	1124 (145)	1.33 (1.05 - 1.70)	1.40 (1.08 - 1.81)
p-trend	4507 (561)	0.02	0.01

* Adjusted for age and sex

** Additionally adjusted for smoking, body mass index, diabetes mellitus, systolic and diastolic blood pressure, total cholesterol and HDL cholesterol

0.20 to 31.50 mg/L (mean 2.69 mg/L, SD 3.08) and in those with late iAMD from 0.20 to 16.80 mg/L (mean 3.04 mg/L, SD 3.18).

The risk of early iAMD increased per SD rise in HsCRP level, adjusted for age and sex, (HR, 1.11; 95% CI 1.02-1.21) also after multivariate adjustment (HR, 1.11; 95% CI 1.02-1.22). Table 2 shows that, the risk of early iAMD also rose with each higher quartile of HsCRP. Additional adjustment for cardiovascular covariates did not substantially change this.

The risk of late iAMD rose per SD increase in HsCRP (HR, 1.31; 95% CI 1.06-1.61) as well as after additional adjustments (HR, 1.28; 95% CI 1.02-1.60). Table 3 shows that

Table 3: Risk of late iAMD for quartiles of baseline HsCRP levels.

Quartiles (range)	n	Hazard ratio (95%CI)*	Hazard ratio (95%CI)**
1st (≤ 0.83)	1028 (16)	1.00 (reference)	1.00 (reference)
2nd (0.84 - 1.72)	1010 (24)	1.34 (0.71 - 2.54)	1.35 (0.70 - 2.58)
3rd (1.73 - 3.22)	999 (30)	1.90 (1.03 - 3.49)	1.96 (1.04 - 3.69)
4th (≥ 3.23)	1006 (27)	1.95 (1.05 - 3.63)	1.79 (0.92 - 3.48)
p-trend	4043 (97)	0.02	0.05

* Adjusted for age and sex

** Additionally adjusted for smoking, body mass index, diabetes mellitus, systolic and diastolic blood pressure, total cholesterol and HDL cholesterol

the risk of late iAMD was higher in all-upper quartiles of HsCRP. However, this only reached statistical significance in the third quartile of the fully adjusted model. The results adjusted for age and sex follow a dose-response pattern, but after additional adjustments this effect was lost probably due to a more limited sample size for late AMD.

DISCUSSION

In this population-based cohort we confirmed data from three clinic-based studies[25-27], that baseline HsCRP levels were associated with both early and late iAMD, with the highest risks in the latter one. This is another support that inflammation is one of the mechanisms involved in the pathogenesis of AMD also in the general population.[24]

Injury to the RPE and possible to the choroid caused by smoking, low antioxidant-intake, light toxicity and obesity may induce AMD through a state of chronic inflammation.[2, 25] Choroidal dendritic cells, which are found to be associated with drusen, respond to locally damaged RPE cells and migrate to the site of tissue damage.[10] Dendritic cells trigger immune-mediated pathways. In persons with AMD the down-regulation of the immune response may be hampered resulting in a state of chronic inflammation of the RPE and damage to the underlying Bruch's membrane leading to progression of AMD.[10]

Evidence is accumulating that inflammatory and immune-associated pathways also play a role in other degenerative diseases associated with advancing age, such as

atherosclerosis and Alzheimer's disease.[33-36] Drusen components were also found in atherosclerotic plaques and deposits in Alzheimer's disease [12] and AMD, atherosclerosis and Alzheimer's disease may partly share a similar inflammatory pathogenesis.

Differential misclassification is unlikely in our study, because AMD graders were masked for HsCRP status and HsCRP data were collected, without knowledge of AMD status. Persons who refused to participate or were lost to follow-up were older and less healthy.[32] If persons with higher HsCRP levels and AMD would selectively not have participated, selection bias would have been introduced. We think this is unlikely because people were unaware of their HsCRP level, and only in late iAMD cases would they be aware of symptoms. We measured HsCRP levels only once. This should not be a major problem because HsCRP has a long half-life of approximately 19 hours[37] and concentrations appear to be fairly stable over at least 5 years in most individuals.[38, 39] Furthermore there is no circadian variation and there is no evidence for seasonal variations in HsCRP.[38, 40-42]

Several large-scale prospective studies have demonstrated that elevated levels of HsCRP were an independent predictor of future cardiovascular events in healthy individuals.[43, 44] In addition to predicting cardiovascular death and myocardial infarction, serum HsCRP is also a predictor of stroke and the development of peripheral arterial disease.[45] Although not yet proven it is hypothesized that CRP directly promotes atherosclerosis and therefore functions as a mediator in the process.[45-49] C-reactive protein-lowering treatments, for example by the use of statins or improvement of lifestyles, were associated with reduced cardiovascular risks.[39, 45, 50, 51]

Atherosclerosis is a known risk factor for AMD, most likely through a decreased choroidal blood flow directly or indirectly impairing the functioning of the RPE.[52, 53] Atherosclerosis is associated with elevated HsCRP levels which could explain the higher risk of AMD.[36] After correction for cardiovascular risk factors the linear trend analysis for early iAMD remained significant, but with late iAMD this became borderline significant. Statistical power due to the lower number of late iAMD cases could have caused the loss of significance, especially since the hazard ratio was still elevated.

CFH is an essential regulator in the complement system. It inactivates C3b and functions therefore as an activation inhibitor of the alternative complement pathway.[54, 55] Due to the CFH Y402H SNP[21-24] complement activation is less suppressed, leading to an increased inflammatory reaction. T his SNP is located in a region that contains the binding sites for heparin and CRP. CFH binds to CRP which may help inhibit the CRP-dependent alternative pathway activation induced by damaged tissue.[54] CFH tends to prevent the assembly of complement complex in the arterial intima.[56] It has been suggested that allele-specific changes in activities of the binding sites for

heparin and CRP modify the protective action of CFH.[22] It is possible that complement-related damage to choroidal vessel walls may lead to wet AMD.[22, 23]

One wonders if reduction of CRP levels would lower the risk of AMD. A substance that can selectively inhibit CRP synthesis has not yet been developed. High body mass index as well as smoking increase CRP levels and moderate alcohol intake, diets with a low glycemic index, statins and multivitamins reduce them.[46, 57] Additional correction for smoking and obesity, also associated with a higher risk of AMD, did not change our point estimates. Nevertheless, reducing both might have a protective effect.

As mentioned, two clinical cross-sectional studies and a longitudinal one found an association between CRP and AMD,[25-27] while a population-based longitudinal study[58] and a population-based cross-sectional study could not confirm this.[59] However these last two studies included fewer cases, especially with late AMD. It has been suggested that differences in results could be contributed to the possibility that inflammation may play a larger role in the pathogenesis of progression to late AMD instead of early AMD.[58] However, in our present study we did not only find an association between HsCRP levels and late iAMD, but also with early iAMD. This is in line with the known progression over the years from stage 0 to stage 4 AMD and supports the inflammatory pathogenesis of both early and late AMD. Finally, clinic-based and cross-sectional studies are more prone to selection bias and thus we think that confirmation by a longitudinal population-based one is important.

In conclusion, persons with a relatively high HsCRP level (> 1.73 mg/L) within the normal range, have a significant higher risk of early and late AMD. We consider HsCRP a potential useful biological marker in profiling the risk of AMD for individual persons.

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2.3

Alcohol consumption and risk of aging macula disorder in a general population

ABSTRACT

Objective: To investigate still controversial associations between overall or specific alcohol consumption and aging macula disorder (AMD) in a general population.
Methods: Alcohol consumption and risk of early or late incident AMD (iAMD) were examined in all participants in the prospective population-based Rotterdam study at risk for AMD of whom we had complete data on alcohol consumption (n = 4229). AMD was graded according to the International Classification and Grading System by the same two trained professionals, who were masked for all other determinants. We used Cox proportional hazards regression analysis to estimate hazard ratios and corresponding 95% confidence intervals (CIs).
Results: During an average follow-up period of 8 years, 600 cases of iAMD were identified, of whom 519 had early and 81 late AMD. After correction for age, sex, smoking, CFH genotype status and other potential confounders, we did not find an association between overall or specific alcohol consumption and risk of early, late dry or late wet iAMD.
Conclusion: Our findings suggest that overall and specific alcohol consumption is not a risk factor for early or late AMD.

INTRODUCTION

Aging macula disorder (AMD), as we now prefer to call age-related macular degeneration,[1] constitutes due to limited treatment options a major burden on the quality of life of the Western population.[2-4] Identification of modifiable risk factors thus is of great importance. Alcohol consumption is one such factor. Alcohol use is quite common in the Netherlands, 80% of the Dutch population older than twelve years consumes alcohol, and 10% is classified as a heavy drinker, consuming six or more glasses of alcohol a day at least once a week.[5]

Moderate alcohol consumption is associated with a decreased risk of cardiovascular disease.[6-8] Increasing evidence that AMD is associated with vascular disease,[9] thus would point to an inverse association between moderate alcohol intake and AMD. On the other hand alcohol is hypothesized to increase oxidative stress or to influence mechanisms that protect against oxidative damage.[10]

Two cross-sectional and three incidence studies found an association between alcohol consumption and AMD.[11-15] Conversely, four different cross-sectional and incidence studies could not confirm these results.[16-19] These inconsistent findings could be attributed to differences in study design, numbers of cases, ethnicity, or in the way AMD was diagnosed. In addition, studies resulting in positive associations between alcohol intake and AMD could not clarify which type of beverage is important. Of all prospective studies investigating this association, the Beaver Dam Study is the only one comparable to our study.[14] Both are large population-based studies using standardized and similar grading for AMD, and were able to perform beverage-specific analyses. Beer consumption is over 10% higher in the Beaver Dam Study compared to our study, but wine consumption is approximately 45% higher in Rotterdam. This enabled us to investigate the association between wine consumption and incident AMD. Thus we studied in this population-based cohort the risk of early or late AMD according to consumption of different levels and types of alcohol.

METHODS

Population

The Rotterdam Study is a prospective, population-based cohort study of cardiovascular-, locomotor-, neurologic- and ophthalmologic diseases in the elderly.[20]

In summary, all inhabitants aged 55 years or older living in Ommoord, a suburb of Rotterdam, the Netherlands were invited to participate in the study. Of the 10,275 eligible individuals, 7983 participated (78%). The ophthalmologic part of the study

started after screening of the participants had begun, leading to 6780 ophthalmic participants, also with 78% response rate. The Rotterdam Study was approved by the medical ethics committee of the Erasmus University and written informed consent was obtained from all participants. Baseline examinations, including a standardized home interview and physical examination at the research center took place between March 1990 and July 1993. Three follow-up examinations took place from September 1993 to the end of 1994, from 1997 to 1999 and from 2000 to the end of 2004.

AMD definition

In order to diagnose AMD, 35° color photographs were taken of the macular area of each eye (Topcon TRV-50VT fundus camera, Topcon Corporation, Tokyo, Japan) after dilatation of the pupils with tropicamide 0.5% and phenylephrine 5%. Fundus transparencies and digitized images from the last follow-up examination[21, 22] were graded with 12.5x magnification according to the International Classification and Grading System for AMD.[23] Two well-trained graders, each having 11 years of experience, graded the fundus images masked for all other participant characteristics. The grading procedures and definitions, as well as the graders, were identical at baseline and at follow-up. We modified the nomenclature from age-related maculopathy[23] into AMD, dividing it in early and late AMD. Late AMD in the present manuscript is similar to AMD in that classification system[23] and is divided in dry (geographic atrophy) and wet (neovascular) AMD.

Next AMD was classified into five mutually exclusive stages 0 to 4 that had an increasing risk of late AMD.[24] No AMD was defined as stage 0, no signs of AMD at all or only small hard drusen (< 63 µm), and stage 1 soft distinct drusen (≥ 63 µm), or pigmentary abnormalities. Because many participants with only one large druse or one hyperpigmentation in this system are classified as stage 1 and because we wanted to separate participants with marked AMD from those with only limited signs, we considered stage 1 as no AMD in the present analyses. Early AMD included stage 2, soft indistinct drusen (≥ 125 µm) or reticular drusen only or soft distinct drusen (≥ 63 µm) with pigmentary abnormalities, and stage 3, soft indistinct drusen (≥ 125 µm) or reticular drusen with pigmentary abnormalities. Late AMD was similar to stage 4. Dry AMD was defined as any sharply demarcated round or oval area of apparent absence of the retinal pigment epithelium (RPE), larger than 175 µm, with visible choroidal vessels, and no wet AMD. Wet AMD was defined as the presence of a serous or hemorrhagic detachment of the RPE and/or a subretinal neovascular membrane and/or subretinal hemorrhage, and/or periretinal fibrous scar. A person was classified according to the highest stage of AMD in either eye, in case of mixed dry and wet classifying wet higher than dry AMD. Early incident AMD (iAMD) was

defined as any sign of early AMD in at least one eye during follow-up among participants with no AMD at baseline in either eye. Persons who were free of late AMD at baseline in both eyes and developed it in at least one eye during follow-up were classified as having late iAMD. Lesions that were considered to be the result of generalized disease, such as diabetic retinopathy, chorioretinitis, high myopia, trauma, congenital diseases, or photocoagulation for reasons other than for wet AMD, were excluded from AMD classification.

Alcohol consumption

Before the baseline center visits, participants received a checklist on which they indicated all foods and drinks they had consumed at least once during the preceding year. The completed checklist formed the basis of an interview, using an extensive semi quantitative food frequency questionnaire at the study center by a trained dietician.[25] The dietary interviews were performed using a computer program that simultaneously checked the data. Participants reported the number of alcoholic beverages they consumed on a weekly basis in each of four categories: beer, wine, moderately strong alcoholic beverages such as port wine or sherry, and liquor. Non-drinkers were considered abstainers. A "drink" was defined as 200 ml of beer that contained 8.0 g of ethanol, 100 ml of wine that contained 10.0 g of ethanol, 75 ml of moderately strong alcohol types that contained 10.5 g of ethanol, or 50 ml of liquor that contained 14.0 g of ethanol.[26] By adding the amounts of ethanol in the four groups we calculated the total consumption of alcohol per participant in grams per day. Because most of the moderately strong alcohol drinks were wine types, this category was combined with the wine category in the analyses leading to three nonexclusive groups of persons drinking beer, wine and liquor. The alcohol consumption was divided into no drinking, use of ≤ 10 g, > 10-≤ 20 g, and > 20 g/day.

Assessment of confounders

Information on all potential confounders was collected at baseline. During a home interview, trained research assistants asked participants for their smoking habits. Smoking was categorized as current, past or never smoker. Systolic and diastolic blood pressures were measured twice at the right brachial artery in a sitting position with a random zero sphygmomanometer. The average of these two measurements was used to determine blood pressure levels. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Non-fasting blood samples were obtained from all participants. Serum total cholesterol and high-density lipoprotein (HDL) levels were measured by an automated enzymatic procedure. Genotypes of complement factor H (CFH Y402H) polymorphism (1277 T/C, rs1061170), were determined in 2-ng genomic DNA extracted according to

standard procedures from leukocytes with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California).

Data analysis

We estimated the risk of iAMD associated with alcohol consumption with Cox's proportional hazard regression analysis. Follow-up time in years was used as time-axis of the model. The associations are presented as hazard ratios (HRs) taking time to event into account, which can be interpreted as relative risks, with 95% confidence intervals (CIs). Non-drinkers were used as reference category. The Cox proportional hazard regression models were repeated for each category of alcoholic beverage. All analyses were initially corrected for age and sex. Analyses were repeated with possible confounders added to the model (smoking, systolic and diastolic blood pressure, total cholesterol and HDL cholesterol, body mass index, and the complement factor H Y402H SNP) using SPSS for Windows, version 11.0 (SPSS Inc., 2001, Chicago, IL).

RESULTS

At baseline, gradable fundus transparencies were available from 6418 participants, of whom 476 (7.4%) had early AMD and 106 (1.7%) late AMD. Of the 5836 persons at risk for any AMD, 4914 (77.9% of those at risk) participated in at least one follow-up examination. Persons did not participate in follow-up examinations due to refusal, death or loss to follow-up. Our study population consisted of 4229 (67.0%) participants from whom data on alcohol consumption were available.

Baseline characteristics of these participants are shown in table 1. The average time between baseline and first follow-up examination was 2.0 years, between baseline and second follow-up 6.5 years, and between baseline and third follow-up 11.1 years. During a mean follow-up time of 8.0 years (range 0.3 to 13.9 years), 600 cases of iAMD were identified, of whom 519 had early iAMD and 81 late iAMD.

For each risk analysis for a subtype of AMD, the other AMD cases were removed from the study population as indicated below tables 2 and 3. Table 2 shows that after correction for age and sex, total alcohol consumption, analyzed in categories, was not associated with early or late iAMD. Because etiologic mechanisms could differ between dry and wet AMD, we analyzed these two end stages separately. Risk of dry iAMD seemed to increase with higher alcohol intake where as for wet iAMD the risk seemed to decrease with higher alcohol consumption (table 3). However, these associations were not statistically significant and 95% CIs were large. We further investigated the results for dry and wet AMD by calculating a trend using alcohol

Table 1: Baseline characteristics and AMD status in 4229 participants

	No AMD n = 3629		Early AMD n = 519		Late AMD n = 81	
Age, years	66.3	(7.2)*	68.0	(7.1)	71.3	(6.4)
Sex, female (%)	2166	(59.7)	295	(56.8)	49	(60.5)
Smoking status (%)						
Never	1218	(33.7)	172	(33.3)	22	(27.5)
Former	1567	(43.4)	230	(44.5)	38	(47.5)
Current	827	(22.9)	115	(22.2)	20	(25.0)
Diabetes Mellitus (%)	318	(8.8)	33	(6.4)	8	(7.4)
Body Mass Index, kg/m^2	26.4	(3.6)	26.3	(3.5)	26.1	(3.1)
Systolic blood pressure, mm Hg	137.1	(21.5)	138.9	(20.3)	137.6	(19.9)
Diastolic blood pressure, mm Hg	73.9	(11.0)	73.5	(11.8)	70.8	(10.8)
Total cholesterol, mmol/L	6.7	(1.2)	6.6	(1.2)	6.7	(1.1)
HDL cholesterol, mmol/L	1.4	(0.4)	1.4	(0.4)	1.4	(0.4)
Complement factor H genotype (%)						
TT	1437	(43.8)	185	(39.7)	16	(20.5)
CT	1469	(44.8)	211	(45.3)	36	(46.2)
CC	372	(11.3)	70	(15.0)	26	(33.3)
Daily alcohol consumption						
Non-drinker	704	(19.4)	90	(17.3)	15	(18.5)
≤ 10 g	1638	(45.1)	235	(45.3)	37	(45.7)
> 10 g - ≤ 20 g	568	(15.7)	82	(15.8)	11	(13.6)
> 20 g	719	(19.8)	112	(21.6)	18	(22.2)

* Means and standard deviations between brackets, unless otherwise indicated

Table 2: Risk of early and late AMD according to level of alcohol consumption*

Daily alcohol consumption	Total participants	Cases	Hazard ratio 95% CI**	Hazard ratio 95% CI***
Early AMD				
Non-drinker	794	90	1.00 (reference)	1.00 (reference)
≤ 10 g	1873	235	1.01 (0.79-1.29)	1.00 (0.76-1.30)
> 10 g - ≤ 20 g	650	82	1.04 (0.76-1.40)	0.98 (0.70-1.36)
> 20 g	831	112	1.11 (0.83-1.48)	1.10 (0.80-1.51)
Late AMD				
Non-drinker	719	15	1.00 (reference)	1.00 (reference)
≤ 10 g	1675	37	0.94 (0.51-1.72)	1.00 (0.53-1.89)
> 10 g - ≤ 20 g	579	11	0.94 (0.43-2.08)	0.77 (0.33-1.80)
> 20 g	737	18	1.26 (0.61-2.60)	1.01 (0.46-2.21)

* Early AMD: n = 4229 - 81 late AMD cases
Late AMD: n = 4229 - 519 early AMD cases

** adjusted for age and sex

*** additionally adjusted for smoking, systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, BMI and CFH genotype status

Table 3: Risk of dry or wet AMD according to level of alcohol consumption*

Daily alcohol consumption	Total participants	Cases	Hazard ratio 95% CI**	Hazard ratio 95% CI***
Dry AMD				
Non-drinker	708	4	1.00 (reference)	1.00 (reference)
≤ 10 g	1648	10	0.93 (0.29 - 2.99)	1.10 (0.32 - 3.80)
> 10 g - ≤ 20 g	573	5	1.58 (0.42 - 6.04)	1.38 (0.31 - 6.16)
> 20 g	731	12	3.09 (0.93 - 10.27)	3.27 (0.88 - 12.19)
Wet AMD				
Non-drinker	715	11	1.00 (reference)	1.00 (reference)
≤ 10 g	1665	27	0.95 (0.47 - 1.92)	0.96 (0.45 - 2.03)
> 10 g - ≤ 20 g	574	6	0.71 (0.26 - 1.96)	0.60 (0.21 - 1.72)
> 20 g	725	6	0.59 (0.21 - 1.68)	0.40 (0.13 - 1.25)

* Dry AMD: n = 4229 - 519 early AMD cases - 50 wet AMD cases
Wet AMD: n = 4299 - 519 early AMD cases – 31 dry AMD cases

** adjusted for age and sex

*** additionally adjusted for smoking, systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, BMI and CFH genotype status

Table 4: Risk of early or late AMD by level of consumption of different types of alcoholic beverages adjusted for age and sex

	Early AMD			Late AMD		
	Total	Cases	Hazard ratio 95% CI	Total	Cases	Hazard ratio 95% CI
Daily beer consumption						
Non-drinker	794	90	1.00 (reference)	719	15	1.00 (reference)
≤ 10 g	598	69	0.79 (0.53 - 1.15)	536	7	0.63 (0.20 - 1.98)
> 10 g - ≤ 20 g	95	8	0.66 (0.31 - 1.41)	88	1	0.82 (0.09 - 7.20)
> 20 g	74	12	1.28 (0.66 - 2.48)	64	2	1.94 (0.35 - 10.67)
Daily wine consumption						
Non-drinker	794	90	1.00 (reference)	719	15	1.00 (reference)
≤ 10 g	1738	214	0.99 (0.78 - 1.27)	1562	38	1.04 (0.57 - 1.89)
> 10 g - ≤ 20 g	377	51	1.18 (0.83 - 1.67)	334	8	1.39 (0.58 - 3.32)
> 20 g	235	35	1.32 (0.89 - 1.96)	202	2	0.60 (0.13 - 2.63)
Daily liquor consumption						
Non-drinker	794	90	1.00 (reference)	719	15	1.00 (reference)
≤ 10 g	740	94	0.90 (0.66 - 1.23)	655	9	0.45 (0.18 - 1.11)
> 10 g - ≤ 20 g	291	34	0.81 (0.54 - 1.23)	264	7	0.92 (0.35 - 2.44)
> 20 g	435	56	0.92 (0.64 - 1.33)	389	10	0.98 (0.40 - 2.40)

intake as a continuous measure in these models. Only in the case of dry AMD the test for trend was significant (fully adjusted model, p-value 0.04). In addition we did not find any beverage-specific associations with early or late iAMD (table 4).

We could not repeat the beverage-specific analyses after stratification by type of late AMD due to loss of power. Correction for additional covariates did not substantially alter our results.

DISCUSSION

In this cohort study we could not detect an association between overall alcohol consumption and early or late iAMD. In addition neither beer, wine nor liquor drinkers were at higher risk for AMD. With higher alcohol consumption, point estimates were increasing for dry iAMD and decreasing for wet iAMD. Although associations did not reach significance, the test for trend was significant for dry AMD. Whether there exists an association between alcohol consumption and dry AMD thus remains inconclusive.

The Beaver Dam Study found cross-sectionally an association between beer consumption in the past year and late AMD. In their 10-years incidence study current heavy alcohol consumption, defined as the consumption of four or more servings of alcoholic beverages daily, corresponding with 46 to 56 g of ethanol, was associated with wet late AMD (RR = 6.51, 95% CI: 1.41-30.2).[11,14] However, because only three current heavy drinkers developed late AMD, and confidence intervals about the risk ratios were large, these results should be interpreted with caution. In the Rotterdam Study only one current heavy drinker, classified in the same way as in the Beaver Dam Study, developed late wet AMD making comparable end stage-specific analyses impossible due to lack of power. The Blue Mountains Eye Study, found cross-sectionally no association with overall alcohol intake and more specifically with beer consumption. Consumption of spirits, however was associated with the presence of early AMD.[16] They suggested that this is likely to be a chance finding because consumption of spirits was not associated with the presence of any large drusen with a diameter greater than 125 µm or late AMD. The NHANES noted cross-sectionally a protective effect of moderate wine consumption on AMD.[17] They did not correct for smoking and the at that time unknown complement factor H Y402H SNP, both strong risk factors for AMD, and recall bias might have resulted in their finding. This protective effect of wine consumption was also cross-sectionally described by the LALES study group, who found in addition a stronger association with AMD in heavy drinkers, and particularly in beer drinkers.[12] This study concerns a Latino population and biological mechanisms, e.g. the levels of alcohol dehydrogenase, could be different from those in a Caucasian population. The prospective Copenhagen Study found a non-significant trend of increased risk of any AMD with increasing daily alcohol intake be it with large CIs.[15] One puzzling aspect in their results was

that they did not find an association between the established risk factor smoking and AMD. Another prospective study found no overall association between alcohol consumption and incidence of AMD, and only among women "excessive" alcohol consumption, defined as 30 g/day, was associated with early AMD and geographic atrophy (RR = 2.04, 95% CI: 1.22 - 3.42).[13] In this study AMD was divided in two separate groups, pooled early and dry AMD and wet AMD, because of possible different etiological mechanisms. Pooling of early and dry AMD is controversial, because both dry and wet AMD are likely to develop in persons with early AMD.[27] A population-based study, with an average follow-up time of 5 years,[19] and the Physicians' Health Study, with an average follow-up time of 12.5 years[18], did not detect an association between alcohol intake and incident AMD. They could not perform beverage-specific analyses.

In contrast with a case-control study, we assessed alcohol consumption before onset of AMD and recall bias is therefore not likely to be involved. In this study, self-reported drinking habits may have introduced misclassification in exposure.[28] In particular, this might have caused underreporting of alcohol consumption among heavy drinkers and have diminished our ability to detect an association. Yet, all other epidemiological studies investigating the possible association between alcohol consumption and AMD used self-reported data on alcohol intake. In addition, with increasing age, most drinkers reduce their alcohol consumption,[29] thereby reducing power. The fact that we did not find an association could partly result from inclusion of former heavy drinkers in the group of no alcohol consumers and persons who did not drink because of their poor health status.[30] However, additional exclusion of persons who had reduced their alcohol consumption in the last five years before baseline did not essentially change our results. The size of the Rotterdam Study, its population-based prospective design and the fact that we have taken a number of important confounders into account, strengthen our findings. Fundus images were graded in a standardized way by the same two well-trained graders at baseline and at all three follow-up visits. In addition alcohol consumption and type of beverages were classified based on a validated food frequency questionnaire. Therefore, we believe that our findings are an important addition to the existing studies on the subject.

In conclusion, neither overall nor specific alcohol consumption was associated with early or late iAMD in our population-based prospective cohort study. Considering the lack of consistent results among studies, we believe that alcohol consumption is not an important risk factor for AMD.

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Chapter 3

Genetic risk factors

3.1

Estrogen receptor alpha gene polymorphisms associated with incident aging macula disorder

ABSTRACT

Purpose: It has been suggested that early menopause increases the risk of aging macula disorder (AMD), the major cause of incurable blindness with a dry and wet late subtype, and that exposure to endogenous or postmenopausal exogenous estrogens reduces this risk. This study was undertaken to investigate whether genetic variations in the estrogen receptor *α* (*ESR1*) gene were associated with incident AMD.

Methods: In the Rotterdam Study, a prospective population-based cohort study of participants aged 55 years and older, associations between *ESR1 PvuII-XbaI* haplotypes and incident early or late AMD were studied in 4571 participants after a mean follow-up time of 7.7 years. Cox proportional hazards regression was used to estimate hazard ratios (HRs) and corresponding 95% confidence intervals (CIs), with adjustment for the most common confounders.

Results: *ESR1 PvuII-XbaI* haplotype 1 was a risk factor for late AMD. Persons with two copies of haplotype 1 were at 3.20 (95% CI: 1.47-6.99) times higher risk for late AMD than non-carriers of haplotype 1, after adjustment for age and sex. This increase was more pronounced for wet AMD (HR = 4.29; 95% CI, 1.47-12.49) after adjustment for age, sex, smoking and complement factor H genotype. Correction for additional confounders, including age at menopause, use of hormone replacement therapy, blood pressure, and body mass index did not essentially alter the findings.

Conclusion: Persons with one or two copies of *ESR1 PvuII-XbaI* haplotype 1 have an increased risk of late AMD, especially of the wet form.

INTRODUCTION

Aging macula disorder (AMD), as we now prefer to call age-related macular degeneration,[1] is a multifactorial disease that affects the center of the retina, the macula lutea, resulting in a central scotoma in the visual field.[2-4] The pathogenesis of AMD is still not clear, and therapeutic strategies are limited. In our rapidly aging population, with a predicted doubling by 2020 of the persons with blindness due to AMD, it is important to identify risk factors for AMD, to develop possible preventive and therapeutic strategies.

Diminished exposure to endogenous estrogens as in early menopause has been cross-sectionally associated with AMD, and post-menopausal hormone replacement therapy seems to protect against the disease.[5-8] Although some studies could not corroborate these findings,[9, 10] the role of estrogen deficiency in the pathogenesis of AMD should be further explored.

Estrogens are steroid hormones that regulate growth, differentiation, and function of male and female reproductive tracts, mammary glands, and the skeletal and cardiovascular systems.[11] They mediate their effects through two distinct intracellular estrogen receptors (ERs): α (ESR1) and β (ESR2). After binding of estrogen to the ERs, they become transcription factors to modulate gene expression.[12] The *ESR1* gene is located on chromosome 6q25. It consists of at least eight exons and spans more than 400 kb (http://www.ncbi.nlm.nih.gov/SNP provided in the public domain by National Center for Biotechnology Information, Bethesda, MD). Many common variations in DNA sequence (polymorphisms) of the *ESR1* gene have been identified, including single nucleotide polymorphisms (SNPs). The two most studied SNPs are the adjacent *PvuII* (rs2234693), a T/C transition in intron 1, and *XbaI* (rs9340799), a G/A transition located 46 bp downstream of the *PvuII* polymorphism. The SNPs are localized in the first intron, 397 and 351 bp upstream of exon 2. It is not yet clear whether they have functional consequences. However, these SNPs, have been associated with several different phenotypes, such as osteoporosis, Alzheimer's disease, and cardiovascular disease.[13-16] Recently it has been demonstrated that, in postmenopausal women, *ESR1 PvuII-XbaI* haplotype 1 is associated with decreased serum estradiol levels in an allele-dose dependent manner.[17]

The ESR1 protein is expressed in a variety of tissues, and the presence of ESR1 has also been demonstrated in the retina, suggesting that estrogens may have a role in retinal biology.[18-20] We set out to study whether SNPs in the *ESR1* gene are a risk factor for AMD.

METHODS

Population

The Rotterdam Study is a prospective, population-based cohort study investigating the incidence and determinants of chronic disabling diseases in the elderly. All inhabitants aged 55 years or older living in one suburb of Rotterdam, the Netherlands were invited to participate in the study.[21] Of the 10,275 eligible individuals, 7983 (78%) participated. The ophthalmologic part of the study started after screening of the participants had begun, leading to 6780 ophthalmic participants-again, a 78% response rate. The study was conducted according to the tenets of the Declaration of Helsinki, and the medical ethics committee of the Erasmus University approved the study protocol. A written informed consent was obtained from all persons. Baseline examinations, including a home interview and physical examination at the research center took place between March 1990 and July 1993. Three follow-up examinations took place from September 1993 to the end of 1994, from 1997 to 1999, and from 2000 to the end of 2004.

AMD definition

To diagnose AMD, 35° color transparencies were taken of the macular area of each eye (TRV-50VT fundus camera; Topcon Corp., Tokyo, Japan) after dilatation of the eyes with tropicamide 0.5% and phenylephrine 5%. These images (digitized from the last follow-up examination on) were graded with 12.5x magnification according to the International Classification and Grading System for age-related maculopathy (ARM) by the same two trained professionals grading AMD from baseline on, who were masked for all other determinants.[21-24] We only changed the terminology from early and late ARM in early and late AMD and categorized the range of AMD fundus signs into five mutually exclusive stages 0 to 4 that had an increasing risk of late AMD.[25] No AMD was defined as stage 0, no signs of AMD at all or only hard drusen (< 63 µm); stage 1 as soft distinct drusen (≥ 63 µm) or only pigmentary abnormalities. Because many participants with only one large druse or one hyperpigmentation in this system are classified as stage 1 and because we wanted to separate participants with marked AMD from those with only limited signs, in the present analyses, we considered stage 1 as no AMD. Thus, we classified as early AMD stage 2, soft, indistinct drusen (≥ 125 µm) or reticular drusen only, or soft, distinct drusen (≥ 63 µm) with pigmentary abnormalities, and as stage 3, soft, indistinct drusen (≥ 125 µm) or reticular drusen with pigmentary abnormalities. Stage 4 was similar to late AMD, subdivided in dry (geographic atrophy) and wet (neovascular) AMD.[25] The disease in each person was classified according to the highest stage of AMD in either eye. Early incident (i)AMD was defined as no AMD at baseline and early AMD in at least

one eye at follow-up. Late iAMD was classified as either no or early AMD at baseline and presence of late AMD in either eye at follow-up. Lesions that were considered to be the result of generalized disease, such as diabetic retinopathy, chorioretinitis, high myopia, trauma, congenital diseases, or photocoagulation for reasons other than for wet AMD, were excluded from the AMD diagnosis.

Genotyping

DNA was extracted from peripheral leukocytes according to standard procedures. All participants were genotyped for the *PvuII* (rs2234693; c.454-397T>C) and the *XbaI* (rs9340799; c.454-351A>G) SNPs. Genotypes were also determined for the complement factor H Y402H polymorphism, which is a major risk factor for AMD (described later). Genotypes were determined in 5 ng genomic DNA with the allelic discrimination assay (Taqman; Applied Biosystems, Foster City, CA). Primer and probe sequences were optimized with the SNP assay-by-design service of Applied Biosystems. Reactions were performed on a sequence detection system (Taqman Prism 7900HT; ABI) 384-well format. We used the genotype data for each of the two *ESR1* SNPs to infer the haplotypes for each individual using the haplotype reconstruction program PHASE.[26] The alleles were defined as haplotypes, such as T-A representing a thymidine (T) nucleotide at the *PvuII* polymorphic site and an adenosine (A) nucleotide at the *XbaI* polymorphic site. We studied haplotypes based on these adjacent polymorphisms, to enhance genetic resolution. Haplotype alleles, based on the combination of these two SNPs, were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population (1, T-A; 2, C-G; 3, C-A; and 4, T-G).[27, 28]

Population for Analysis

At baseline, gradable fundus transparencies were available for 6418 participants, of whom 476 (7.4%) had early AMD and 106 (1.7%) late AMD. Of the 6312 persons at risk for any AMD, 4914 (77.9% of those at risk) participated in at least one follow-up examination and in 4571 (72.4%) of them, haplotype data were available. Persons did not participate in follow-up examinations due to refusal, death, or loss to follow-up. Haplotype data were missing for persons who at baseline only had an interview and did not visit the research center, refused blood sampling, or from whom no blood was available due to various logistic reasons. For each risk analysis for a subtype of AMD, the other AMD cases were removed from the study population as indicated in table footnotes.

Assessment of Confounders

Information on all potential confounders was collected at baseline. During a home interview, participants were asked by trained research assistants about smoking habits, and women about age at menopause and use of hormone replacement therapy. Smokers were categorized as current, past, or never. In the research center, systolic and diastolic blood pressures were measured twice at the right brachial artery with a random zero sphygmomanometer with the participant in a sitting position. The average of these two measurements was used to determine blood pressure levels. Body mass index was calculated as weight in kilograms divided by height in meters squared. Genotypes of complement factor H (CFH Y402H) polymorphism (1277 T/C, rs1061170), were determined in 2-ng genomic DNA extracted according to standard procedures from leukocytes with the allelic discrimination assay (Taqman; ABI). Confounder data for smoking, body mass index, and systolic and diastolic blood pressure were missing in 1% of the participants, and data for CFH genotype in 10%. In women, confounder data on hormone replacement therapy and age at menopause were missing in 4% of the participants.

Data analysis

One-way analysis of variance (ANOVA), for continuous variables, and Pearson's chi-square for dichotomous variables were used to compare possible confounders between participants grouped by the haplotype of interest. The observed genotype distributions were compared using Pearson's chi-square test to determine whether they were in Hardy Weinberg equilibrium. The associations between *ESR1* haplotype stratified on allele copy number (0, 1 or 2) and early or late iAMD, stratified on type of end stage were investigated with the Cox proportional hazards model to compute hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). To calculate a trend, *ESR1* haplotype 1 was used as a continuous measure in the model. As a reference group, we used persons with only stage 0 or 1 AMD at baseline and at follow-up. Follow-up time in years was used as time axis of the model. HRs can be interpreted as relative risks. All analyses were adjusted for age and sex. To account for potential confounding, additional adjustments were made for smoking, and the complement factor H Y402H SNP, major risk factors associated with AMD.[29-32] Further adjustment for blood pressure and body mass index did not essentially alter our results (data not shown). Based on previous analyses we chose haplotype 1 as the risk allele.[15, 28, 33, 34] To evaluate the effect of sex we performed sex-specific analyses for early and late iAMD. In women, analyses were additionally adjusted for the potential confounding effects of age at menopause and any use of HRT. All analyses were performed with commercial software (SPSS, ver. 11.0; SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics of the 4571 participants are shown in Table 1. *ESR1* haplotype 1 was not associated with any of the baseline characteristics, including age at menopause and use at any time of hormone replacement therapy. After an average

Table 1: Baseline characteristics of 4571 participants, stratified on number of allele copies of *ESR1* haplotype 1 (T-A).

Number of allele copies *ESR1* haplotype 1	0		1		2		p-value
Number of participants (%)	1008	(22.1)	2285	(50.0)	1278	(28.0)	
Age (y)	67.1	(7.6)	67.1	(7.6)	66.9	(7.7)	0.7
Sex, female (%)	581	(57.6)	1322	(57.9)	739	(57.8)	0.9
Smoking status (%)							
Never	346	(34.5)	760	(33.6)	416	(32.9)	
Current	229	(22.8)	496	(21.9)	305	(24.1)	0.5
Past	428	(42.7)	1008	(44.5)	545	(43.0)	0.7
Systolic blood pressure, (mm HG)	137.0	(21.2)	137.7	(21.4)	137.9	(21.8)	0.6
Diastolic blood pressure, (mm HG)	73.7	(11.0)	73.7	(11.0)	74.1	(11.2)	0.5
Body Mass Index (kg/m^2)	26.3	(3.6)	26.3	(3.6)	26.4	(3.7)	0.9
Complement Factor H Y402H SNP (%)							
Noncarrier	422	(43.6)	939	(42.9)	520	(42.4)	
Heterozygous	429	(44.4)	977	(44.6)	556	(45.3)	0.9
Homozygous	116	(12.0)	274	(12.5)	151	(12.3)	0.9
Age at menopause (y)	48.7	(4.9)	48.8	(5.1)	49.1	(4.8)	0.3
Ever use of HRT (%)	84	(15.1)	199	(15.7)	101	14.2)	0.7

Data are the means with standard deviations in parentheses, unless otherwise indicated.

Table 2: Risk of early or late iAMD according to *ESR1* haplotype 1 (T-A).

Number of copies of *ESR1* haplotype 1	Total participants	Cases (%)	Risk adjusted for age and sex	Risk adjusted for age, sex, smoking and CFH genotype
Early AMD				
0	1000	116 (11.6%)	1.00 (reference)	1.00 (reference)
1	2228	265 (11.9%)	1.07 (0.86 - 1.33)	1.04 (0.83 - 1.30)
2	1248	163 (13.1%)	1.18 (0.93 - 1.50)	1.16 (0.91 - 1.48)
p-trend			0.16	0.21
Late AMD				
0	892	8 (0.9%)	1.00 (reference)	1.00 (reference)
1	2020	57 (2.8%)	3.39 (1.62 - 7.11)	3.07 (1.46 - 6.46)
2	1115	30 (2.7%)	3.20 (1.47 - 6.99)	2.85 (1.30 - 6.25)
p-trend			0.01	0.02

Early AMD: n = 4571 - 95 late AMD cases

Late AMD: n = 4571 - 544 early AMD cases

Table 3: Risk of dry or wet late iAMD according to *ESR1* haplotype 1 (T-A).

Number of copies of *ESR1* haplotype 1	Total participants	Cases (%)	Risk adjusted for age and sex	Risk adjusted for age, sex, smoking and CFH genotype
Dry AMD				
0	888	4 (0.5%)	1.00 (reference)	1.00 (reference)
1	1989	26 (1.3%)	3.13 (1.09 - 8.98)	2.76 (0.95 - 8.01)
2	1093	8 (0.7%)	1.72 (0.52 - 5.71)	1.41 (0.41 - 4.85)
p-trend			0.57	0.79
Wet AMD				
0	888	4 (0.5%)	1.00 (reference)	1.00 (reference)
1	1994	31 (1.6%)	3.72 (1.31 - 10.55)	3.50 (1.23 - 9.94)
2	1107	22 (2.0%)	4.71 (1.62 - 13.66)	4.29 (1.47 - 12.49)
p-trend			0.001	0.01

Dry AMD: n = 4571 - 544 early AMD cases - 57 wet AMD cases
Wet AMD: n = 4571 - 544 early AMD cases - 38 dry AMD cases

Table 4: Risk of early or late iAMD according to *ESR1* haplotype 1 (T-A) stratified on sex.

Number of copies of *ESR1* haplotype 1	Total participants	Cases (%)	Risk adjusted for age	Risk adjusted for age, smoking and CFH genotype*
Men				
Early AMD				
0	423	53 (12.5%)	1.00 (reference)	1.00 (reference)
1	935	114 (12.2%)	1.01 (0.73 - 1.40)	1.02 (0.73 - 1.42)
2	530	74 (14.0%)	1.14 (0.80 - 1.63)	1.15 (0.80 - 1.66)
p-trend			0.43	0.42
Late AMD				
0	374	4 (1.1%)	1.00 (reference)	1.00 (reference)
1	849	28 (3.3%)	3.22 (1.13 - 9.19)	3.02 (1.05 - 8.72)
2	465	9 (1.9%)	1.88 (0.58 - 6.09)	1.71 (0.51 - 5.71)
p-trend			0.49	0.58
Women				
Early AMD				
0	577	63 (10.9%)	1.00 (reference)	1.00 (reference)
1	1293	151 (11.7%)	1.12 (0.83 - 1.50)	1.10 (0.81 - 1.49)
2	718	89 (12.4%)	1.21 (0.88 - 1.68)	1.13 (0.81 - 1.61)
p-trend			0.24	0.48
Late AMD				
0	518	4 (0.8%)	1.00 (reference)	1.00 (reference)
1	1171	29 (2.5%)	3.51 (1.23 - 9.99)	3.10 (1.07 - 8.90)
2	650	21 (3.2%)	4.55 (1.56 - 13.26)	3.52 (1.18 - 10.46)
p-trend			0.01	0.03

* Plus age at menopause and use (ever) of HRT in women.

follow-up time of 7.7 years (range 0.3-13.9 years), 639 (14.0%) persons had iAMD, of whom 544 (11.9%) had early iAMD, and 95 (2.1%) had late iAMD, of whom 38 had dry and 57 wet iAMD. For early iAMD cases the average follow-up time from baseline to the development of AMD was 4.5 years and for late iAMD cases 6.2 years. We observed the four possible *PvuII-XbaI* haplotype alleles in the following frequencies: haplotype 1 (T-A) 53.0%, haplotype 2 (C-G) 35.0%, haplotype 3 (C-A) 12.0% whereas haplotype 4 (T-G) was not present in our study population. Genotype and allele distributions were in Hardy-Weinberg Equilibrium.

ESR1 haplotype 1 was associated with late iAMD (Table 2). Persons with one copy of haplotype 1 were at 3.39 (95% CI, 1.62-7.11) times higher risk and for persons with two copies, this was 3.20 (95% CI, 1.47-6.99). After stratification by type of end stage (Table 3), it seemed that particularly wet iAMD was responsible for this association. Individuals with one copy had a 3.72 (95% CI, 1.31-10.55) times higher risk of wet iAMD, whereas this was 4.71 (95% CI, 1.62-13.66) for individuals with two copies. HRs were also increased for dry iAMD, but they did not reach significance. Early iAMD was not associated with *ESR1* haplotype 1. Additional correction for smoking and complement factor H also did not essentially alter our results. After stratification on sex (Table 4), HRs were increased in the men with one or two copies of haplotype 1, but this only reached statistical significance in the men with one copy and the risk of late iAMD. In the women we found an association with late iAMD: women with one copy of haplotype 1 had a 3.10 (95% CI, 1.07 - 8.90) times higher risk and this was for the women with two copies 3.52 (95% CI, 1.18 - 10.46). Further stratification on type of end stage was not possible due to lack of power. Multivariate adjustment, including age at menopause and any use of HRT, did not substantially change these HRs.

Additional stratified analysis by genotypes of the CFH Y402H polymorphism to test for gene-gene interaction and by smoking status to test for gene-environmental interaction did not show any significant associations (data not shown).

DISCUSSION

In this large, prospective population-based study, we demonstrated that the *ESR1 PvuII-XbaI* haplotype 1 was strongly associated with late iAMD. After stratification by type of end stage, wet iAMD showed highly significant HRs. Although point estimates were elevated in dry iAMD, there was no significant effect visible, probably due to lack of power. Therefore, it remains inconclusive whether an association with *ESR1 PvuII-XbaI* haplotype 1 exists.

The Rotterdam Study is a large study with a population-based prospective design, which increases the reliability of the associations found. Fundus images were grad-

ed in a standardized way by the same two well-trained graders at baseline and at all three follow-up visits. Differential misclassification is unlikely, because AMD graders were masked for the presence of *ESR1 PvuII-XbaI* haplotype 1 status and haplotype data were collected without knowledge of AMD status. There was loss to follow-up due to the older age of the participants, and thus only the healthier persons were able to participate in the follow-up visits. This leads to an underestimation of the strength of the associations. If especially persons with one or two copies of *ESR1* haplotype 1 with iAMD had not participated, selection bias would have been introduced. We think this is unlikely because people were unaware of their *ESR1* haplotype 1 status, and only in case of late iAMD would they be aware of symptoms. Genetic association studies can be influenced by population heterogeneity. In our study, 99% of the participants were Dutch whites and represent an ethnically homogeneous and representative sample of the population from The Netherlands.

We could not replicate the association between *ESR1 PvuII-XbaI* haplotype 1 and late AMD in our prevalent data. The possibility exists that our associations are a chance finding. However, the Rotterdam Study is a large study with a population-based prospective design, which increases the reliability of the associations found. We hypothesized that selective mortality may be involved. Persons with one or two copies of *ESR1* haplotype 1 have a higher risk of AMD, and if these persons also die earlier, it could explain the discrepancy between prevalent and iAMD. An independent incidence study is needed to confirm our findings.

How could these *ESR1* SNPs influence the risk of iAMD? A hypothesis regarding functionality of these polymorphisms is that expression of ESR1 is changed through altered binding of transcription factors, perhaps as a direct result of the *PvuII* and/or *XbaI* polymorphisms or through linkage disequilibrium with one or more functional sequence variations elsewhere in the *ESR1* gene within the linkage disequilibrium block. In support of this hypothesis, it was recently demonstrated that the *PvuII* C-allele produces a functional binding site for the transcription factor B-myb and the *PvuII* T-allele eliminates this site, suggesting a potential functional polymorphism.[35] The presence of this allele may result in a substantially lower ESR1 transcription or production of isoforms with different features; therefore, if a decreased amount of ESR1 is present or ESR1 with an altered sensitivity for estrogen, estrogen signaling may be less effective. This resembles a situation in which estrogen activity is decreased.[15, 35] Carriers of the T-allele would be at higher risk for diseases associated with low estrogen levels, such as AMD.

Recently an association was found between *ESR1 PvuII-XbaI* haplotype 1 and decreased estradiol levels in a small randomly selected subset of postmenopausal women (n = 631) in the Rotterdam Study.[17] We could not analyze the association between iAMD and estradiol levels due to low power. It was hypothesized that the

lower ESR1 expression caused by the *PvuII* T-allele leads to a lower expression of an enzyme in the estrogen synthesis pathway and thus reduces estrogen levels.[17] In all men estrogen serum levels are substantially higher than in postmenopausal women and this could partially mask the genotypic reduction. This fits with the fact that *ESR1 PvuII-XbaI* haplotype 1 was the most strongly related with iAMD in the women. Lower serum levels of estrogen in men and especially in postmenopausal women, particularly if they have one or two copies of this haplotype, could lead to reduced transcription of the ESR1.[18] Whether these *ESR1* variants have functional consequences must be investigated, as we lack definite evidence at this moment. In addition, we could not detect an association between HRT and iAMD, probably due to the low number of women taking HRT (n = 69) at baseline.[36]

Several studies have demonstrated the presence of ERs in human, bovine, and rat retinas, principally in the retinal pigment epithelium and choroid.[18-20] Retinal pigment epithelium ERs are functional, and transcriptional active and estrogen-mediated regulation of genes is important in regulating the turnover of extracellular matrix and in maintaining its basement (Bruch's) membrane.[19] Dysregulation in the production of matrix metalloproteinase-2, a gelatinase regulated by estrogens that degrades extracellular matrix components, is thought to contribute to the formation of deposits around Bruch's membrane.[12, 37] Because *ESR1* haplotype 1 was not associated with early iAMD, we assume that this haplotype influences more the progression of early AMD toward wet AMD instead of the initiation of drusen. Deposits around Bruch's membrane may promote a proangiogenic response, by binding to integrins or by modifying integrin expression on endothelial cells. This response stimulates the growth of choroidal neovascularisation,[38] the basic mechanism of wet AMD.

In conclusion, we showed an increased risk of wet iAMD in persons with one or two copies of *ESR1 PvuII-XbaI* haplotype 1. This association was not explained by additional risk factors, marking *ESR1 PvuII-XbaI* haplotype 1 as a possible independent risk factor for wet iAMD. This study supports a pathophysiological role of estrogen in the development of AMD.

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3.2

Insulin-like growth factor-1 gene polymorphism and risk of aging macula disorder

ABSTRACT

Background/aims: Pro-angiogenic factors are believed to play a role in the pathogenesis of choroidal neovascularization. Insulin-like growth factor (IGF-1) is a known pro-angiogenic factor that participates in ocular neovascularization. The purpose of our study was to examine if there exists an association between a micro satellite repeat polymorphism in the promoter region of the *IGF-1* gene, which is associated with serum IGF-1 levels, and incident aging macula disorder (iAMD).

Methods: In the Rotterdam Study, a prospective population-based cohort study of participants aged 55 years and older, associations between this polymorphism in the *IGF-1* gene and early or late incident AMD (iAMD) were studied in 4821 participants. After a mean follow-up time of 7.7 years, 591 cases of early and 99 cases of late iAMD were identified. We used Cox proportional hazards regression to estimate hazard ratios (HRs) and corresponding 95% confidence intervals (CIs).

Results: We did not find an association between this polymorphism in the *IGF-1* gene and risk of early (HR 1.11; 95% CI 0.84-1.47) or late (HR 0.97; 95% CI 0.50-1.90) iAMD. In particular no association was found with wet iAMD (HR 0.96; 95% CI 0.42-2.24).

Conclusion: Our findings do not support involvement of genetic variation in the *IGF-1* gene in the development of AMD, and especially not of wet AMD.

INTRODUCTION

Age-related macular degeneration is one of the over 20, often descriptive, names for an affliction known for over 130 years,[1] according to the pathophysiological thinking at a certain period. We prefer to name this now aging macula disorder, also abbreviated as AMD.[2] At present treatment options are limited and are restricted to patients with wet AMD, the neovascular form of late AMD.[3] Vascular growth factors are major effectors in this process. It is assumed that angiogenic growth factors produced by the RPE, such as insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF) play a role in the development of choroidal neovascularisation.[4-6] IGF-1 stimulates VEGF expression in RPE cells.[7] Synthesis of IGF-1 and its receptor, and IGF-1 receptor protein were found in ocular neovascular membranes of patients with AMD.[6] This makes the *IGF-1* gene a candidate gene for especially wet AMD.
IGF-I is a ubiquitous polypeptide that regulates growth, differentiation and proliferation of many types of cells, including human RPE cells. It is the product of the *IGF-1* gene, which has been mapped to chromosome 12.[8] A polymorphism in the *IGF-1* gene 5'promoter region of the *IGF-1* gene is associated with circulating serum IGF-1 levels.[9] This polymorphism contains a variable length of a cytosine-adenosine (CA)-repeat sequence. Homozygous carriers of the most common 192 bp allele had the highest serum levels, while its absence was significantly associated with 20% lower circulating IGF-1 levels.[9] Serum levels of IGF-1 are influenced by various factors such as age, growth hormone, insulin, diet and physical activity.[10] This implies that this polymorphism in the *IGF-1* gene could reflect chronic exposure to IGF-1 throughout the body more accurately than an incidental measurement of IGF-1 levels.
We set out to test our hypothesis that persons homozygous for the 192 bp allele would be at higher risk for AMD and especially for wet AMD.

METHODS

Population

The Rotterdam Study is a prospective, population-based cohort study of cardiovascular-, locomotor-, neurologic- and ophthalmologic diseases in the elderly.[11]
In summary, all inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands were invited to participate in the study. Of the 10,275 eligible individuals, 7983 participated (78%). The ophthalmologic part of the study started after screening of the participants had begun, leading to 6780 ophthalmic participants, also with 78% response rate. The Rotterdam Study was approved by the medical ethics committee of the Erasmus University and written informed consent was obtained

from all participants. Baseline examinations, including a standardized home interview and physical examination at the research center took place between March 1990 and July 1993. Three follow-up examinations took place from September 1993 to the end of 1994, from 1997 to 1999 and from 2000 to the end of 2004.

AMD definition

To diagnose AMD, 35° color photographs were taken of the macular area of each eye (TRV-50VT fundus camera, Topcon Corporation, Tokyo, Japan) after dilatation of the pupils with tropicamide 0.5% and phenylephrine 5%. The fundus images and the digitized images from the last follow-up examination[12] were graded with 12.5x magnification according to the International Classification and Grading System for AMD.[13] In this system, all AMD fundus signs within a standard circle (diameter 6 mm) around the fovea are recorded. We deviated from this system by changing the name age-related maculopathy in AMD. Two well-trained graders, each having 11 years of experience, graded the fundus images masked for most determinants of the participants. The grading procedures and graders were identical at baseline and at follow-up.[12]
We next categorized eyes into five mutually exclusive stages 0 to 4 according to fundus signs that had an increasing risk of late AMD. No AMD was defined as stage 0, no signs of AMD at all or only hard drusen (< 63 µm), and stage 1 soft distinct drusen (≥ 63 µm), or only pigmentary abnormalities. Because many participants with only one large druse or one hyperpigmentation in this system are classified as stage 1 and because we wanted to separate participants with marked AMD from those with only limited signs, in the present analyses, we considered stage 1 as no AMD. Thus, we classified as early AMD stage 2, soft indistinct drusen (≥ 125 µm) or reticular drusen only, or soft distinct drusen (≥ 63 µm) with pigmentary abnormalities, and stage 3, soft indistinct drusen (≥ 125 µm) or reticular drusen with pigmentary abnormalities. Stage 4 was similar to late AMD with a dry and a wet form. Dry AMD was defined as any sharply demarcated round or oval area of apparent absence of the RPE, larger than 175 µm, with visible choroidal vessels, and no wet AMD. Wet AMD was defined as the presence of a serous or hemorrhagic detachment of the RPE and/or a sub retinal neovascular membrane and/or sub retinal hemorrhage, and/or periretinal fibrous scarring, even with patches of dry AMD. A person was classified according to the highest stage of AMD in either eye and in case both dry and wet AMD were present, as wet AMD. Early incident (i)AMD was defined as no AMD at baseline and early AMD in at least one eye at follow-up. Late iAMD was classified as either no or early AMD at baseline and presence of late AMD in either eye at follow-up. Lesions that were considered to be the result of other disease, such as diabetic retinopathy, chorioretinitis, high myopia, trauma, angioid streaks, or photocoagulation for reasons other than for wet AMD, were excluded from AMD diagnosis.

Measurements

Information on all potential confounders was collected at baseline. During a home interview, participants were asked for their smoking habits by trained research assistants. Smoking was categorized as current, past or never smoker. Systolic and diastolic blood pressures from the right upper arm were measured twice with a random zero sphygmomanometer with the participant in a sitting position. The average of these two measurements was used to determine blood pressure levels. Body mass index was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Diabetes mellitus was considered to be present when persons currently used oral blood-glucose-lowering medication or insulin, or had a non-fasting or post-load glucose level above 11.0 mmol/L.

Genotype determinations

Polymerase chain reaction (PCR) using genomic DNA from peripheral white blood cells was performed using oligonucleotide primers designed to amplify the polymorphic $(CA)_n$ repeat 1 kb upstream from the transcription site of the *IGF-1* gene.[9] Earlier, we identified 10 different alleles in the promoter region of the *IGF-1* gene in a sample of 900 persons of the Rotterdam Study.[9] Of these 88.4% were homozygous or heterozygous for a 192-bp allele, suggesting that this is the common allele from which all other alleles originated. Consequently, the frequency of the other nine alleles was low. Based on this observation, using the common allele, our study population was divided into three possible genotypes: carriers homozygous for the 192 bp allele (reference group), carriers heterozygous for the 192 bp allele and non-carriers of the 192 bp allele.

Genotypes of complement factor H (CFH Y402H) SNP (1277 T/C, rs1061170), were determined in 2-ng genomic DNA extracted according to standard procedures from leukocytes with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California).

Population for analysis

At baseline, gradable fundus transparencies were available on 6418 participants, of whom 476 (7.4%) had early AMD and 106 (1.7%) late AMD. Of the 6312 persons at risk for any AMD, 4914 (77.9% of those at risk) participated in at least one follow-up examination. Persons did not participate in follow-up examinations due to refusal, death or loss to follow-up. Our study population consisted of 4821 (76.4% of those at risk) participants on whom blood specimens were available for IGF-1 genotyping. Genotype data were missing for persons who at baseline only had an interview and did not visit the research center, refused blood sampling, or of whom no blood was available due to random logistic reasons.

Data analysis

The observed genotype distributions were compared using a Pearson's chi-square test to determine if they were in Hardy Weinberg equilibrium. General characteristics of the total study population, stratified by genotype, were compared using one-way analysis of variance (ANOVA) for continuous variables, and Pearson's chi-square for dichotomous ones. We used Cox proportional hazards regression analysis to estimate relative risks and 95% confidence intervals (95% CIs). Follow-up time in years was used as time axis of the model. Hazard ratios can be interpreted as relative risks. Analyses were initially adjusted for age and sex. To account for potential confounding additional adjustments were made for smoking and the complement factor H Y402H SNP, major risk factors associated with AMD.[14-17] All analyses were carried out using SPSS 11.0 (SPSS Inc., Chicago, USA).

RESULTS

During a mean follow-up time of 7.7 years (range 0.3 to 13.9 years), 690 cases of iAMD were identified, of which 591 (7.4%) had early iAMD and 99 (1.7%) late iAMD. Table 1 presents the baseline characteristics of the 4821 participants for each genotype group. Based upon the genotype classification, 2127 (44.1%) participants were homozygous, 2117 (43.9%) were heterozygous for the 192-bp allele and 577

Table 1: Baseline characteristics of 4821 participants at risk for AMD, stratified by IGF-1 genotype

	Homozygous carriers 192-bp allele		Heterozygous carriers 192-bp allele		Non-carriers 192-bp allele		p-value
Numbers (%)	2127	(44.1)	2117	(43.9)	577	(12.0)	
Age, years	67.1	(7.7)*	66.9	(7.6)	67.3	(8.0)	0.4
Sex, female (%)	1238	(58.2)	1237	(58.4)	343	(59.4)	0.9
Smoking status (%)							
Current	489	(39.9)	489	(41.8)	108	(35.6)	0.1
Past	882	(54.5)	925	(57.6)	270	(58.1)	0.1
Diabetes mellitus (%)	189	(9.1)	189	(9.2)	49	(8.6)	0.9
Systolic blood pressure, mm Hg	137.8	(21.6)	137.6	(21.4)	137.2	(21.7)	0.8
Diastolic blood pressure, mm Hg	73.8	(11.0)	74.1	(11.3)	73.1	(10.9)	0.2
Body Mass Index, kg/m^2	26.4	(3.7)	26.3	(3.6)	26.3	(3.6)	0.5
CFH Y402H SNP (%)							
Heterozygous	868	(50.9)	862	(51.6)	225	(50.1)	0.8
Homozygous	234	(21.9)	241	(22.9)	64	(22.2)	0.9

* Means with standard deviations in parentheses, unless otherwise indicated.

Table 2: Associations between *IGF-1* genotypes and incident early or late AMD

192-bp allele	Total participants	Cases (%)		Model 1* Hazard ratio (95%CI)	Model 2** Hazard ratio (95%CI)
Early AMD					
Homozygous carriers	2086	243	(11.6)	1.00 (reference)	1.00 (reference)
Heterozygous carriers	2073	266	(12.8)	1.14 (0.95 - 1.35)	1.12 (0.93 - 1.35)
Non-carriers	565	74	(13.1)	1.15 (0.89 - 1.49)	1.11 (0.84 - 1.47)
Late AMD					
Homozygous carriers	1884	41	(2.2)	1.00 (reference)	1.00 (reference)
Heterozygous carriers	1851	44	(2.4)	1.16 (0.76 - 1.77)	0.96 (0.61 - 1.50)
Non-carriers	503	12	(2.4)	1.09 (0.57 - 2.07)	0.97 (0.50 - 1.90)

* Adjusted for age and sex

** Additionally corrected for smoking and CFH Y402H SNP carrier status

Table 3: Associations between *IGF-1* genotypes and incident dry or wet late AMD

192-bp allele	Total participants	Cases (%)		Model 1* Hazard ratio (95%CI)	Model 2** Hazard ratio (95%CI)
Dry AMD					
Homozygous carriers	1858	15	(0.8)	1.00 (reference)	1.00 (reference)
Heterozygous carriers	1826	19	(1.0)	1.37 (0.70 - 2.70)	0.94 (0.45 - 1.95)
Non-carriers	495	4	(0.8)	0.96 (0.32-2.90)	0.91 (0.30 - 2.76)
Wet AMD					
Homozygous carriers	1869	26	(1.4)	1.00 (reference)	1.00 (reference)
Heterozygous carriers	1832	25	(1.4)	1.03 (0.60 - 1.79)	0.95 (0.54 - 1.67)
Non-carriers	499	8	(1.6)	1.14 (0.52 - 2.52)	0.96 (0.42 - 2.24)

* Adjusted for age and sex

** Additionally corrected for smoking and CFH Y402H SNP carrier status

(12.0%) were classified as non-carriers. Genotype distributions were in Hardy-Weinberg Equilibrium. Table 2 shows that the presence of the 192-bp allele was not significantly associated with early or late iAMD. Because we particularly expected an association between wet iAMD and presence of the 192-bp allele, we stratified late iAMD in the dry and wet forms. Table 3 demonstrates that the presence of the 192-bp allele was associated with neither of them.

DISCUSSION

We found that a polymorphism in the *IGF-1* gene was no risk factor for early or late iAMD. Also no indication could be found that participants who were homozygous

for this allele, with presumably the highest serum levels of IGF-1 were at risk for wet iAMD.

Strengths of our study are its prospective population-based design, the large study sample, which increases the reliability of our findings, the standardized way in which fundus pictures were graded for diagnosing AMD, and the masking of the examiners for the determinants and confounders collected at baseline. Limitations are loss to follow-up, due to the fact that older and diseased persons were less likely to participate, resulting in a healthier study population. This may lead to an underestimation of the strength of the associations. Genetic association studies can be influenced by population heterogeneity. In our study, approximately 99% of the participants were white persons from the Netherlands and therefore represent an ethnically homogeneous and representative sample of the Dutch population. Potential confounding factors were dealt with in the analyses, but they did not substantially change the point estimates.

We can only speculate why we did not find an association between this polymorphism and iAMD. It is possible that there exists no association or that we can't find it because of loss of power and a small effect. AMD probably is a complex disease, controlled by several genes with minor effects interacting with environmental factors, thereby influencing disease expression.[18-20] Consequently the effect of a genetic polymorphism may be small and may not reach significance in a genetic association study.[21] Compared with early AMD, fewer persons developed late AMD during follow-up, so loss of power could affect our analyses, particularly our end-stage-specific analyses. If IGF-1 would influence only wet AMD and the effect of this polymorphism is small, an association might not be detected.

The pathogenesis of retinal neovascularization is better understood than the pathogenesis of choroidal neovascularization. Retinal hypoxia, due to occlusion of retinal vessels, is the driving force for retinal neovascularisation.[22] IGF-1 influences angiogenesis directly[23] or through VEGF, which is an acknowledged stimulatory factor for retinal neovascularisation.[7] VEGF is also involved in the development of choroidal neovascularisation.[24] IGF-1 as well as hypoxia are thought to stimulate expression of VEGF in RPE cells.[25, 26] RPE cells express high levels of VEGF and a potent antiangiogenic factor, the pigment epithelium-derived factor (PEDF). It is hypothesized that a critical balance between these two factors is important to prevent the development of choroidal neovascularisation.[27] Immunoreactivity of PEDF is lower in choroids of AMD patients compared with that in control persons, and VEGF immunoreactivity is similar in AMD patients and controls.[28] The vitreous of patients with choroidal neovascularization due to AMD contains lower levels of PEDF compared to that of age-matched controls; this was not found for VEGF.[29] It might be possible that a

decline in PEDF levels is more important in the pathogenesis of wet AMD, than an increase in VEGF levels.

In this population-based cohort of elderly aged 55 years and older a polymorphism in the *IGF-1* gene, was not a risk factor for iAMD and particularly not for wet AMD. These results do not support our hypothesis that this polymorphism contributes to the genetic susceptibility to AMD.

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3.3

Polymorphisms in the vascular endothelial growth factor gene and risk of aging macula disorder

The Rotterdam Study

ABSTRACT

Purpose: Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis and a target for inhibition therapy in wet aging macula disorder (AMD). The purpose of our study was to examine whether genetic variation in the *VEGF* gene is associated with AMD and, especially, with its wet end stage.

Methods: In this prospective population-based cohort study among men and women aged 55 years and over, AMD was classified according to the modified International Classification System on fundus color images. We determined genotypes and haplotypes for three functional *VEGF* single nucleotide polymorphisms (SNPs): C-2578A, G-1154A, and G-634C. Cox proportional hazards regression analyses were used to investigate possible associations between the individual SNPs and incident AMD (iAMD). We used the program Haplo.Stats to test the associations between *VEGF* gene haplotypes and iAMD.

Results: Of 4228 participants at risk for early or late iAMD, for whom blood specimens were available for *VEGF* genotyping, 514 developed early iAMD and 89 late iAMD (35 dry and 54 wet) after a mean follow-up of 7.4 years. None of the SNPs showed a significant association with early or late iAMD, especially not with wet iAMD. Haplotype analyses also detected no associations.

Conclusion: Our a priori hypothesis that three common SNPs in the *VEGF* gene would be a risk factor for AMD, especially the wet form, could not be confirmed.

INTRODUCTION

Early fundus signs of aging macula disorder (AMD), as we now prefer to call age-related macular degeneration,[1] are yellow extra cellular deposits under the retinal pigment epithelium (RPE), called drusen, and pigmentary abnormalities. It is a progressive disorder, ultimately leading to either dry (geographic atrophic) or wet (neovascular) late AMD, end stages of AMD that usually are associated with severe visual acuity loss. The dry variant of late AMD is characterized by gradual degeneration over the years of the RPE, leading to a loss of photoreceptors.[2] Wet AMD is characterized by leakage from or growth of abnormal blood vessels under the retina leading to RPE detachment or subretinal hemorrhage, resulting in fibrovascular disciform scarring. AMD is the leading cause of irreversible blindness in the Western world,[3-5] and its prevalence is estimated to double by 2020.[6] Loss of central visual function results in the inability to perform daily activities such as, reading, identifying faces, fine detailed working or driving. The etiology of AMD is still not clear. Research points to AMD as a complex disorder in which genetic and environmental factors interact.

Of the few available treatments, laser photocoagulation, photodynamic therapy or intraocular injection of vascular endothelial growth factor (VEGF) inhibitors can delay the progression of beginning wet AMD in a limited number of patients. An increased expression of VEGF was demonstrated in the RPE and choroid of postmortem eyes with early AMD.[7] Recently, VEGF inhibitors, such as pegaptanib sodium (Macugen), ranibizumab (Lucentis), and bevacizumab (Avastin) became available for wet AMD. VEGF inhibitors can prevent the development of sub retinal neovascular membranes, and decrease the permeability of their blood vessels.[8] This could delay vision loss, and even improve the visual function of patients with beginning wet AMD.

VEGF (also referred to as VEGF-A) is an endothelial cell-specific mitogen, that induces normal and abnormal angiogenesis, and is a potent mediator of vascular permeability.[9] The *VEGF* gene is located on chromosome 6p21.3 and the coding region spans approximately 14 kb.[10, 11] Alternative exon splicing produces several VEGF isoforms, named according to the number of amino acids, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ (the most common isoform), $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$.[12] The $VEGF_{121}$, $VEGF_{183}$, and $VEGF_{189}$ isoforms are expressed in various tissues, while $VEGF_{165}$ is the most abundantly expressed isoform. In contrast, $VEGF_{145}$ and $VEGF_{206}$ are less abundant.[13] The different isoforms have varying degrees of angiogenic properties; it has been suggested that either $VEGF_{121}$ or $VEGF_{165}$ has the strongest angiogenic properties.[13] Pegaptanib sodium inhibits only the $VEGF_{165}$ isoform, while ranibizumab and bevacizumab should inhibit all VEGF isoforms.[14] Several different single nucleotide

polymorphisms (SNPs) have been described in the *VEGF* gene, including three relatively common SNPs that may influence VEGF expression. The C-2578A (rs699947) and G-1154A (rs1570360) SNPs are located in the promoter region and the G-634C (rs2010963) SNP is located in the 5' untranslated region of the gene. These SNPs in the *VEGF* gene are associated with VEGF production,[15-17] and with several different diseases, such as diabetic retinopathy,[15] atherosclerosis,[18] chronic heart failure,[19] ankylosing spondylitis,[20] Alzheimer's disease,[21] and cancer.[22-24]
VEGF plays a central role in the regulation of angiogenesis. Therefore, we hypothesized that individual functional *VEGF* SNPs and haplotypes would be associated with early and especially wet late AMD.

METHODS

Population

The Rotterdam Study is an ongoing prospective, population-based cohort study investigating the incidence and determinants of chronic disabling diseases in the elderly. All inhabitants aged 55 years or older living in a suburb of Rotterdam, the Netherlands were invited.[25] Of the 10,275 eligible individuals, 7983 (78%) participated. The ophthalmologic part of the study started after screening of the participants had begun, leading to 6780 (also 78% response) ophthalmic participants. The tenets of the Declaration of Helsinki were followed and the appropriate medical ethics committees approved of the study. All participants signed an informed consent and gave permission to retrieve information from medical records. Baseline examinations, including a home interview and physical examinations at the research center, took place from 1990 till 1993 and were followed by three examinations from 1993 to 1994 (response rate 88%), from 1997 to 1999 (response rate 80%) and from 2000 till the end of 2004 (response rate 74%).

AMD definition

For the diagnosis of AMD, 35° color pictures were taken of the macular area of each eye (Topcon TRV-50VT fundus camera, Topcon Corporation, Tokyo, Japan) after dilatation of the pupils with tropicamide 0.5% and phenylephrine 5%. These transparencies, and, in the last follow-up, digitized images, were graded with 12.5x magnification according to the International Classification and Grading System [26] by the same two trained professionals grading AMD from baseline on, who were masked for all other determinants.[27, 28] In this system, all AMD fundus signs within a standard circle (diameter 6 mm) around the fovea are recorded. We only changed the terminology from early and late ARM in early and late AMD and categorized the

range of AMD fundus signs into five mutually exclusive stages 0 to 4 that had an increasing risk of late AMD.[28]

No AMD was defined as stage 0, no signs of AMD at all or only hard drusen (< 63 μm), and stage 1 as soft distinct drusen (≥ 63 μm), or pigmentary abnormalities. Because for our analyses we wanted to have a clear separation between participants with no AMD and the numerous participants with only one or two soft distinct drusen classified as stage 1, we considered stage 1 as no AMD in the present analyses. We included as early AMD stage 2, soft indistinct drusen (≥ 125 μm) or reticular drusen only or soft distinct drusen (≥ 63 μm) with pigmentary abnormalities, and stage 3, soft indistinct drusen (≥ 125 μm) or reticular drusen with pigmentary abnormalities. Stage 4 was similar to late AMD and was subdivided in dry (geographic atrophy) and wet (neovascular) AMD.[28] A person was classified according to the highest stage of AMD in either eye and in case of both dry and wet AMD in either eye as wet AMD. Early incident AMD (iAMD) was defined as no AMD at baseline and early AMD in at least one eye at follow-up. Late iAMD was classified as either no or early AMD at baseline and presence of late AMD in either eye at follow-up. Lesions that were considered to be the result of other disease, such as diabetic retinopathy, chorioretinitis, high myopia, trauma, angioid streaks, or photocoagulation for reasons other than wet AMD, were excluded from AMD classification.

Genotyping

All participants were genotyped for the C-2578A, G-1154A, and G-634C SNPs of the *VEGF* gene. These polymorphisms have been described at http://www.ncbi.nlm.nih.gov/SNP under the identification numbers rs699947 (C-2578A), rs1570360 (G-1154A), and rs2010963 (G-634C). Genotyping was performed, regardless of disease status, on baseline blood samples that were acquired by venapuncture and stored at –80°C. DNA was isolated according to standard procedures. Genotypes were determined in 2-ng genomic DNA using Taqman allelic discrimination assays. Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. For details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384 wells format. We also determined haplotypes, to enhance genetic resolution. Haplotype alleles present in the population were inferred by means of the haplo.em function of the program Haplo.Stats (http://cran.r-project.org/src/contrib/Descriptions/haplo.stats.html), which computes maximum likelihood estimates of haplotype probabilities.[29, 30] Haplotypes were considered rare when their frequency was 1% or less. This resulted in four common haplotypes that describe > 99% of our population. Haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population: coding from C-2578A, G-1154A, and G-634C, haplotype 1 = C-G-C, 2 = A-A-G, 3 = A-G-G and 4 = C-G-G.

Assessment of confounders

Information on all potential confounders was collected at baseline. During a home interview, trained research assistants asked participants for their smoking habits. Smoking was categorized as current, past or never smoker. Systolic and diastolic blood pressures were measured twice at the right brachial artery in a sitting position with a random zero sphygmomanometer. The average of these two measurements was used to determine blood pressure levels. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Non-fasting blood samples were obtained from all participants. Serum total cholesterol and high-density lipoprotein (HDL) levels were measured by an automated enzymatic procedure. Genotypes of the complement factor H (CFH Y402H) SNP (1277 T/C, rs1061170), were determined in 2-ng genomic DNA extracted according to standard procedures from leukocytes with the Taqman allelic discrimination assay (Applied Biosystems, Foster City, California).

Population for analysis

At baseline, gradable fundus transparencies were available on 6418 participants, of whom 476 (7.4%) had early AMD and 106 (1.7%) late AMD. Of the 6312 persons at risk for any AMD, 4914 (77.9% of those at risk) participated in at least one follow-up examination. Persons did not participate in follow-up examinations due to refusal, death or loss to follow-up. Our study population consisted of 4228 (67.0% of those at risk) participants from whom blood specimens were available for *VEGF* genotyping. Genotype data were missing for persons who at baseline only had an interview and did not visit the research center, refused blood sampling, or of whom no blood was available due to random logistic reasons.

Data analysis

The observed genotype distributions were compared using Pearson's chi-square test to determine if they were in Hardy Weinberg equilibrium. The Common allele was defined as the allele with the highest frequency. Persons homozygous for the common allele were used as the reference category. Cox proportional hazards regression analyses were used to investigate possible associations between the individual C-2578A, G-1154A, and G-634C SNPs, and iAMD. Follow-up time in years was used as the time axis of the model. All analyses were adjusted for age and sex, and were performed using SPSS 11.0 (SPSS Inc., Chicago, USA). To test the association of *VEGF* gene haplotypes and iAMD, we used the program Haplo.Stats.[29, 30] The probability for each haplotype pair in each individual was assigned and next an individual's phenotype was directly modelled as a function of each inferred haplotype pair, weighted by their estimated probability, to account for haplotype ambiguity. The haplo.glm function of Haplo.Stats was used to investigate these associations by computing

odds ratios. The most frequent haplotype in our population served as the reference category. All analyses were adjusted for age, sex and follow-up time. Haplo.em and haplo.glm were implemented in the Haplo.Stats software using the R language.

RESULTS

Table 1 shows the baseline characteristics of the study population on whom genotype data of the *VEGF* C-2578A, G-1154A, and G-634C SNPs were available. After

Table 1: Baseline characteristics of the study population at risk for AMD (n = 4228)

Variable		
Age, years	67,0	(7.6)
Sex, female (%)	2456	(58.1)
Smoking status (%)		
Never	1397	(33.3)
Former	1837	(43.4)
Current	956	(22.8)
Diabetes Mellitus (%)	354	(8.4)
Body Mass Index, kg/m^2	26.3	(3.6)
Systolic blood pressure, mm Hg	137.6	(21.4)
Diastolic blood pressure, mm Hg	73.8	(11.0)
Total cholesterol, mmol/L	6.7	(1.2)
HDL cholesterol, mmol/L	1.3	(0.4)
Complement Factor H Y402H SNP (%)		
Non-carrier	1781	(42.9)
Heterozygous	1850	(44.6)
Homozygous	519	(12.5)
C-2578A SNP (%)		
CC	1068	(25.3)
CA	2095	(49.6)
AA	1065	(25.2)
G-1154A SNP (%)		
GG	1911	(45.2)
GA	1832	(43.3)
AA	485	(11.5)
G-634C SNP (%)		
GG	1825	(43.2)
GC	1939	(45.9)
CC	464	(11.0)

* Means and standard deviations between brackets, unless otherwise indicated

Table 2: SNPs in the *VEGF* gene and the risk of early or late iAMD, stratified by type of late iAMD.

Hazard ratios adjusted for age and sex (95% CI)				
	Early iAMD	Late iAMD	Dry iAMD	Wet iAMD
VEGF SNP				
C-2578A				
CC	1.00	1.00	1.00	1.00
CA	0.99 (0.78-1.26)	0.83 (0.47-1.46)	0.86 (0.33-2.22)	0.80 (0.39-1.62)
AA	0.94 (0.77-1.17)	0.82 (0.50-1.35)	1.02 (0.46-2.27)	0.71 (0.38-1.32)
G-1154A				
GG	1.00	1.00	1.00	1.00
GA	0.93 (0.78-1.12)	0.78 (0.50-1.22)	1.04 (0.52-2.09)	0.64 (0.36-1.14)
AA	0.81 (0.60-1.09)	0.87 (0.43-1.72)	0.76 (0.22-2.62)	0.91 (0.40-2.09)
G-634C				
GG	1.00	1.00	1.00	1.00
GC	0.96 (0.80-1.15)	1.00 (0.64-1.56)	1.17 (0.59-2.32)	0.91 (0.51-1.62)
CC	1.02 (0.77-1.35)	1.00 (0.50-2.00)	0.52 (0.12-2.29)	1.32 (0.59-2.93)

Table 3: Haplotypes of the *VEGF* gene and the risk of early or late iAMD, stratified by type of late iAMD.

Odd ratios adjusted for age, sex, and follow-up time (95% CI)				
	Early iAMD	Late iAMD	Dry iAMD	Wet iAMD
Haplotype 1 (C-G-C)	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Haplotype 2 (A-A-G)	0.93 (0.79-1.10)	0.93 (0.64-1.36)	1.06 (0.59-1.93)	0.87 (0.54-1.40)
Haplotype 3 (A-G-G)	1.10 (0.91-1.34)	0.93 (0.59-1.48)	0.96 (0.46-2.02)	0.92 (0.52-1.64)
Haplotype 4 (C-G-G)	1.00 (0.82-1.22)	1.11 (0.72-1.73)	1.30 (0.66-2.56)	1.02 (0.58-1.79)

an average follow-up time of 7.4 (SD 3.9) years, 603 (14.3%) persons had iAMD, 514 (12.2%) early iAMD, and 89 (2.1%) late iAMD, of whom 35 were dry and 54 wet iAMD. All genotype distributions were in Hardy-Weinberg equilibrium. Using the HapMap website (htpp://www.hapmap.org), the SNPs were found to lie in one linkage disequilibrium block. The frequencies of *VEGF* genotypes were: -2578 CC 25.3%, CA 49.6%, AA 25.2%; -1154 GG 45.2%, GA 43.3%, AA 11.5%; and -634 GG 43.2%, GC 45.9%, CC 11.0%. Table 2 shows that genotypes were not associated with early or late iAMD. Because we particularly expected an association between genotypes and wet iAMD, we stratified late iAMD into the dry and wet forms. Genotypes were associated with neither (Table 2). Additional adjustment for smoking, systolic and diastolic blood pressure, total and HDL cholesterol, body mass index, and the complement factor H Y402H SNP did not essentially change our results (data not shown). Haplotype alleles were present in the following frequencies: haplotype 1 (C-G-C) in 33.8%, haplotype 2 (A-A-G) in 33.0%, haplotype 3 (A-G-G) in 16.9%, haplotype 4 (C-G-G) in 16.1%, and the remaining haplotypes in less than 0.2%. *VEGF* haplotypes were neither associated with early or late iAMD, dry or wet type (Table 3).

DISCUSSION

In our cohort study, we were unable to demonstrate an association between three common SNPs in the *VEGF* gene and iAMD. We also studied haplotypes because susceptibility to complex diseases is likely attributable to multiple alleles whose collective effects may be better predicted by determining the full haplotype information.[31] Also haplotype analyses demonstrated no association. Adjusting for possible confounders did not alter these findings.

Strengths of our study are its population-based design, the large study sample, which increases the reliability of our findings, and the standardized way in which fundus pictures were graded for diagnosing AMD. In contrast to case-control studies, the prospective nature of our study makes our results less prone to survival bias. Limitations are loss to follow-up, due to the fact that older and diseased persons were less likely to participate, resulting in a healthier study population. This may lead to an underestimation of the strength of the associations. Genetic association studies can be influenced by population heterogeneity. In our study, approximately 99% of the participants were white and, therefore, represent an ethnically homogeneous and representative sample of the population from the Netherlands.

The SNPs in the *VEGF* gene studied in our study were selected because they were reported to be associated with the production of VEGF.[15-17] Several other studies investigated whether these SNPs were associated with a variety of diseases in which angiogenesis plays an important role. Although these three functional SNPs in the *VEGF* gene were not associated with iAMD, we cannot exclude that other SNPs located on the *VEGF* gene might be so. However, most other SNPs that were reported so far in the *VEGF* gene are nonfunctional. The *VEGF* gene is quite polymorphic and, therefore, when the effect of a single SNP is not large, the effect of several SNPs may determine the production of VEGF, and thus its clinical manifestations.[20] Although our study population is large, we cannot exclude that we missed small effects of the *VEGF* SNPs and haplotypes that we studied, especially in our end stage-specific analyses. Replication in other large population-based studies is therefore necessary. Another possibility is that the true associations between these SNPs and AMD may not be conspicuous because of unknown gene-gene or gene-environment interactions.[32] That is why we stratified the individual SNP analyses on smoking and CFH, two major risk factors for AMD, but found no associations (data not shown).

Two more studies recently examined the association between SNPs in the *VEGF* gene and AMD. In a family-based and case-control dataset linkage was found.[33] Their *VEGF* SNP hCV8311614 is identical to the G-634C (rs2010963) SNP, also known as C+405G. They also found a significant association with the G-634C SNP in the *VEGF* gene and wet AMD in the case-control dataset.[33] A clinic-based case-control

study could not find an association between the individual G-634C SNP and wet AMD.[34] However, they combined nine SNPs in the promoter and 5'UTR region in order to form haplotypes, including the G-634C SNP, and found an association with wet AMD (OR = 18.24, 95% CI 2.25-148.25), although with large confidence limits. In addition, haplotype analyses of five intronic SNPs demonstrated two haplotypes associated with wet AMD and one haplotype associated with the control group.

In conclusion, we did not find evidence for a role of three functional SNPs (C-2578A, G-1154A, and G-634C) or reconstructed haplotypes in the *VEGF* gene in the development of AMD. Analyzing additional SNPs in the *VEGF* gene deserves further attention in other large population-based studies, because interactions between different *VEGF* SNPs or haplotypes resulting in wet AMD cannot be ruled out for the moment.

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Chapter 4

General Discussion

GENERAL DISCUSSION

The main objective of the studies described in this thesis was to identify environmental and genetic factors that are involved in the pathogenesis of AMD. All studies were performed within the context of the Rotterdam Study, a large prospective population-based cohort study in participants aged 55 years and older. In this final chapter, I will discuss some of the methodological issues in clinical and genetic epidemiology, summarize the main findings with their clinical relevance and provide suggestions for future research topics.

METHODOLOGICAL CONSIDERATIONS

Clinical epidemiology

Using a large prospective population-based cohort has many advantages. Because of the prospective design, in which the assessment of the risk factor occurred before the onset of the disease, a temporal relationship can be determined. This supports causality, given that possible biases are adequately dealt with. Our follow-up duration was long, and extensive information on several potential confounders was available on all participants.

Precision determines the reproducibility of measurement of the point estimates. The large cohort increases the reliability of the associations found, by minimizing random error, or increasing precision. After stratification by type of end stage, the number of people who developed dry and wet late incident AMD (iAMD) was limited, therefore precision could be a concern in our end stage-specific analyses.

The validity of a study can be divided into external, which refers to generalizability, and internal, which involves restricting systematic error or bias. External validity can only be addressed if internal validity is established. A study is internally valid if it represents the truth for the individuals studied, and the results cannot be explained by bias or confounding.

In general, three types of bias can be identified: selection bias, information bias, and confounding bias. Selection bias occurs if the relation between exposure and disease is different for persons who participate compared to those who are theoretically eligible, including non-participants. Of the 10,275 eligible individuals at baseline, 7983 (78%) participated. The ophthalmologic part of the study started after screening of the participants had begun, leading to 6780 (also 78% response) ophthalmic participants. We have a good response rate, thereby limiting the possibility of selection bias. In cohort studies, loss to follow-up is another important source for selection bias. In our study, it is particularly important because our cohort

was not continuously monitored and therefore participation at the follow-up visits was crucial. Data from medical files from ophthalmologists or general practitioners often were not detailed enough to make a diagnosis of AMD according to our criteria. Persons who refused to participate or were lost to follow-up were older and less healthy.[1] This means that we investigated a relatively healthier cohort, and this could cause an underestimation of the associations found. However, we think that the direction of the associations we found was not affected by this.

Another threat to validity is information bias, which results from misclassification of disease or determinant. This misclassification can be non-differential or differential. Non-differential misclassification, which is random, generally leads to a dilution of the effect. We made great effort to avoid this by using from baseline on the same two well-trained professionals to grade AMD in a standardized way. Differential misclassification is a distortion of findings that results from misclassification of the outcome that is related to the determinant or vice versa. This could either exaggerate or underestimate an effect. Differential misclassification of the determinant is unlikely, because in our prospective study the exposure was measured before the onset of AMD. This also holds for missing data caused by failure of equipment, absence of a research assistant, or refusal of persons to participate in specific examinations. These data were collected at baseline, and therefore their absence cannot be associated with iAMD. Differential misclassification of AMD is also unlikely, because the persons who graded AMD were masked for exposure status of the cases.

Confounding may be considered a confusion of the effect under study with that of an extraneous factor. Something can only be a confounder when it is associated with both the determinant and the outcome under study. In addition, a confounder may not be an intermediate step in the causal pathway between exposure and disease. In our studies we dealt with confounding by adjusting for potential confounders. Age is probably the most important confounder in studies investigating associations with AMD. We therefore included age as a potential confounder in all analyses. Although it is not likely that sex is associated with AMD, we considered it to be a potential confounder by convention. Because smoking and the complement factor H Y402H SNP are major risk factors associated with AMD, we also included these potential confounders in our analyses. Although we adjusted for several potential confounders, the possibility of insufficient measurement of confounders (residual confounding) or the presence of unknown confounders should always be considered in observational studies.

Genetic epidemiology

The basic goal of genetic epidemiology is to understand the role of specific genes in the etiology of a certain disease. Traditionally, the family-based study design has

been used to investigate genetic components of disease, especially in monogenetic diseases. Although a variant in the CFH gene explains a considerable number of AMD cases, AMD still is considered to be a complex disease, in which multiple genes and environmental factors play a role in the pathogenesis. To study genetic factors of complex disorders, such as AMD, the most commonly used strategy is the genetic association study. In this approach, candidate genes are selected, based on their biological plausibility in the pathogenesis of the disease, their location or because they were found in previous association studies. After selection of the candidate gene, it is tested whether a potentially interesting polymorphism in this gene occurs more frequently in cases than in controls. Because in this thesis we used this approach in identifying genetic risk factors for AMD, we will only discuss methodological considerations of genetic association studies.

The main advantage of these association studies is the ability to detect the influence of genes with moderate to small effects. In addition, unrelated persons are studied instead of families, thereby facilitating data collection substantially. On the other hand, cohorts for association studies on late-onset diseases are less often available. Potential cases may have died or did not yet show the clinical signs of AMD, while they might develop the disease in the near future leading to false-negative results.[2] Spurious associations may occur when a variant is in linkage disequilibrium with a nearby variant, which is the truly causative disease polymorphism.[3] Even in well-performed studies the question remains whether a polymorphism itself causes the disease, or the association can be explained by linkage equilibrium. An important problem in genetic association studies is inconsistency in results and absence of reproducibility of initial positive findings.[4] In general, this can be attributed to lack of statistical power, effect modification by other genetic or environmental factors, and population stratification. Complex diseases, such as AMD, are believed to result from several genes with usually individual small effects interacting with environmental risk factors. Consequently the effect of a genetic polymorphism may be small and may not reach significance in a genetic association study.[5] Because we used data from the Rotterdam Study, a large study population for our analyses, this problem may have partly been overcome. However in our end stage-specific analyses the number of cases was small and therefore we could not exclude the possibility of false-negative findings. Statistical power can also be increased by formation of haplotypes, that describe the total common variation of a gene.[6] Differences in effect size could be attributed to effect modification by other genetic or environmental factors, if these factors differ between study populations.[4] Therefore it is important to have reliable data on specific environmental risk factors, which may interact with genetic factors, when performing a genetic association study. Population stratification is an important problem in genetic association studies. When the

study population consists of a mixture of two or more subpopulations that have different allele frequencies and disease risks, associations between genotype and outcome could be confounded by population stratification.[4] By using restriction or matching by demographic background, population stratification can be controlled for. In the Rotterdam Study confounding by population stratification is not likely, because approximately 99% of the participants were white persons from the Netherlands and therefore represent an ethnically homogeneous and representative sample of the Dutch population.

When these issues are addressed, genetic association studies offer a powerful approach in identifying genetic risk factors for complex diseases.

MAIN FINDINGS AND THEIR CLINICAL RELEVANCE

Antioxidants

Oxidative stress, which refers to cellular damage caused by reactive oxygen intermediates, has been implicated in the pathogenesis of many age-related disorders such as AMD.[7, 8] The retina is especially vulnerable for damage caused by oxidative stress because of the high oxygen consumption, the cumulative exposure to light, and the high amount of polyunsaturated fatty acids and photosensitizers.[9] In the multicenter randomized placebo-controlled Age-Related Eye Disease study, supplements containing 5 to 13 times the recommended daily dose of beta carotene, vitamins C and E, and zinc significantly reduced the risk of progression to late AMD. To further explore this hypothesis, we studied dietary intake of antioxidant micronutrients, including carotenoids, vitamins, and trace elements, in relation to the risk of AMD. We found that beta carotene**,** vitamins C and E, and zinc have a protective effect on the development of AMD. We believe that based on our findings and on data from the Age-Related Eye Disease study, it is justified to give dietary advice and nutritional supplementation if dietary intake is expected to remain insufficient in elderly persons with an increased risk of AMD, such as those with early AMD or with several sibs with AMD.

CRP

It has been suggested that inflammation plays a role in the etiology of AMD.[2, 10-12] CRP is a non-specific marker of inflammatory or infectious processes. Two clinic-based cross-sectional studies and one similar longitudinal study reported associations between CRP levels and AMD.[13-15] We attempted to confirm these data in a population-based cohort study. We found that persons with a relatively high HsCRP level (> 1.73 mg/L) within the normal range, have a significant higher risk of early and late

iAMD. Reducing levels of CRP might have a favourable impact on the development of AMD. High body mass index as well as smoking increase CRP levels and moderate alcohol intake, diets with a low glycemic index, statins and multivitamins reduce them.[16, 17] By optimizing these lifestyle factors, the risk of AMD should decrease. In the future, CRP levels potentially could give clinically relevant predictive information, in addition to other known risk, in profiling the risk of AMD in individuals. At this moment we believe that further research is warranted and that a possible predictive role of serum CRP levels should be critically evaluated.

Alcohol consumption

Moderate alcohol consumption may protect against AMD, because of its positive effect on cardiovascular disease.[18-22] Conversely, alcohol itself may be a source of oxidative stress, or may interfere with mechanisms which protect against oxidative damage.[22] Several studies have investigated associations between alcohol consumption and AMD, with conflicting results.[23-31] Because of the importance of identifying modifiable risk factors for AMD we also examined this potential association. We were unable to find an association between overall or specific alcohol consumption and early or late iAMD. After stratification by type of end stage, with higher alcohol consumption, point estimates were increasing for dry iAMD and decreasing for wet iAMD. However, associations did not reach significance. The lack of consistent findings among studies suggests that it is not likely that alcohol consumption is markedly associated with AMD. Patients should be warned, however, that high alcohol consumption is not beneficial for their general health.

Estrogen receptor 1

Diminished exposure to endogenous estrogens, like in early menopause, have been associated with an increased risk of AMD and exposure to endogenous or postmenopausal exogenous estrogens reduces this risk,[32-35] although other studies refute this.[36, 37] The effect of estrogen is mediated through intracellular estrogen receptors. We examined the role of two polymorphisms in the estrogen receptor 1 (*ESR1*) gene on the risk of AMD in men and women. Recently *ESR1* haplotype 1 was associated with serum estradiol levels in postmenopausal women,[38] We demonstrated an increased risk of late iAMD, and especially wet late iAMD in persons carrying *ESR1* haplotype 1. After stratification on sex, this association was most pronounced in women. This confirms a possible pathophysiological role for estrogen in the development of AMD. The clinical relevance of this finding is less obvious at this time.

Insulin-like growth factor-1

It has been suggested that pro-angiogenic growth factors, such as insulin-like growth factor-1 (IGF-1) are involved in the development of wet late AMD.[39-41] IGF-I is a ubiquitous polypeptide that regulates growth, differentiation and proliferation of many types of cells, including human RPE cells. We investigated whether a polymorphism in the *IGF-1* gene, which is associated with circulating IGF-1 levels,[42] is also associated with AMD, particularly with wet AMD. This specific polymorphism in the *IGF-1* gene was not associated with early or late iAMD. After stratification by type of end stage we also could not demonstrate an association with dry or wet late iAMD. In case the effect of this polymorphism is small, we would not find an association, especially in our end stage-specific analyses, because of lack of power. Our study suggests that this polymorphism in the *IGF-1* gene is no major contributor to the genetic susceptibility to AMD.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a glycoprotein and is a growth factor specific for endothelial cells.[43] It induces normal and abnormal angiogenesis and is a potent inducer of vascular permeability.[44] Recently, VEGF inhibitors became available for the treatment of wet late AMD. We investigated whether three polymorphisms in the VEGF gene, associated with VEGF levels,[45-47] and several different diseases, such as diabetic retinopathy, atherosclerosis, chronic heart failure, ankylosing spondylitis, Alzheimer's disease, and cancer,[48-54] are also associated with AMD, and especially wet AMD. Neither the individual functional polymorphisms nor the constructed haplotypes showed an association with early, late dry, or wet iAMD. Our study suggests that these three SNPs are not associated with the pathogenesis of AMD.

SUGGESTIONS FOR FUTURE RESEARCH

Therapeutical strategies are still limited, although over the last decade we obtained more insight in the etiology of AMD. A major challenge in the next years is to further unravel the mechanisms responsible for the development of drusen, and dry as well as wet AMD. In this way the development of treatments that may slow down, stop, or preferably prevent the disease will be stimulated.

Only a few modifiable risk factors for AMD have been identified thus far. Exposure to risk factors for AMD might occur early in life and over a long period of time. This could explain why associations are not recognised in studies with a short follow-up time and of older persons. In order to identify environmental risk factors for AMD

it is important to study large populations with a long-term follow-up. It is often difficult to compare studies due to different classifications of exposure and AMD. Therefore studies should use standardized definitions of AMD and the exposures studied. Even large studies, such as the Rotterdam Study, suffer from the problem of relatively small numbers of persons with late AMD. A solution would be collaboration between researchers in order to pool their data. This would improve statistical power, and consequently will give more precise point estimates.

Several studies have attempted to identify susceptibility genes for AMD. Until recently, there have been relatively few breakthroughs in our understanding of the genetics of AMD. In the past year, researchers have identified a major risk variant in the complement factor H (*CFH*) gene,[55-58] and recent reports suggest that SNPs in the *LOC387715*, *C2/FB* and *HTRA1* genes are also major risk factors for AMD.[59-62] Besides CFH, genetic association studies have been mostly inconclusive. Verification of reported associations is therefore an area of interest for the future. Both positive, as well as negative studies should be replicated, preferably in large population-based cohorts. Most genetic association studies have investigated only one or two gene variants. Future studies should attempt to examine as many polymorphisms as possible while adjusting for multiple testing.[63] Genetic association studies seem to be a useful strategy in identifying genetic risk factors for AMD. However, only the genetic variation in a candidate gene is studied, so genetic loci associated with AMD which are not in linkage disequilibrium with the gene studied, remain undetected. The genome-wide association study, which is planned for all Rotterdam Study participants, might be a good approach to circumvent this disadvantage of genetic association studies. An important question is how much is already explained by known genes and how to find new genes. It remains unclear whether we have to search for common variants with small effects, or rare ones with large effects.

The finding that late AMD is more prevalent in whites than blacks combined with the fact that genotype frequencies of CFH are similar in whites and blacks, suggests that this gene alone cannot explain the pathogenesis of AMD. Previously, most studies have investigated the effect of only genetic or environmental factors. Because AMD is most likely to be a complex disease, epidemiological studies should focus their attention on gene-environmental interactions. Because of differences in genotype distributions and environmental factors across populations, studies investigating these interactions should be performed in different ethnic groups.

At this moment, there are no animal models that faithfully imitate all phases of AMD. The development of advanced animal models that capture the complex interactions between genes and environment would help to elucidate its pathogenesis and the development of therapeutic strategies. Until such complex animal models

are developed, simple animal models and in vitro models of specific characteristics of AMD are still necessary.

In conclusion, if and when the complex pathogenesis of AMD will be elucidated remains unanswered. However, considerable progress has been made over the last 25 years and promising results are emerging continuously. Better understanding of the genetics of AMD, longer follow-up from large population-based studies, and if possible clinical trails should ultimately lead to the development of therapeutic strategies which can prevent or treat AMD.

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Chapter 5

Summary / Samenvatting

Summary

Aging macula disorder (AMD) is the leading cause of irreversible blindness among persons above age 65 years in the Western world. Due to the growing elderly population it is becoming an important issue in health care. AMD affects the central part of the retina, known as the macula lutea, ultimately leading to loss of visual function. Despite intensive research in the last decades, only in a minority of the cases treatment strategies are available that can partially restore visual function. Currently, AMD is considered to be a multifactorial disease, in which environmental and genetic risk factors interact. The main objective of the research in this thesis was to identify new risk factors for this blinding disease. The studies mentioned were all embedded in the Rotterdam Study. This is a large prospective population-based cohort study among 7983 persons aged 55 years and older living in a suburb of Rotterdam, in which most potential risk factors were assessed at baseline between 1990 and 1993. At the research centre fundus photographs were made, which were graded according to an International Classification and Grading System for AMD by two trained professionals in order to identify participants with no, early or late AMD.

Chapter 1 is a brief introduction to the work presented in this thesis.

Chapter 2 deals with the relation between environmental factors and risk of AMD. In **Chapter 2.1** the relationship between diet and AMD is presented. At baseline, participants were asked about their dietary habits, and subsequently these data were converted to specific nutrient intake per day. High dietary intake of beta carotene, vitamins C and E, and zinc was associated with a reduced risk of AMD. In **Chapter 2.2** the influence of a serum marker of inflammation on the risk of AMD was investigated. Higher serum C-reactive protein levels were associated with an increased risk of AMD. This finding confirms earlier observational clinic-based studies that inflammation is involved in the pathogenesis of AMD in the general population. **Chapter 2.3** describes the relation between alcohol consumption and AMD. Moderate alcohol consumption is associated with a decreased risk of cardiovascular disease. Because vascular disease has been associated with AMD, moderate alcohol consumption could also protect against the development of AMD. On the other hand, alcohol can cause oxidative damage. Alcohol consumption seemed not to be a modifiable risk factor for AMD.

Studies between genetic polymorphisms, or common DNA sequence variations, and AMD are presented in **Chapter 3.** In the past we found in the Rotterdam Study that estrogen might be involved in the pathogenesis of AMD. Therefore in **Chapter 3.1** the association between polymorphisms in the estrogen receptor 1 (*ESR1*) gene,

related to serum estradiol levels in postmenopausal women, and AMD was investigated. Certain polymorphisms in the *ESR1* gene were associated with an increased risk of AMD, and this was most pronounced in postmenopausal women. In **Chapter 3.2** the role of a cytosine-adenosine (CA) repeat polymorphism in the promoter region of the insulin-like growth factor 1 (*IGF-1*) gene, related to serum IGF-1 levels, and AMD is examined. Pro-angiogenic factors, such as IGF-1, are believed to play a role in the pathogenesis of AMD. No association was found between this polymorphism and AMD. Vascular endothelial growth factor (VEGF) induces angiogenesis, and is a target for therapeutic inhibition of wet AMD. In **Chapter 3.3** the role of three polymorphisms in the *VEGF* gene, associated with several different diseases and VEGF production, on the development of AMD was investigated. No association was found between these three polymorphisms in the *VEGF* gene and AMD.

In the general discussion in **Chapter 4**, several methodological issues in clinical and genetic epidemiology are commented on in order to facilitate a proper interpretation of the study results that are described in this thesis. Furthermore, I discussed the main findings and their clinical relevance and finally provided suggestions for future research on AMD.

Samenvatting

Ouderdoms macula aandoening (OMA) is de belangrijkste oorzaak van irreversibele blindheid bij mensen boven de 65 jaar in de Westerse wereld. Door de toenemende vergrijzing zal het een belangrijke kwestie in de gezondheidszorg worden. OMA tast het centrale deel van het netvlies, de macula lutea, aan en veroorzaakt in het eind stadium van OMA ernstig verlies van zicht. Ondanks recente wetenschappelijke doorbraken, zijn er maar voor een kleine groep patiënten behandelingen beschikbaar die het verlies van zicht gedeeltelijk kunnen herstellen. Volgens de huidige inzichten wordt OMA beschouwd als een multifactoriële aandoening die het gevolg is van wisselwerking tussen erfelijke aanleg en omgevingsfactoren. De doelstelling van het onderzoek beschreven in dit proefschrift, was het identificeren van nieuwe risicofactoren voor deze blindmakende ziekte. Alle studies beschreven in dit proefschrift zijn gebaseerd op data afkomstig uit het Erasmus Rotterdam Gezondheid en Ouderen (ERGO) onderzoek, internationaal bekend als "the Rotterdam Study". Dit is een groot prospectief bevolkingsonderzoek, waaraan 7.983 mannen en vrouwen van 55 jaar en ouder, wonend in de Rotterdamse wijk Ommoord, deelnemen. Bij aanvang van de studie tussen 1990 en 1993 zijn de deelnemers uitgebreid onderzocht en geïnterviewd en zijn er gegevens verzameld over verschillende potentiële risicofactoren. Op het onderzoekscentrum zijn foto's gemaakt van het netvlies, die later beoordeeld zijn door twee vakkundige medewerksters, volgens een internationaal classificatie en graderingsysteem speciaal ontwikkeld voor OMA.

Hoofdstuk 1 is een korte inleiding op het werk gepresenteerd in dit proefschrift.

Hoofdstuk 2 behandelt de relatie tussen externe factoren en het risico op OMA. In **Hoofdstuk 2.1** wordt de invloed van voeding op het risico op OMA onderzocht. Bij aanvang van de studie werd deelnemers gevraagd naar hun voedingsgewoonten, waarna uit deze data de gemiddelde consumptie van specifieke bestanddelen is berekend. Een relatief grote consumptie van bèta-caroteen, vitamine C en E en zink verlaagd het risico op OMA. In **Hoofdstuk 2.2** is de invloed van een ontstekingsmarker in het bloed onderzocht. Hogere concentraties C-reactief proteïne (CRP) waren geassocieerd met een hoger risico op OMA. Dit bevestigt de eerder in een klinisch onderzoek aangetoonde rol van ontsteking bij het ontstaan van OMA, ook in de algemene bevolking. **Hoofdstuk 2.3** beschrijft de relatie tussen alcohol inname en OMA. Matige alcohol consumptie was geassocieerd met een licht verlaagd risico op hart- en vaatziekten. Omdat OMA geassocieerd is met vaatziekten, zou matige alcohol consumptie mogelijk ook een beschermende werking kunnen hebben op de ontwikkeling van OMA. Aan de andere kant kan alcohol oxidatieve schade veroorzaken. Alcohol consumptie leek geen noemenswaardige risicofactor voor OMA.

Hoofdstuk 3 bevat enkele studies waarin associaties worden bestudeerd tussen genetische polymorfismen, of veelvoorkomende variaties in DNA volgorde, en OMA. Wij vonden in het verleden in het ERGO onderzoek dat een gebrek aan vrouwelijke geslachtshormonen, oestrogenen, een rol speelt in het ontstaan van OMA. Polymorfismen in het oestrogeen receptor 1 (*ESR1*) gen zijn gerelateerd aan lagere oestradiol spiegels in het bloed van vrouwen na de overgang. In **Hoofdstuk 3.1** is daarom de associatie tussen deze *ESR1* polymorfismen en OMA onderzocht. Bepaalde genetische variaties in het *ESR1* gen leidden tot een verhoogd risico op OMA, en in het bijzonder bij postmenopauzale vrouwen. In **Hoofdstuk 3.2** wordt de rol van een cytosine-adenosine (CA) repeat polymorfisme in het promoter gebied van het insuline-achtige groeifactor 1 (*IGF-1*) gen, wat gerelateerd is aan circulerende IGF-1 spiegels, en OMA onderzocht. Er wordt gedacht dat pro-angiogene factoren, zoals IGF-1, een rol spelen in de pathogenese van OMA. Dit polymorfisme was niet geassocieerd met de kans op OMA. Vasculaire endotheliale groeifactor (VEGF) induceert angiogenese en VEGF-remmers worden gebruikt in de behandeling van natte OMA. In **Hoofdstuk 3.3** is de rol van drie polymorfismen in het *VEGF* gen, die geassocieerd zijn met verschillende aandoeningen en de productie van VEGF, op de ontwikkeling van OMA onderzocht. Tussen deze drie polymorfismen in het *VEGF* gen en OMA is geen associatie gevonden.

In de algemene discussie in **Hoofdstuk 4** worden enkele methodologische problemen van de klinische en genetische epidemiologie besproken, om een juiste interpretatie van de onderzoeken in dit proefschrift te vergemakkelijken. Daarnaast bespreek ik de belangrijkste bevindingen en hun klinische relevantie en geef ik suggesties gegeven voor toekomstig onderzoek naar OMA.

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Sharmila

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Sharmila Sabina Boekhoorn was born on November 19th, 1978 in Schiedam, the Netherlands. She graduated in 1997 at the "Scholengemeenschap Spieringshoek" in Schiedam (Gymnasium). That same year she started her medical studies at the Erasmus University in Rotterdam. She obtained her medical degree cum laude in 2003. In July 2003, she started the work described in this thesis under supervision of Prof.dr. P.T.V.M. de Jong and Prof.dr. A. Hofman at the department of Epidemiology and Biostatistics. In 2005, she obtained a Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences (NIHES). On April 1st 2007 she started her residency in Ophthalmology at the Eye Hospital, Rotterdam (prof.dr. J.C. van Meurs).