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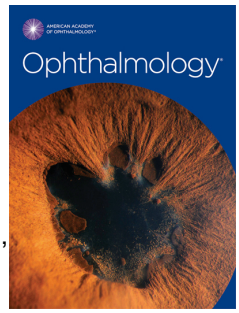

Genetic risk, lifestyle, and AMD in Europe. The EYE-RISK consortium

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Genetic risk, lifestyle, and AMD in Europe. The EYE-RISK consortium

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ABSTRACT

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

Design: Pooled analysis of cross-sectional data from the E3 consortium.

Participants: 17.174 individuals aged 45+ participating in 6 population-based cohort studies, 2 clinic based studies, 1 case-control study.

Methods: AMD was diagnosed and graded based on fundus photographs. Data on genetics, lifestyle, and diet were harmonized and completed where necessary. Minor allele frequencies and population attributable fraction (PAF) were calculated per single nucleotide polymorphism (SNP). A total genetic risk score (GRS) and pathway-specific risk scores (complement, lipid, extra-cellular matrix, other) were constructed based on the dosage of SNPs and conditional beta's; a lifestyle score was constructed based on smoking and dietary intake.

Results: The risk variants with the largest difference between late AMD cases and controls, and the highest PAFs were located in ARMS2 (rs3750846) and CHF (rs570618 and rs10922109). Both risk increasing and protective variants had the highest PAFs. Combining all genetic variants, the total genetic risk score ranged from -3.50 to 4.63, was normally distributed and increased with AMD severity. Of the late AMD cases, 1581/1777 (89%) had a positive total GRS. The complement pathway and ARMS2 were by far the most prominent genetic pathways contributing to late AMD (positive GRS 90% of late cases), but risk in three pathways was most frequent (35% of late cases). Lifestyle was a strong determinant of the outcome in each genetic risk category; unfavorable lifestyle increased the risk of late AMD at least twofold.

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

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ABBREVIATIONS

AMD = Age-related macular degeneration; **AREDS**= Age Related Eye Disease Study, **CORRBI** = Combined Ophthalmic Research Rotterdam Biobank; **EUGENDA** = European Genetic Database; **GA** = Geographic Atrophy; **GWAS**= Genome wide association study; **HRC** = Haplotype Reference Consortium; **OR** = Odds Ratio; **RPE** = retinal pigment epithelium; **RS**= Rotterdam Study; **SNP** = Single Nucleotide Polymorphism; **WARGMS**= Wisconsin Age Related maculopathy Grading System.

Keywords: Age-related macular degeneration (AMD), genetics, population, pathways, Europe

Précis: Age-related macular degeneration is driven by complement and ARMS2, but caused in most by multiple genetic pathways. Someone's genetic effect can be severely reduced by healthy lifestyle.

This article contains additional online-only material. The following should appear online-only: Figure 3, 4 and 7, Tables 1-3 and cohort descriptions.

INTRODUCTION

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

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

In this study, we aimed to investigate the contribution of genetic variants to AMD risk in Europe using data from the large European Eye Epidemiology (E3) consortium. We aimed to determine the

contribution of each disease pathway in AMD, and investigated whether lifestyle changes can reduce the risk of late AMD, in particular in individuals with a high genetic risk of AMD.

METHODS

Study population:

The E3 consortium is a European collaboration of studies with epidemiologic data on common eye disorders; a detailed description on the consortium can be found elsewhere[9]. All data on AMD were harmonized and collected in the EYE-RISK database (version 6.0). Nine studies from France, Germany, the Netherlands, and Portugal had data on AMD genotype and phenotype available for analysis, and were enrolled as a pooled dataset in the current study. The cohort descriptions of the included studies are available at External link http://www.aaojournal.org. CORRBI, MARS, and EUGENDA were clinic-based studies, the remaining were population-based (RSI, RSII & RSIII, Alienor-3C, Montrachet-3C and CES (Coimbra Eye Study)). Persons aged 45 years and older were included in the analyses; various analyses only included controls aged 75 years or older. All studies were performed in accordance with the Declaration of Helsinki for research involving human subjects and the good epidemiological practice guideline, and had written informed consent from all participants.

Clinical examination:

The phenotype of AMD was determined on fundus photographs centered on the macula; individuals received the diagnosis of the worst eye. AMD features were graded locally by clinicians or experienced graders; classifications were grouped into three severity classes. Controls did not display AMD, aside from only small drusen or only pigment irregularities; persons with early or intermediate AMD had soft indistinct (large) drusen and/or reticular drusen, with or without pigmentary irregularities, and were further referred to as intermediate AMD. Persons with late AMD had GA, or CNV. Persons with both end stages were diagnosed as CNV.

Lifestyle factors including smoking and dietary habits were assessed by questionnaire.

Genetic analyses and risk scores

AMD genetic risk variants were ascertained from the EYE-RISK/E3 database[5, 9]. Studies had used various platforms to determine the 52 known risk variants, such as whole exome sequencing, exome chip (Illumina HumanExome BeadChip), genomic SNP arrays (Illumina 550K (duo) chip or Illumina 610 quad), or Taqman assays, and a custom-made AMD genotyping platform using single molecule molecular

inversion probes (smMIPs) with next generation sequencing; the EYE-RISK genotype assay[10], see cohort descriptions. If variants had been determined by multiple methods which included direct genotyping, we used data from the latter method. When no direct genotyping was available, genotypes were dosages derived from Haplotype Reference Consortium (HRC) imputation or 1000G. Three (rs71507014, rs67538026, rs142450006) of the 52 known AMD risk variants could not be included in our analysis since genotypes were not available for multiple cohorts.

Genetic risk scores (GRS) were calculated for the 17,174 individuals for whom the five major risk variants (*CFH* rs10922109, *CFH* rs570618, *C2* rs429608, *C3* rs2230199, *ARMS2* rs3750846) were available. Complete genotype data on minor risk alleles were available in 62.3% persons; 85.1% individuals had 47/49 variants. GRS were calculated by multiplying the conditional beta of the AMD risk variant[5] with the allele dosage. Subsequently, all calculations were summed. Pathway-specific GRS were constructed in the same manner. For the complement GRS, we included all risk variants in the *CFH, CFI, C9, C2, TMEM97/VTN* and *C3* genes. For the lipid GRS, variants in *ABCA1, LIPC, CETP, APOE* were included. For the extra-cellular matrix (ECM) GRS, variants in *COL4A3, ADAMTS9-AS2, COL8A1, VEGFA* and *SYN3/TIMP3* were included. The remaining variants were included in 'other' GRS. The function of *ARMS2* was mostly considered unsettled. However, as recent evidence suggests a role in the complement pathway[11], we analyzed this gene as a stand-alone pathway GRS as well as part of the complement pathway GRS.

Lifestyle score

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

Statistical analysis

The population attributable fraction (PAF) was calculated for each variant using the formula of Miettinen *et al*.[12] PAF = Pc * ((OR−1)/OR); where OR is the odds ratio, and Pc is the proportion of exposed cases among the cases. The pooled dataset formed the basis for all analysis. We calculated the discriminative

accuracy between late AMD cases and controls for our model of genetic factors using the Saddle Point Signature software version 2.8.3 (Saddle Point Science Ltd., Worcester Park, United Kingdom) in a batch multivariate regression analysis. Results were cross-validated by the leave one out principle. Prediction performance at each iteration was quantified by counting errors of persons assigned to the wrong category (controls or cases). The dataset was fully balanced between controls and cases; the regression equations corresponded to a pseudo dataset, in which the outcome classes were equal in size but the other statistical features were identical to the true dataset. Missing values were not set to zero but imputed to the mean. Covariates were selected based on error expectation minimization.

Where appropriate, comparisons were made with Pearson chi-square test, Jonckheere-Terpstra test for ordered alternatives, or independent sample t-test. Interaction of genetic and lifestyle risk was assessed by a univariate ANOVA. Graphical outputs were constructed with GraphPad Prism 5 (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com"). Histograms and a receiver operator characteristic curve were constructed with SPSS (IBM Corp. Released 2012 IBM SPSS Statistics for Windows, Version 25.0 Amonk, NY: IBM Corp).

RESULTS

We identified a total of 17,174 individuals aged 45 years and older with data on genetics and AMD; 13,324 persons without AMD, 2,073 with intermediate AMD and 1,777 individuals with late AMD. Of the persons with late AMD, 309 had developed GA and 1,468 CNV. Age ranged from 45 to 101 years old with a mean of 68.7 years (SD 10.4), the proportion of women was 58.5%, current smoking 16.8% (*n*=2,888), former smoking 39.5% *(n*=6,786). For risk calculations, we aimed to ensure a true phenotype of no AMD, and therefore included only controls aged 75+ years (*n*=3,167)in these analyses. . The proportion of women in this subset (controls 75+ and intermediate and late AMD cases) was 61.3%, current smoking 9% (*n*=630) and former smoking 36.2% (*n*=2,541).

Single variants

First, we focused on frequency distributions of the 49 single risk variants in the three phenotype groups, and ranked variants according to frequency differences between late and no AMD (**Figure 1a**). SNPs from the complement pathway and *ARMS2* showed the largest difference in frequency between cases and controls (rs10922109, rs61818925 and rs570618 (*CFH*), rs429608 (*C2*), rs2230199 (*C3*), rs3750846 (*ARMS2*)). Among the first ten variants, five variants had a lower frequency in cases, corresponding to a protective effect on AMD. Next, we calculated the population attributable fraction (PAF) for each single

variant. *ARMS2* variant rs3750846 was associated with a high PAF (0.3) for late AMD, while variants in *CFH* exhibited both the largest PAF (0.33) (rs570618) and the largest inverse PAF (-0.37) (rs10922109) (**Figure 1b**). A similar pattern with smaller PAFs was observed for intermediate AMD. Only variant rs11080055 in *TMEM97/VTN*, showed a higher PAF for intermediate (0.063) than for late AMD (0.024). Only four (0.2% or 4/1777) late AMD cases did not carry any of the five major risk SNPs, compared to 33 (1% or 33/3167) of controls.

Genetic risk score for AMD

We subsequently combined all genetic variants in a GRS and assessed its distribution. In the population-based cohort studies (*n*= 13,194), the score ranged from -3.50 to 4.63 (mean 0.40, standard deviation (SD) 1.24) and had a normal distribution (**Figure 2a**). With respect to the distribution per phenotype, the GRS in controls ranged from -3.03 to 3.94 (mean 0.26, SD 1.16), in intermediate AMD from -3.11 to 4.71 (mean 0.83, SD 1.33), and in late AMD from -3.00 to 6.23 (mean 1.64, SD 1.32) (**Figure 2b**). Although the lowest GRS value was similar for all phenotypes, the entire distribution showed a significant increase with increasing AMD severity (Jonckheere-Terpstra test for ordered alternatives; p-value <0.0001). When stratifying late AMD into GA and CNV, slightly higher scores were noted for CNV (**Figure 2c**): GA ranged from -2.72 to 4.87 (mean 1.46, SD 1.41) and CNV ranged from -3.00 to 6.23 (mean 1.67, SD 1.30, independent sample t-test p-value=0.01). We estimated the discriminative accuracy of a score based on the 49 AMD-associated genetic variants (Supplementary **Figure 3** and **4** available at External link http://www.aaojournal.org) for identification of late AMD; the area under the curve (AUC) was 0.838. We identified a minimal set of variants by using the leave one out principle, and found an almost identical AUC (0.837) when including 27 AMD-associated variants (score is available in the Supplementary material at External link http://www.aaojournal.org).

Genetic risk scores per pathway

Next, we constructed pathway-specific GRS; for the complement, lipids, extra-cellular matrix, age-related maculopathy susceptibility 2 (*ARMS2*) and 'Other'. The complement pathway score ranged from -3.15 to 3.64 in the population-based studies, and 55% of participants scored above 0 for this pathway. The ARMS2 score ranged from 0 to 2.15 as only one risk variant determines this score. The lipid pathway had GRS ranging from -1.44 to 0.49, the ECM pathway from -0.92 to 1.46, and 36% and 33%, respectively, had a score higher than zero. The pathway 'Other' ranged from -1.06 to 1.45; 61% had a positive score.

The distribution of all pathway GRS in our total study population showed a positive shift with increasing AMD severity (Jonckheere-Terpstra test for ordered alternatives, p-value<0.0001, supplementary **Table 1** available at External link http://www.aaojournal.org and **Figure 5**), but the complement and ARMS2 GRS demonstrated the largest increase for late AMD, especially when combined (shift of mean GRS from 0.39 to 1.59).

Frequency of positive GRS

We studied the proportion of individuals with a positive (>0) GRS for each of the pathways, as this indicates more genetic risk than protection from that particular pathway. Positive GRS for all pathways were most frequent in late AMD (**Figure 6**). Positive GRS the for complement and 'other' pathways were most prevalent in all phenotypes. The largest increase per phenotype severity was found for the complement and ARMS2; the proportion of persons with positive GRS in the complement pathway rose from 51% in controls to 77% (26% increase) in late AMD cases and ARMS2 rose from 35% in controls to 65% (30% increase) in late AMD cases (Pearson Chi-Square 2-sided test, p-value <0.0001 for both). Not one pathway GRS was above zero in all late AMD cases, but 90% had a positive GRS for the combination of complement and ARMS2. Upon closer inspection of the remaining 10% (n=152), these late AMD cases did carry risk alleles in these two pathways but had a high frequency of protective variants which resulted in a GRS below zero (supplementary **Table 2** available at External link http://www.aaojournal.org). Subsequently, we examined the risk SNPs in greater detail by investigating the proportion of persons with at least one risk allele per pathway (supplementary **Figure 7**, available at External link http://www.aaojournal.org). 99% of persons with late AMD had a risk SNP in either the complement or 'Other' pathway, but this was also the case for controls. For ARMS2, lipid and ECM pathway this was less frequent.

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

Next, we calculated the distribution of pathways with a GRS above zero (see **Figure 8**). The majority of participants had two to four pathways with a GRS above zero (85%). A small proportion (7%) of individuals had a GRS in only one pathway above zero, and an even smaller proportion (1%; n=23) of individuals had a GRS below or equal to zero for all pathways.

Combining genetics with lifestyle

Data on lifestyle factors were available for a subset of the study population (n=3,525). In these subjects, we investigated the AMD lifestyle factors smoking, and dietary intake of vegetables, fruit and fish. Cases were more often current smokers (OR 1.39), consumed less vegetables (OR 0.40), less fruit (0.35) and less fish (OR 0.17, all with a p-value<0.0001, supplementary **Table 3** available at External link http://www.aaojournal.org). We composed a lifestyle score based on these variables, and stratified the score into tertiles: favorable, intermediate, and unfavorable lifestyle. For each GRS category (also tertiles) we observed that, the more unfavorable the lifestyle, the higher the risk of late AMD. Lifestyle increased the risk 2-2.3 times depending on the genetic risk. In the highest genetic risk group, the OR increased from 14.9 to 35.0 in individuals with an unfavorable lifestyle (**Figure 9**).

DISCUSSION

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

Our results need to be seen in light of the strengths and limitations of this study. An important strength was the very large number of Europeans included in this study. From the E3 consortium, we included nine studies with genetic data, i.e., population studies from the Netherlands, France, and Portugal, as well as case-control studies from the Netherlands and Germany. Data were harmonized and entered into a single database, which allowed us to perform in depth analyses on combinations of phenotype, genotype, and lifestyle in the pooled dataset. Grouping genes into pathways and calculating pathway-specific genetic susceptibility enabled us to study molecular drivers and personalized risks. A limitation of our study was the incompleteness of data on several determinants in some studies. We focused on 49 genetic variants that were individually associated with AMD[5], of which only few were rare. Hence, we

cannot elaborate on risks provided by most of the currently known rare variants. The studies providing the greater part of cases were case-control studies without follow-up data, and we were therefore restricted to cross-sectional analyses.

A positive GRS indicated more causative genetic risk than protection by genetic variants. As this was present in (2546/4044) 63% of the population, we conclude that genetic susceptibility to AMD is highly prevalent. Among cases with late AMD, the proportion of a positive GRS rose to (1581/1777) 89%. We investigated this in greater detail, and found that the five major risk alleles were absent in only 66 (1%) persons, indicating that 99% of the study population carried at least one major risk allele. By contrast, on average 2.5 major risk alleles were present among late AMD cases and were absent in only 0.2% (4/1777). A set of 27 risk variants was enough to reach discriminative accuracy 0.84 for late AMD versus no AMD. Adding more variants did not improve this further, and the AUC was in line with previous studies[13, 14]. It should be emphasized that such high discrimination based solely on genetic variants is exceptional for a complex disorder, although this is still challenging at mean GRS levels.

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

The rs3750846 (or its proxy rs10490924, A69S) variant in the *ARMS2* locus carried the highest risk of late AMD, and the second highest attribution to overall AMD occurrence in our study (Figure 1). In East Asia,

this allele is twice as common (MAF 0.40 in East Asia vs 0.19 in Europeans), but the risk of late AMD for carriers appears comparable (OR 2.94 in India vs OR 3.06 in Europe)[18, 19]. The function of *ARMS2* is subject of ongoing research. Recently, Micklisch *et al*. showed *in vitro* that ARMS2 functions as a surface complement regulator by binding to the cell membrane of apoptotic and necrotic cells, and subsequently binds properdin and activates complement[11]. This provides evidence that ARMS2 can be an initiator of complement. We considered two different scenarios for the pathway of *ARMS2*: a function in the complement pathway and as a an independent function. When regarded as a complement gene, the vast majority (90%) of late AMD had an increased genetic risk in this pathway, making complement the main driver of late AMD. As a stand-alone, *ARMS2* also provided a significant contribution, as it was present in two thirds of late AMD.

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

We further investigated the impact of the most important lifestyle factors, smoking and diet, in relation to genetic risk. As expected, persons with AMD had lower intake of vegetables, fish, and fruit, and higher rates of smoking (Supplemental Table 3)[20-26]. Together, we showed that a more unfavorable lifestyle almost doubled the risk of late AMD. This occurred in all genetic risk strata but the OR increase was most prominent in those at high genetic risk. These findings confirm previous reports from the Rotterdam Study[27, 28] and AREDS, which demonstrated interaction between single nutrients and *CFH* and *ARMS* risk variants *2*, a protective role of diet in those with a high GRS[29]. The current study analyzed a more comprehensive set of risk variants, and found that a healthy diet and non-smoking was also beneficial in persons with low genetic risk. Oxidative stress is the most recognized molecular effect of smoking in the pathogenesis of AMD[30], and antioxidants the most important contribution of a healthy diet. Oxidative stress with abundant reactive oxygen species, peroxidation of lipids, proteins, RNA, and DNA in the retina can lead to cytotoxic effects and inflammation, enhancing the development of AMD[31]. Unfortunately, a healthy diet consisting of sufficient fruits, vegetables, and fatty fish is consumed by only a minority of elderly[28], and smoking is still twice as high among those with late AMD (Supplement Table 3). This asks for more rigorous measures for prevention, and training of doctors in behavioral change techniques may be part of this.

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

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The EYE-RISK Consortium

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FIGURE LEGENDS

Figure 1A. Minor allele frequency of cases and controls for 49 AMD associated genetic variants. The variants are ranked according to the difference in allele frequencies between late AMD cases and controls, with the most discriminative variants on the left side of the graph.
B. Population attributable fraction of 49 AMD-associated genetic variants for intermediate (light blue) and late (green) AMD. CFH_rs121913059 is not included for intermediate AMD since it was too rare to make useful calculations.

Figure 2. A. Distribution of the total AMD GRS (genetic risk score) in the European population. **B.** Distributions of the total AMD GRS, top panel showing the controls (aged ≥75 years), middle panel intermediate AMD and bottom panel late AMD. **C.** Distributions of the total AMD GRS, left panel (light blue) showing the frequency of geographic atrophy (GA) for each total AMD GRS and the right panel (green) showing the frequency of choroidal neovascularization (CNV) for each total AMD GRS, both on log scale.

Figure 5 Distributions of the genetic risk scores for the complement (**A**), lipids (**B**), extra-cellular matrix (**C**), ARMS2 (**D**) and the other pathway (**E**) and complement with ARMS2 combined (**F**) in controls and late AMD cases.

Figure 6. Percentage of individuals with a GRS above zero for each of the pathways. Dark blue = the controls 75 years and older, light blue = intermediate AMD cases, green = late AMD cases. The asterisk (*) indicated statistical differences in a Pearson Chi-Square test (2-sided) with p-value <0.0001, Bonferroni correction for multiple testing is p=0.0028.

Figure 8. Distribution of late AMD cases according to pathway scores above zero, numbers inside the bars indicate the frequency.

Figure 9. Odds ratio of risk for late AMD stratified by GRS and lifestyle risk. CI = Confidence interval.

Table 1. Difference in the mean of each pathway score per AMD stage

	Complement	ARMS2	Lipid	ECM	Other	Complement+AMRS2
Controls ≥75 years	-0.01	0.4	-0.12	-0.09	0.08	0.39
Intermediate	0.29	0.58	-0.09	-0.06	0.10	0.88
Late	0.65	0.94	-0.06	-0.03	0.14	1.59
p-value*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

***** Jonckheere-Terpstra test for ordered alternatives

Table 2 Frequency of SNPs in 152 late AMD cases with complement pathway score below 0 and no ARMS2 risk allele. Sorted by frequency.

SNP	%	Freq	OR	OR Fritsche *et al*	SNP	%	Freq	OR	OR Fritsche *et al*	SNP	%	Freq	OR	OR Fritsche *et al*
CFH_rs10922109	96	146	0.40	0.38	TNFRSF10A_rs79037040	37	56	0.94	0,9	CFH_rs187328863	3	4	1.20	2.27
LIPC_rs2043085	86	131	1.06	0.87	CFH_rs61818925	36	55	0.56	0,6	ACAD10_rs61941274	3	4	0.94	1.51
C2_rs943080	74	112	0.85	0.88	PILRB_rs7803454	36	54	1.18	1,13	CFH_rs191281603	2	3	1.41	1.07
CFI_rs10033900	73	111	1.11	1.15	C2_rs429608	34	52	0.52	0,57	COL8A1_rs140647181	2	3	1.53	1.59
TMEM97_rs11080055	73	111	1.05	0.91	KMT2E_rs1142	34	51	1.17	1,11	CFH_rs148553336	1	1	0.32	0.29
ADAMTS9_rs62247658	69	105	1.14	1.14	C2_rs114254831	32	48	1.07	1,13	C2_rs144629244	1	1	1.12	1.39
C3_rs2230199	69	105	1.31	1.43	LIPC_rs2070895	30	45	0.86	0,87	C2_rs181705462	1	1	1.03	1.55
RAD51B_rs61985136	65	99	0.87	0.9	SLC16A8_rs8135665	29	44	1.25	1,14	C3_rs147859257	1	1	2.82	2.86
NPLOC4_rs6565597	59	89	1.07	1.13	CFH_rs570618	29	44	2.40	2,38	C9_rs62358361	1	1	2.00	1.8
MIR6130_rs10781182	54	82	0.99	1.11	RDH5_rs3138141	29	44	1.15	1,16	CFH_rs35292876	0	0	2.11	2.42
CETP_rs17231506	49	74	1.10	1.16	SYN3_rs5754227	26	40	0.75	0,77	CFH_rs121913059	0	0	2.43	20.28
B3GALTL_rs9564692	47	71	0.83	0.89	COL8A1_rs55975637	24	37	1.28	1,15	CFI_rs141853578	0	0	57.92	3.64
TGFBR1_rs1626340	46	70	0.86	0.88	RAD51B_rs2842339	19	29	1.10	1,14	ARMS2_rs375084 6	0	0	3.06	2.81
COL4A3_rs11884770	45	69	0.90	0.9	APOE_rs429358	18	28	0.77	0,7					
APOE_rs73036519	45	69	0.92	0.91	CTRB2_rs72802342	9	14	0.79	0,79					
ABCA1_rs2740488	44	67	0.86	0.9	PRLR_SPEF2_rs114092250	8	12	0.88	0,7					
ARHGAP21_rs12357257	40	61	1.04	1.11	C20orf85_rs201459901	6	9	0.64	0,76					
CETP_rs5817082	39	60	0.81	0.84	C3_rs12019136	5	8	0.34	0,71					

Table 3 Comparison of controls versus late AMD cases with a logistic regression corrected for age and sex, in EUGENDA, RSI & RSIII and Alienor.

	Controls ≥75	Late AMD	OR	CI 95%	p-value
Never Smoked **Former smoker** **Current smoker**	N=1029 N=757 N=185	N=435 N=533 N=152	1.39	1.23-1.57	**<0.0001**
Vegetables medium servings per day	0.94 (SD 0.18) N=1535	0.89 (SD 0.25) N=939	0.40	0.27-0.58	**<0.0001**
Fruit medium servings per day	0.92 (SD 0.22) N=1535	0.84 (SD 0.32) N=941	0.35	0.25-0.47	**<0.0001**
Fish medium servings per day	0.24 (SD 0.23) N=1534	0.17 (SD 0.16) N=938	0.17	0.11-0.27	**<0.0001**

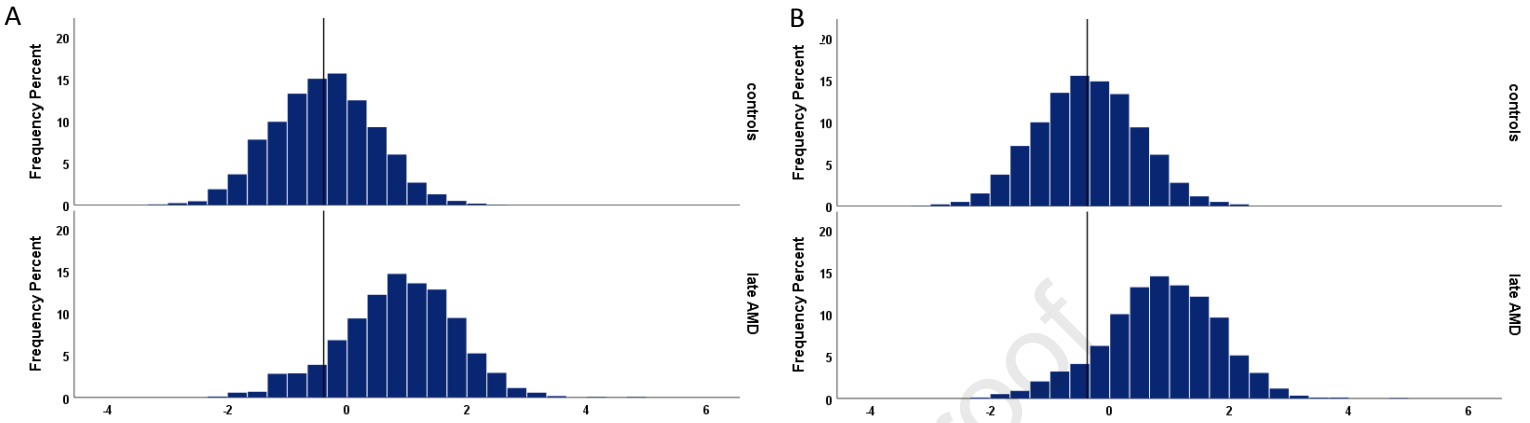


Figure 3. a Showing the distribution of the predictive score for controls and late AMD including 49 AMD associated variants. **b**. Distribution of the predictive score with the minimal set of 27 variants for controls and late AMD.

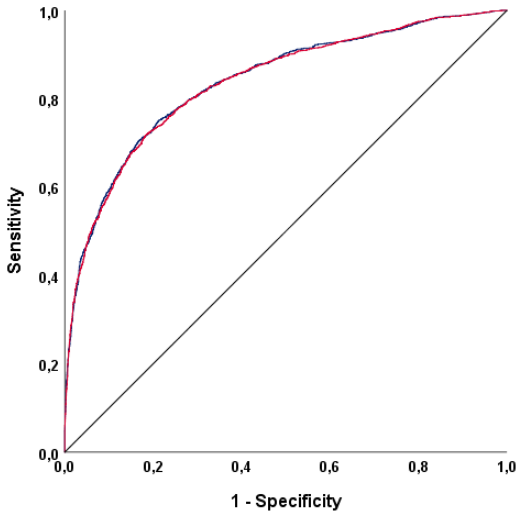


Figure 4. Receiver operator curve for predictive risk scores to differentiate between late AMD cases and controls. The blue line indicates the GRS including all 49 AMD-associated variants (AUC 0.838), the red line indicates the GRS for the minimal set of 27 AMD-associated genetic variants (AUC 0.837).

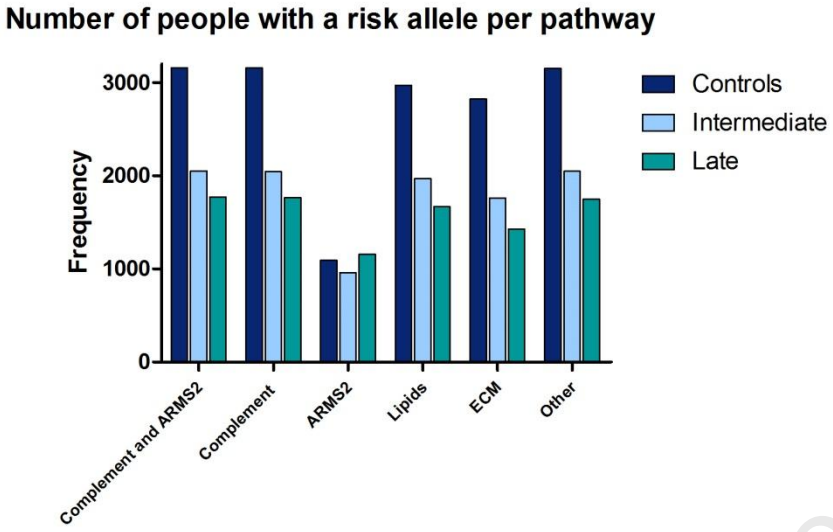
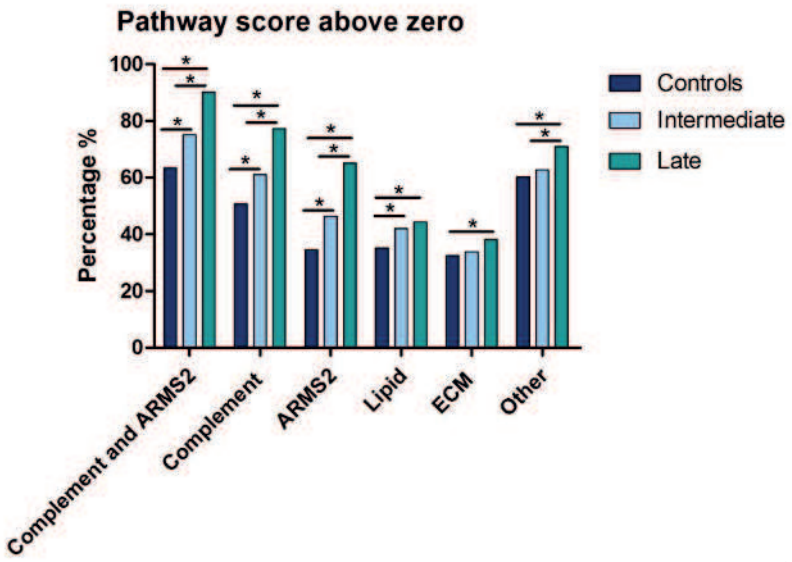
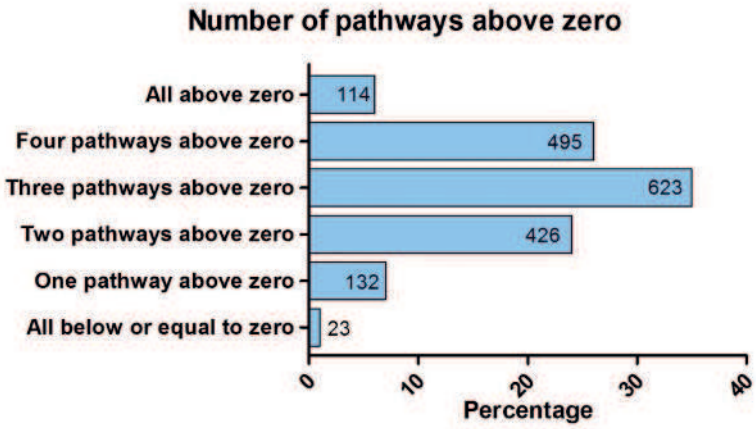
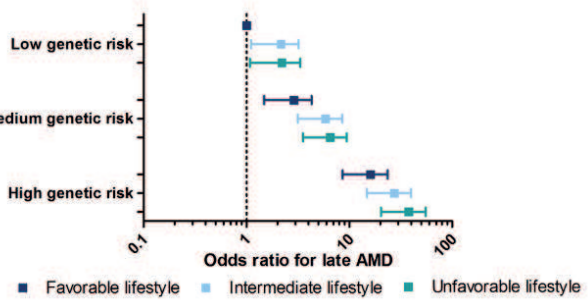


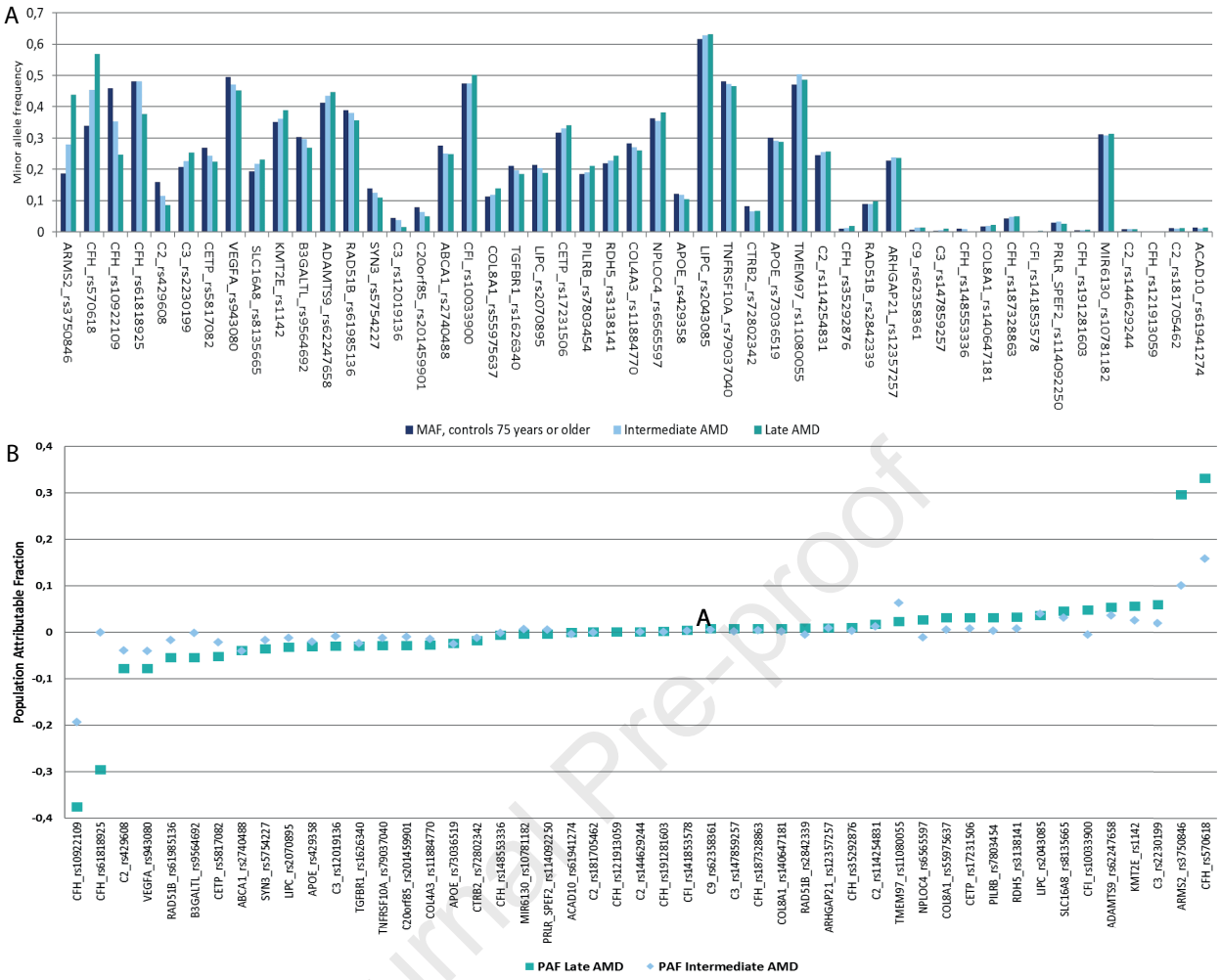
Figure 7. The number of people with a risk allele, per pathway. Dark blue = the controls 75 years and older, light blue = intermediate AMD cases, green = late AMD cases.

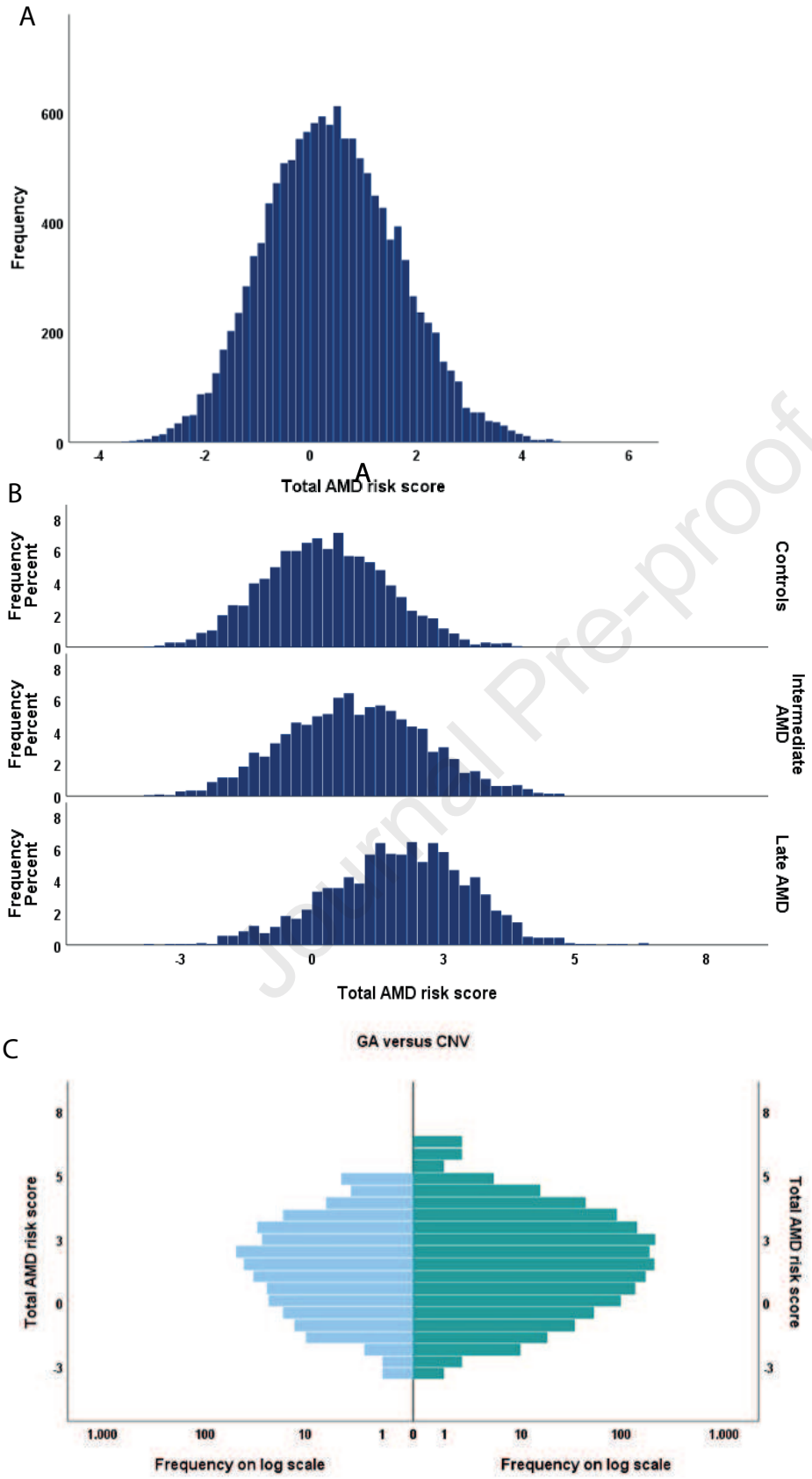


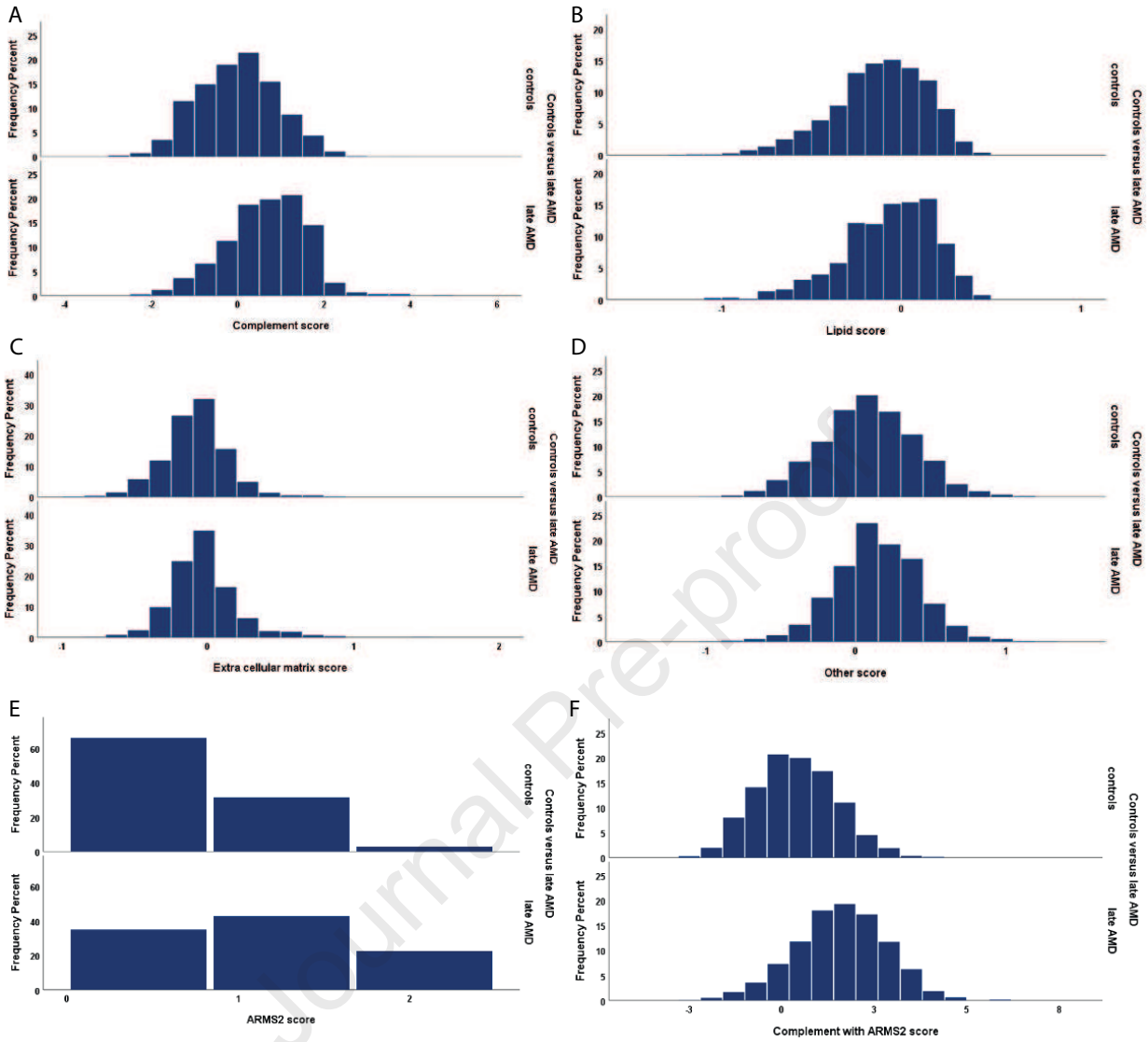


Subgroup	Cases/Controls	Odds Ratio	CI 95%	p-value
Low genetic risk				
Favorable lifestyle	27/292	1 reference		
Intermediate lifestyle	46/250	1.99	1.30-3.30	0.007
Unfavorable lifestyle	37/198	2.02	1.19-3.43	0.009
Intermediate genetic risk				
Favorable lifestyle	51/207	2.67	1.62-4.39	<0.0001
Intermediate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









Description of included studies, earlier described by AP Khawaja *et al.*[1], KM Williams *et al.*[2] , JM Colijn *et al.*[3] and by C Delcourt *et al.*[4]

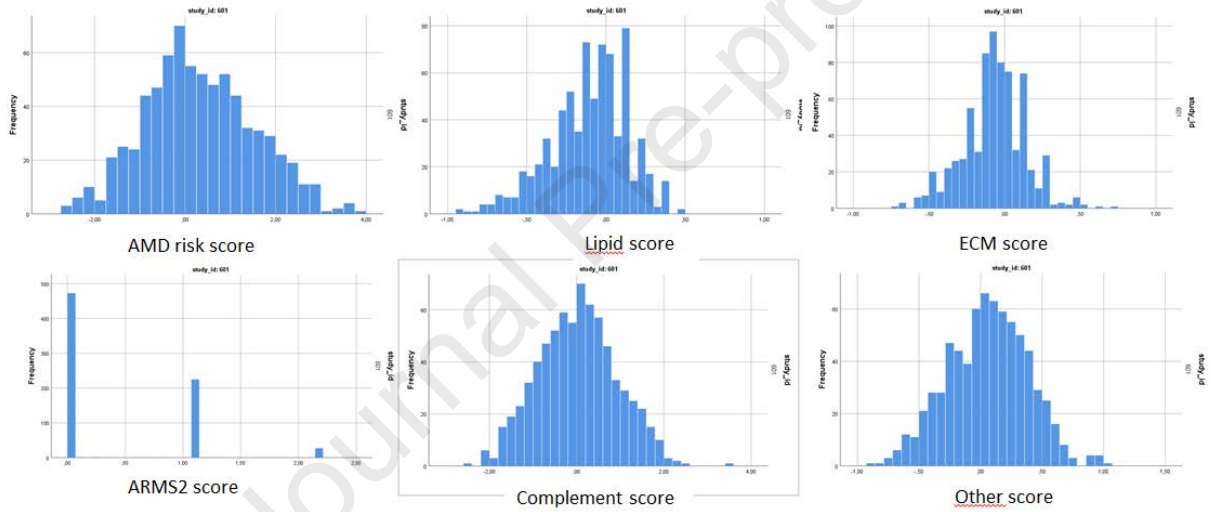
Studies included in the analysis

Region	Study	Data collection period	Total participants	Total participants with controls >75 years	Mean age (SD)	Gender, % Male	No AMD/early AMD/late AMD (N)	Smoking % former/% current
France	Alienor-3C	2006-2008	728	674	81 (4.2)	37.1	508/123/43	30.9/4.7
France	Montrachet-3C	2009-2013	978	978	82 (3.8)	36.8	756/200/22	31.2/2.0
Germany	MARS	2001-2003	763	575	77 (8.9)	42.3	49/231/295	33.7/8.2
Germany/Netherlands	EUGENDA	2007-2012	3143	2344	77 (8.9)	40.0	384/683/1277	40.4/7.6
Netherlands	RS-I	1990-1993	5632	1612	79 (6.6)	35.2	1098/432/82	36.2/16.0
Netherlands	RS-II	2000-2002	2065	367	77 (7.9)	43.3	231/123/13	51.8/15.3
Netherlands	RS-III	2005-2008	2918	199	69 (11.4)	42.7	67/125/7	52.8/18.6
Netherlands	CORRBI		74	54	79 (8.0)	55.6	10/10/34	-
Portugal	MIRA	2012-2013	873	214	71 (7.7)	39.3	64/146/4	4.2/0.9
Total			17174	7017	78 (7.9)	38.7	3167/2073/1777	36.2/9.0

Alienor-3C

Subjects of the Alienor Study were recruited from a population-based study, the Three-City (3C) Study[5], assessing the associations of age related eye diseases with nutritional factors. The 3C Study included subjects aged 65 years or older from three French Cities (Bordeaux, Dijon and Montpellier). The Alienor Study eye examinations are offered to all participants of the 3C cohort in Bordeaux since the third follow-up visit (2006-2008), of which 963 (66.4%) participated in the baseline eye examination.[5]

Eye examinations included, for each eye, two 45° non mydriatic color retinal photographs (one centered on the macula, the other centered on the optic disc) (TRC NW6S, Topcon, Japan), AMD was classified using international classifications.[6, 7] The Alienor Study also takes into account gene polymorphisms and environmental factors. The methods of this study have been published elsewhere[2]. Genetic polymorphisms were determined by the Lille Génopôle, from DNA samples collected at the first visit in Bordeaux (1999–2001) using genotyping assays (Taqman; Applied Biosystems, Inc., [ABI], Foster City, CA). Smoking habits and medical history were examined by interview. The design of this study was approved by the Ethical Committee of Bordeaux (Comité de Protection des Personnes Sud-Ouest et Outre-Mer III) in May 2006.

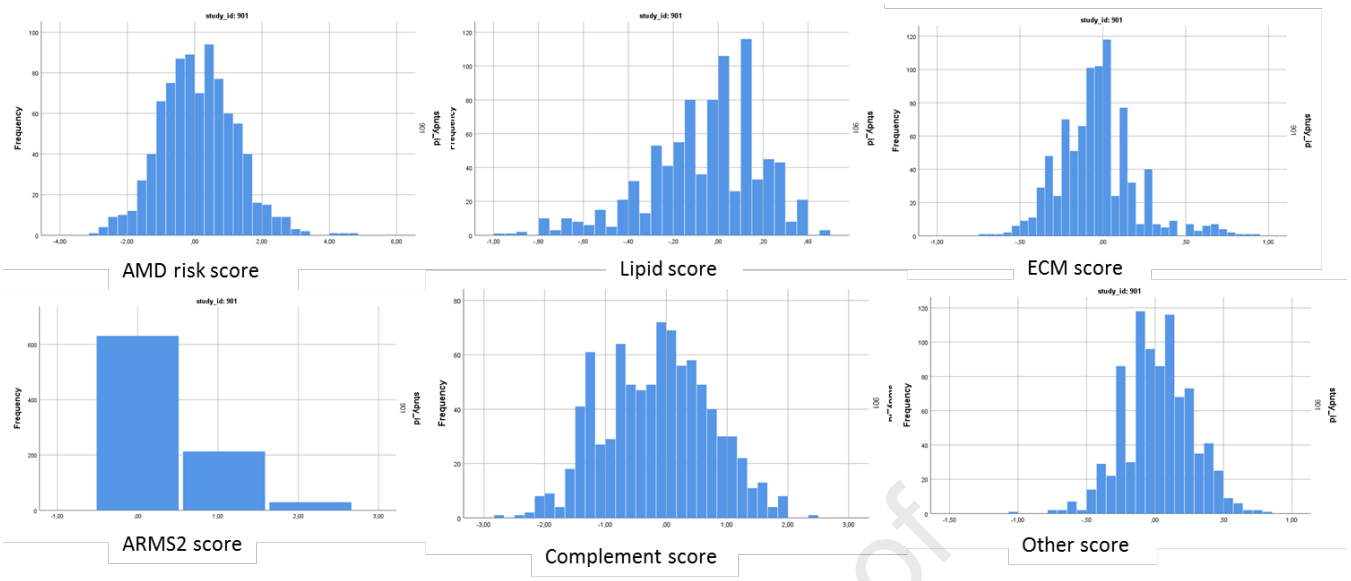


Coimbra – MIRA study

The Coimbra study is a Portuguese population-based study, including people aged 55 years and older. The subjects who were recruited from a Portuguese primary health-care center of the coastal town (Mira) between August 2009 and April 2011, (*N*=2975) were included in this current study.

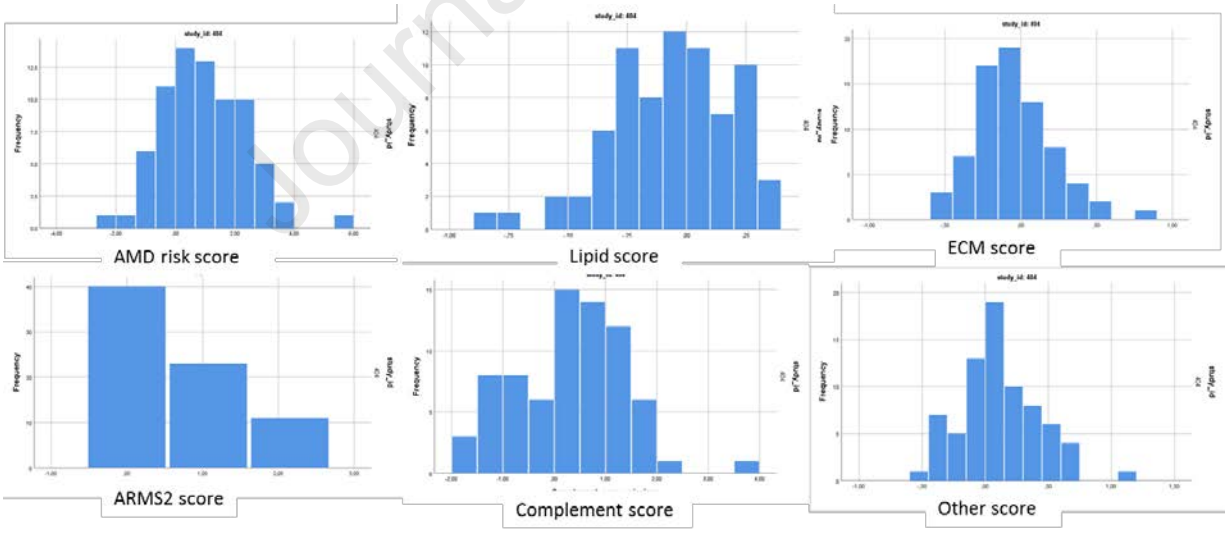
All participants had fundus photographs taken from the optic disk, macula and temporal to the macula using a digital mydriatic Topcon® fundus camera (TRC-50EX; Topcon Corporation, Tokyo, Japan). Images were graded step-wise by a centralized reading centre (Coimbra Ophthalmology Reading Centre, CORC - AIBILI). AMD was graded following The International Classification and Grading System (ICGS), signs of disease were stratified into 5 severity stages using the Rotterdam classification. This AMD grading was facilitated by software from Retmarker AMD Research (Critical Health, SA, Portugal). [8, 9]

Smoking habits, alcohol consumption, medical history and other variables were collected by interview. Genotyping was performed using the assay developed by the RadboudUMC, Nijmegen[10]. This cohort was not included in the calculation of the minor allele frequencies and population attributable risks.



CORRBI - Combined Ophthalmic Research Rotterdam Biobank

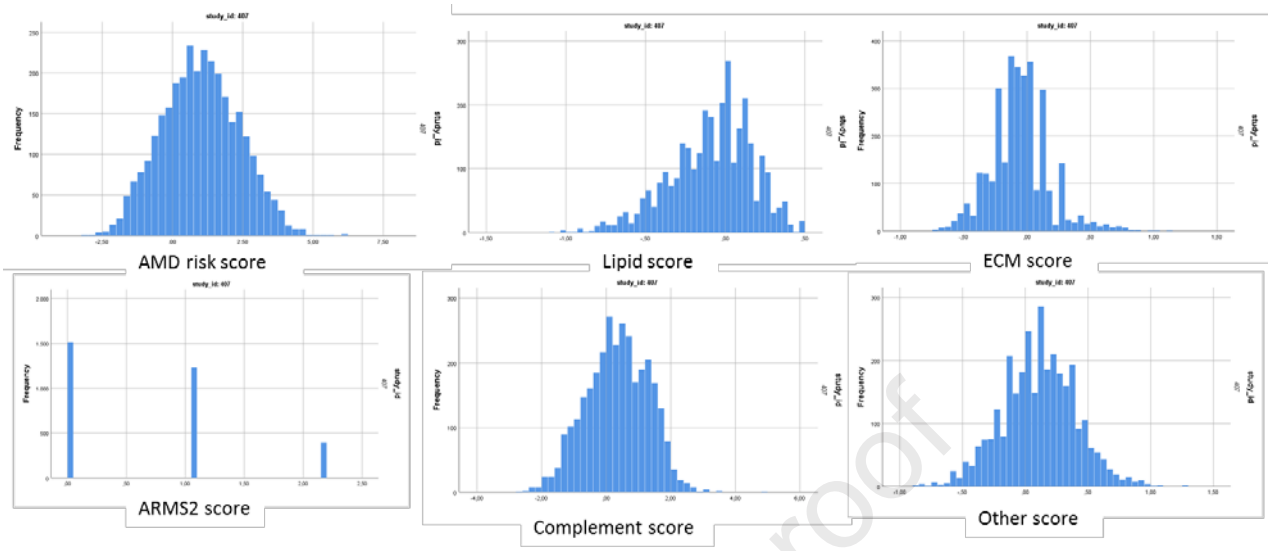
The Combined Ophthalmic Research Rotterdam Biobank (CORRBI) is a biobank from the Ophtalmology department of the Erasmus Medical Center and the Rotterdam Eye Hospital, Rotterdam, The Netherlands. The biobank started collecting biological samples and clinical data from electronic medical records from 2012 onwards. Genotyping for the current study was performed using the assay developed by the RadboudUMC, Nijmegen[10]. No environmental factors were collected, therefore for these analyses CORRBI was excluded, as well as in the minor allele frequency calculations and population attributable risks. Written informed consent was obtained from all patients.



EUGENDA

The EUGENDA (European Genetic Database) is a case-control study focusing on genetic and non-genetic factors in age-related macular degeneration (AMD)[11]. Subjects were recruited from the clinic in Nijmegen (Netherlands) and Cologne (Germany). Color fundus photos, SD-OCT and fluorescein angiography were used by two independent graders to grade AMD following a standard protocol from the Cologne Image Reading Center and Laboratory (CIRCL). Nutrition and lifestyle variables were assessed by questionnaire. Genotyping was performed using the assay developed by the

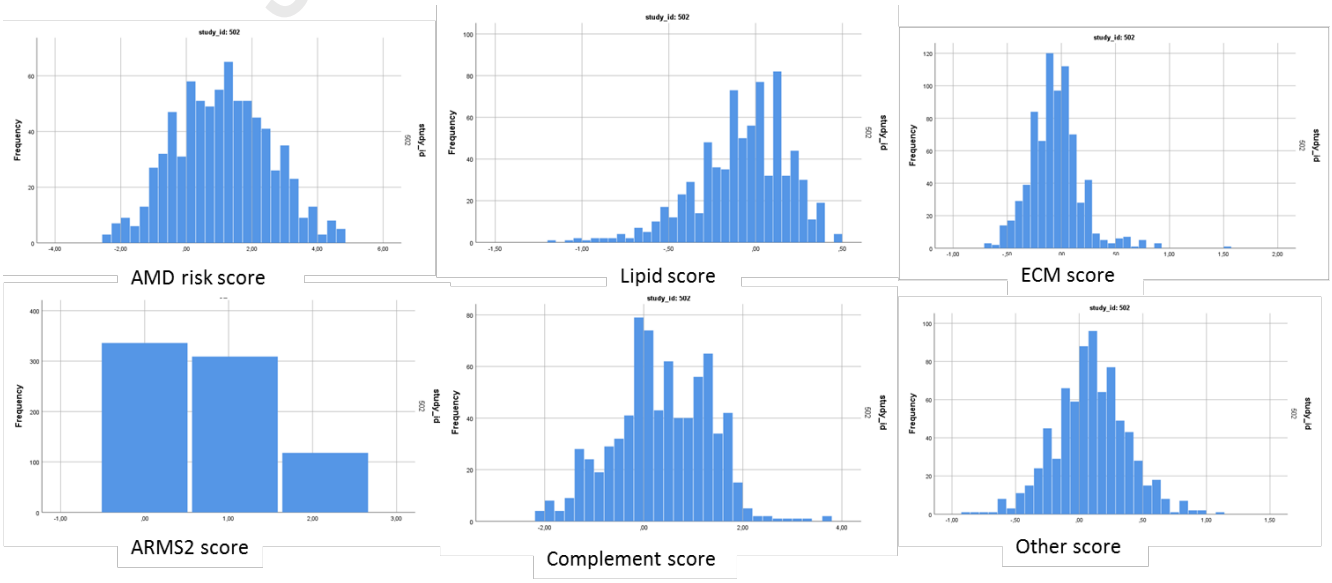
RadboudUMC, Nijmegen[10]. The study was approved by the ethics committees in both Cologne and Nijmegen.



MARS- Muenster aging and retina study

The MARS Study is follow-up study focussing on the progression of AMD. From June 2001 to October 2003, residents from the Muenster (Germany) region were recruited (N=1060) following the eligibility criteria described previously[12, 13]. In short, patients aged between 60-80 years with drusen and/or retinal pigment epithelial changes in at least one eye and clear visibility of the retina. Control subjects were partners, volunteers, and people coming to the clinic to help and guide AMD patients who had no signs of AMD themselves.

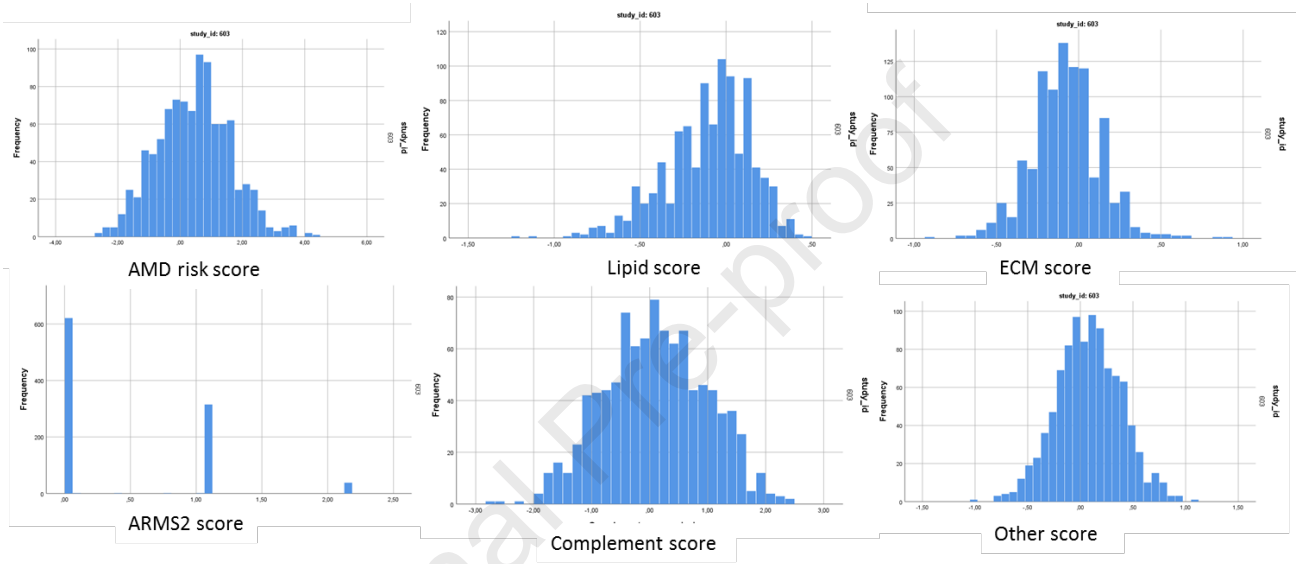
Lifestyle, smoking and medical history were obtained by interview using a standardized questionnaire. Bloodsamples were taken at the first examination for genetic analyses. Genotyping was performed using the assay developed by the RadboudUMC, Nijmegen[10]. The study was approved by the Institutional Review Board of the University of Muenster, and written informed consent was obtained from all study participants, in compliance with the Declaration of Helsinki.



Montrachet-3C

Subjects of the MONTRACHET (Maculopathy Optic Nerve nuTRition neurovAsCular and HEarT diseases) study were recruited a population-based study, the Three-City Study(3C)[5], earlier described in the cohort Alienor-3C. The participants aged 65 years and older were selected from electoral rolls. From 2009 onwards (the fifth follow-up visit) eye examinations were included in the examination of participants in Dijon.

The eye examination was conducted in the Department of Ophthalmology, University Hospital Dijon, France. The examination included OCT imaging and 45° non mydriatic color retinal photographs of the macula and the optic nerve head. AMD was graded according to the international classification[5]. Participants were asked to fill in a questionnaire on lifestyle, environmental factors and nutrition. Blood samples were drawn and genotyping was performed with the Illumina Human 610-Quad BeadChip, imputation was performed with 1000 Genomes Phase I integrated variant set (March2012).

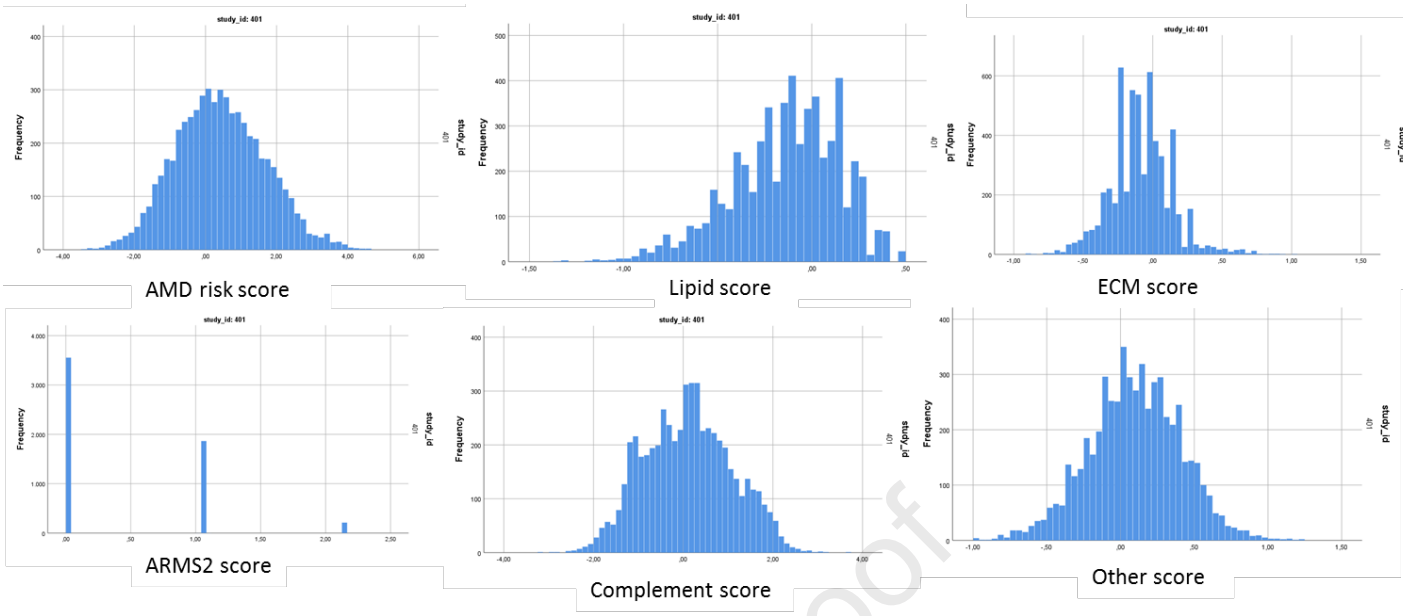


Rotterdam Study I/II/III

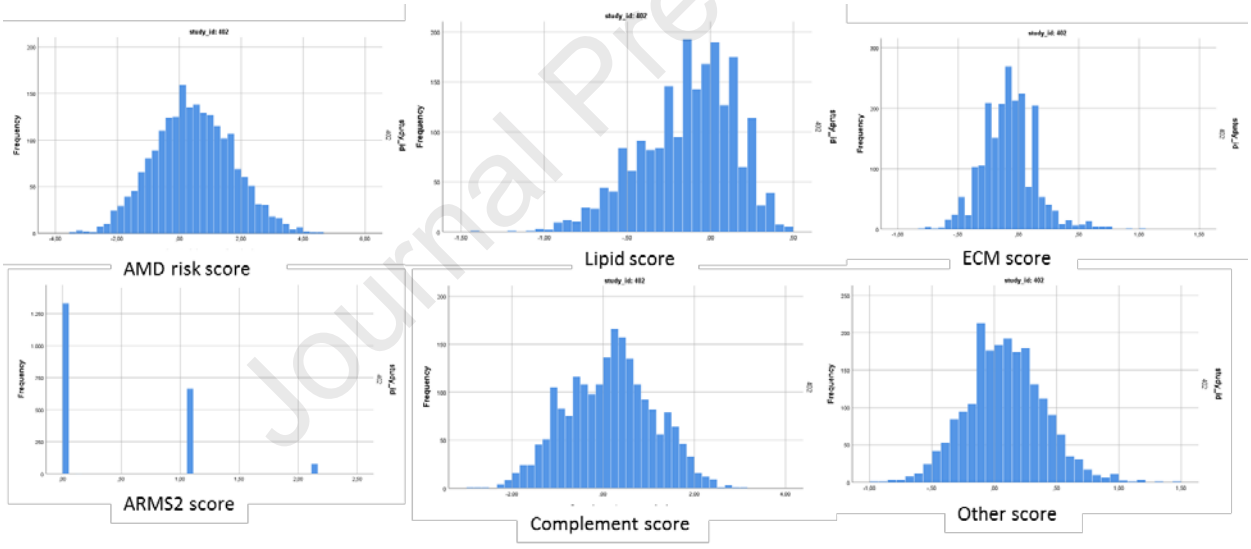
The three Rotterdam Studies are all prospective cohort studies of people living in Ommoord, a district of the city of Rotterdam. The first cohort started recruiting participants aged 55 years and older in 1990 (N=7983, response rate of 78%). The second cohort started recruiting in 2000 (N=3011, response rate of 67.3%), and the third cohort included participants from 45 years and older (N=3932, response rate 64.9%) starting in 2006.

Participants underwent an extensive physical examination at a research center including questionnaires for smoking and dietary habits. During the eye examination mydriatic color fundus photographs were taken of the macula and the optic nerve head[14, 15]. Signs of AMD were graded according to the Rotterdam classification by experienced graders. All photographs with uncertain diagnoses were evaluated by three retina specialists. Genotyping was performed using the Illumina HumanExome BeadChip for exome chip analysis in RS I, Nimblegen SeqCap EZ V2 capture kit on an Illumina Hiseq2000 sequencer for whole exome sequencing, for imputation studies Illumina 550K (duo) chip or Illumina 610 quad was used and imputed with Haplotype Reference Consortium (HRC) imputation or 1000Genomes. All participants provided written informed consent to participate in the study and to have their information obtained from treating physicians.

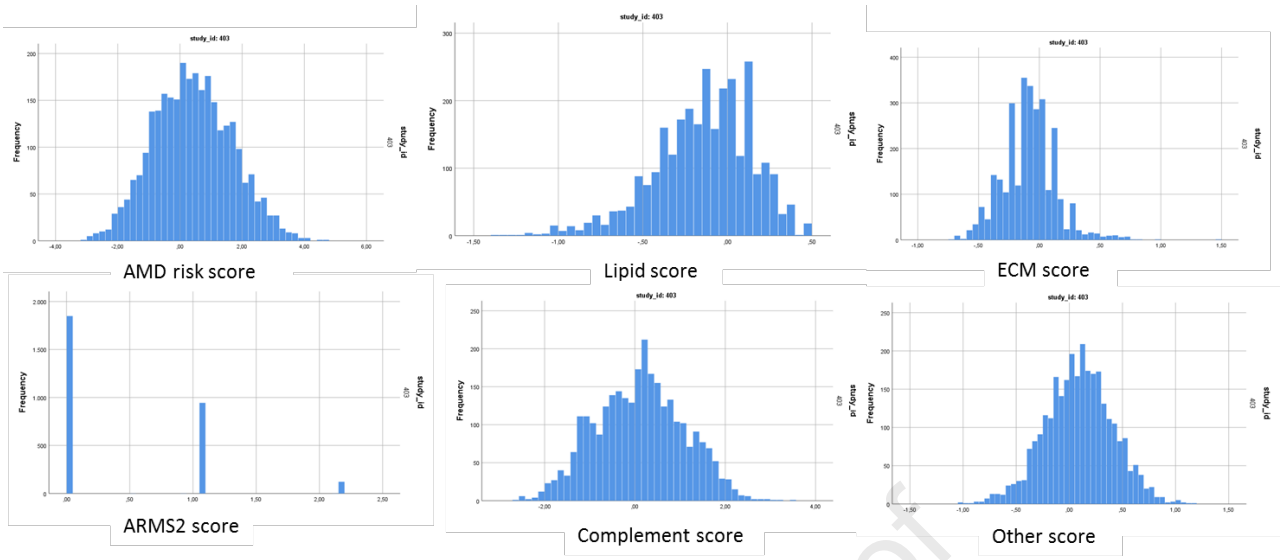
Rotterdam study I



Rotterdam study II



Rotterdam study III



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TITLE OF ARTICLE: Genetic risk, lifestyle, and AMD in Europe. The EYE-RISK consortium

AUTHORS: J.M. Colijn, , M Meester, T Verzijden, A de Breuk, R Silva, B.M.J. Merle, A. Cougnard-Grégoire, CB Hoyng, S Fauser, T Coolen, C Creuzot-Garcher, HW Hense, M Ueffing, C Delcourt, A.I. den Hollander, CCW Klaver

AUTHOR NAME	RESEARCH DESIGN	DATA ACQUISITION AND/OR RESEARCH EXECUTION	DATA ANALYSIS AND/OR INTERPRETATION	MANUSCRIPT PREPARATION
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T Verzijden	☒	☒	☒	☐
A de Breuk	☐	☒	☐	☐
R Silva	☐	☒	☐	☐
B Merle	☐	☒	☐	☐
A Cougnard-Gregoire	☐	☒	☐	☐
C Hoyng	☐	☒	☐	☐

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S Fauser	☐	☒	☐	☐
T Coolen	☐	☒	☒	☐
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HW Hense	☐	☒	☐	☐
M Ueffing	☐	☒	☐	☒
C Delcourt	☒	☒	☒	☐
A den Hollander	☒	☒	☒	☐
C Klaver	☒	☒	☒	☒

OTHER CONTRIBUTIONS:

Précis: Age-related macular degeneration is driven by complement and ARMS2, but caused in most by multiple genetic pathways. Someone's genetic effect can be severely reduced by healthy lifestyle