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A Deep Phenotype Association Study reveals specific phenotype associations with genetic variants in age-related macular degeneration:

Age-Related Eye Disease Study 2 (AREDS2) Report No. 14.

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Abstract

Purpose—Age-related macular degeneration (AMD), a multifactorial disease with variable phenotypic presentation, was associated with 52 single nucleotide polymorphisms (SNPs) at 34 loci in a Genome-Wide Association Study (GWAS). These genetic variants could modulate different biological pathways involved in AMD, contributing to phenotypic variability. To better understand the effects of these SNPs, we performed a Deep Phenotype Association Study (DeePAS) in Age-Related Eye Disease Study 2 (AREDS2), followed by replication using AREDS participants, to identify genotype associations with AMD and non-AMD ocular and systemic phenotypes.

Design—Cohort study.

Participants—AREDS and AREDS2 participants.

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No conflicting relationship exists for any author.

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This article contains additional online-only material. The following should appear online-only: Supplementary table 1 and supplementary table 2.

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Methods—AREDS2 participants (discovery cohort) had detailed phenotyping for AMD, other eye conditions, cardiovascular, neurological, gastro-intestinal and endocrine disease, cognitive function, serum nutrient levels and others (total of 139 AMD and non-AMD phenotypes). Genotypes of the 52 GWAS SNPs were obtained. The DeePAS was performed by correlating the 52 SNPs to all phenotypes using logistic and linear regression models. Associations that reached Bonferroni-corrected statistical significance were replicated in AREDS.

Main outcome measures—Genotype-phenotype associations.

Results—A total of 1776 AREDS2 participants had 5 years follow-up; 1435 AREDS participants had 10 years. The DeePAS revealed a significant association of the rs3750846 SNP at the *ARMS2/HTRA1* locus with subretinal/sub-retinal pigment epithelial (RPE) hemorrhage related to neovascular AMD (OR 1.55 (1.31–1.84), $p=2.67 \times 10^{-7}$). This novel association remained significant after conditioning on participants with neovascular AMD ($p=2.42 \times 10^{-4}$). Carriers of rs3750846 had poorer visual acuity during follow-up ($p=6.82 \times 10^{-7}$) and were more likely to have a first-degree relative with AMD ($p=5.38 \times 10^{-6}$). Two SNPs at the *CFH* locus, rs10922109 and rs570618, were associated with drusen area in the ETDRS grid ($p=2.29 \times 10^{-11}$ and $p=3.20 \times 10^{-9}$, respectively) and the center subfield ($p=1.24 \times 10^{-9}$ and $p=6.68 \times 10^{-8}$, respectively). SNP rs570618 was additionally associated with the presence of calcified drusen ($p=5.38 \times 10^{-6}$). Except for positive family history of AMD with rs3750846, all genotype-phenotype associations were significantly replicated in AREDS. No pleiotropic associations were identified.

Conclusion—The association of the SNP at the *ARMS2/HTRA1* locus with subretinal/sub-RPE hemorrhage and poorer visual acuity and of SNPs at *CFH* locus with drusen area may provide new insights in pathophysiological pathways underlying different stages of AMD.

Introduction

Age-related macular degeneration (AMD) is a common retinal disease affecting millions of people worldwide.¹ Drusen are considered the hallmark feature of AMD, but may be accompanied by other characteristics such as pigment abnormalities, atrophic changes or neovascularization. Drusen themselves present in various shapes and sizes, varying from small to large, and from reticular pseudodrusen to calcified drusen.² Altogether, phenotypic presentation of AMD is highly variable and multiple distinct AMD features can be recognized.

AMD is a multifactorial disease where different environmental and genetic risk factors contribute to clinical manifestations. Genetic predisposition is thought to account for 40–70% of disease development³, and a great deal of effort and resources have resulted in the identification of susceptibility loci. Single nucleotide polymorphisms (SNPs) in complement genes, such as *CFH*, *C2/CFB*, *CFI* and *C3*, have been consistently associated with AMD and have implicated the complement pathway as a major contributor to AMD.^{4–7} SNPs at other loci suggest the involvement of additional biological pathways, such as lipid and extracellular matrix, in AMD pathology.⁸ The most recent Genome-Wide Association Study (GWAS), including 16,144 late AMD-patients and 17,832 controls, has identified 52 single nucleotide polymorphisms (SNPs) in 34 loci, augmenting our understanding and identification of pathways that might impact AMD phenotypes.⁹ Nonetheless, how genetic

variants at these loci influence AMD is unclear. Exploring the relationship of SNPs at genetic loci to AMD and non-AMD phenotypes can lead to novel insights in disease pathology.

The Age-Related Eye Disease Study (AREDS) was a randomized clinical trial that studied the effects of zinc and antioxidant supplements on the progression of AMD in persons ranging from having no AMD to late AMD in one eye. The supplements reduced the progression to late AMD in persons with intermediate AMD or late AMD in one eye.¹⁰ After the completion of AREDS, the Age-Related Eye Disease Study 2 (AREDS2) was designed to investigate the effect of omega-3 fatty acids, substitution of beta-carotene with lutein/zeaxanthin, and lowering the zinc dose on AMD progression.¹¹ The AREDS2 results lead to the substitution of beta-carotene with lutein/zeaxanthin. In contrast to AREDS, AREDS2 enrolled participants having either bilateral intermediate AMD with large drusen or unilateral late AMD. They represent a high-risk group at the far end of the AMD spectrum, yet with highly variable phenotypic presentation. In addition to detailed AMD phenotyping, the AREDS2 participants have been thoroughly screened for other conditions, such as cardiovascular diseases, cognitive function, and other ocular disorders. AREDS2, therefore, presents a unique population to study the relationship between SNPs involved in AMD pathogenesis, phenotypic variability, and pleiotropy.

The Phenome Wide Association Study (PheWAS) was developed to investigate the relationship of one genotype with respect to many phenotypes and is thus an ideal method to identify pleiotropy of genetic variants.^{12, 13} PheWAS phenotypes are usually recorded as a broad range of disease descriptions in International Classification of Disease (ICD9) codes, thus lacking specific information at a granular level. In AREDS2, emphasis was not on broad phenotyping, but rather on standardized and reproducible detailed phenotyping (deep phenotyping) such as fundus photographic grading of lesion characteristics of AMD. The data collection did not encompass the entire phenome. Therefore, the term PheWAS was not appropriate for this design. To emphasize the use of deep phenotyping, we adopted the term “Deep Phenotype Association Study” for a better description of the methodology.

In this Deep Phenotype Association Study (DeePAS), we apply a PheWAS approach to gain insights into the role of reported AMD-related genetic variants in contributing to phenotypic variability and possible disease mechanisms through pleiotropic associations with non-AMD phenotypes.

Methods

Population

The discovery cohort in this study consisted of participants from the AREDS2 trial, which was a randomized, double-masked, placebo-controlled trial that enrolled 4203 participants between 2006 and 2012. A more detailed design of the clinical trial has been described previously.¹⁴ AREDS2 was designed to evaluate the effect of adding omega-3 fatty acids (docosahexaenoic acid 350 mg and eicosapentaenoic acid 650 mg) and lutein/zeaxanthin (10 mg/2 mg) to the AREDS formulation on the progression of AMD. Additionally, in a secondary optional randomization step, participants were further assigned to elimination of

beta-carotene and/or lowering of the zinc dosage from 80 mg to 25 mg. In the current study, we combined all treatment groups, as AREDS2 did not have a true placebo arm (all participants were given some form of the AREDS supplements) and previous analyses suggested no interaction between genotype and response to treatment.¹⁵ AREDS2 exclusively enrolled people at high risk of progression to late AMD, selecting for those with bilateral large drusen or unilateral large drusen with late AMD in the fellow eye. Participants were followed up to a median of 5 years.

Ethics statement

Institutional Review Board approval was obtained previously for both the AREDS and AREDS2 populations. All participants provided written informed consent. This research was HIPAA-compliant and adhered to the tenets of the Declaration of Helsinki.

Phenotypes

In a PheWAS, phenotypes will commonly utilize general diagnoses of diseases, such as ICD9 codes for “macular degeneration” or “acute myocardial Infarction”, but would usually lack possibly important further details. Here, we evaluated the association of several detailed phenotypes within and outside of the AMD spectrum. Therefore, this study design was termed DeepPAS. All AREDS2 participants underwent baseline and yearly eye examinations, including stereoscopic fundus photography of the macula and optic nerve. A central reading center performed masked grading of all fundus photographs¹⁶, as well as fundus autofluorescence (FAF) images of AREDS2 participants¹⁷, providing deep AMD-phenotyping for each individual. In-depth phenotyping for other characteristics for each participant was provided by a detailed medical history at every study visit and during telephone contacts occurring 6 months after each study visit. Medical history of cardiovascular and neurological events, as well as hospital admissions or death were validated by review of medical records by a trained morbidity and mortality committee consisting of cardiologists, neurologists and internal medical specialists.¹⁸ Additionally, 3741 participants took part in the ancillary cognitive function study and completed at least 1 cognitive function test. The results of a battery of 8 different cognitive function tests were converted into *z* scores and subsequently into one composite *z* score, where a higher score represents better cognitive function. The details of the cognitive tests and creation of the cognitive function composite score are described in a previous AREDS2 report.¹⁹ The grading forms and study medical history forms were assessed by two independent reviewers (FvA and MS) to extract phenotype information corresponding to a clinically distinct phenotype. Redundant variables were identified and removed or combined when appropriate. When possible, phenotypes were converted into dichotomous variables indicating that the phenotype was either ‘present’ or ‘absent’. A phenotype was considered ‘present’ if it had been recorded at least once during the course of AREDS2. Variables that could not be captured in a ‘present’ or ‘absent’ format (such as serum vitamin levels or drusen area) and variables lacking a clinically relevant cut-off (such as the cognitive function composite *z* score) were analyzed as continuous variables. In case a continuous variable was measured multiple times over the 5-year follow-up, the worst score was used. For the cognitive function score we used the last known test result and for the serum levels we used the measurement at baseline. Eye phenotypes were considered present if they were recorded

in either eye. For continuous variables, the worst score in either eye was used. Phenotypes were placed into 8 phenotype groups: AMD, RETINA, EYE, COGNITIVE, CARDIOVASC, NEURO, GI/ENDO (gastro-intestinal and endocrine disorders), OTHER and SERUM. All 139 phenotypes that were reported in at least 10 cases and were included in the analysis are described in supplementary table 1 (available at <http://www.aaojournal.org>).

Genotypes

The 52 SNPs in 34 loci, associated with AMD in a large GWAS from the International AMD Genomics Consortium (IAMGDC)⁹, were selected for this study. AREDS and AREDS2 participants had been genotyped for these 52 SNPs as part of the GWAS, using a custom Illumina HumanCoreExome array as described.⁹ Only individuals of Caucasian descent were included in this study.

Statistical analysis

The DeePAS analysis was performed using the R-package “PheWAS”.¹³ Dichotomous phenotypes were analyzed with binary logistic regression and associations were reported using odds ratios (OR) with 95%-confidence intervals (95%-CI). Continuous phenotypes were assessed by linear regression models reporting the beta coefficient and standard error (SE). All genotypes were coded as 0, 1 or 2 according to the number of SNP alleles present per individual and an additive model was assumed for all genotypes. A total of $52 \times 139 = 7228$ genotype-phenotype associations were assessed. Genotype-phenotype associations were considered statistically significant if they reached the Bonferroni corrected p-value of $< 6.9 \times 10^{-6}$. Significant genotype-phenotype associations were re-analyzed after adjusting for age, gender, smoking and education. DeePAS analyses were performed on a person-level as opposed to eye-level. To evaluate incident occurrence of subretinal/sub-retinal pigment epithelium (RPE) hemorrhage we performed Cox-proportional hazards modelling using both eyes as the unit of analysis and corrected for the dependency between eyes.

Replication

The replication cohort consisted of participants from AREDS, for which study design and outcomes have been published previously.^{10, 20} AREDS was a randomized controlled trial following 4757 participants with various stages of AMD over a median period of 10 years. For replication purposes, only AREDS participants with AREDS AMD category 3 (at least one large druse, extensive intermediate-sized drusen, or non-central GA) or 4 (late AMD in one eye) at baseline were included. As in AREDS2, all treatment arms in AREDS were combined. Phenotypes that were required for replication in AREDS were obtained using the same definitions as for AREDS2 and over the full 10-year follow-up time of AREDS. Associations that reached Bonferroni corrected significance in the primary analysis of AREDS2, were tested for replication in the AREDS cohort using the same statistical methods.

Results

Population characteristics

The discovery cohort included 1776 AREDS2 participants with genotyping data for the 52 AMD-associated SNPs. The replication cohort consisted of 1435 individuals from AREDS that were graded as AREDS AMD category 3 or 4 at baseline. The patient characteristics for the discovery and the replication cohort are summarized in table 1.

Deep Phenotype Association Study (DeePAS)

The DeePAS included 52 genetic variants and 139 phenotypes. A total of 7209 out of 7228 potential genotype-phenotype association tests were performed. The remaining 19 correlations could not be performed due to a non-varying genotype. In those cases, the phenotype in question had been assessed in only a subset of individuals and some of the rare genotypes were not present in that subset, precluding any association testing. All phenotype-genotype correlations are reported in supplementary table 2 (available at <http://www.aaojournal.org>). The p-values from the DeePAS analysis are presented as a Manhattan plot (figure 1). Several phenotype-genotype associations reached Bonferroni adjusted significance (table 2). The *ARMS2/HTRA1* rs3750846 SNP was significantly associated with subretinal/sub-retinal pigment epithelial (RPE) hemorrhage ($p=2.67 \times 10^{-7}$), ETDRS visual acuity ($p=6.82 \times 10^{-7}$), hemorrhage characteristic of AMD ($p=7.59 \times 10^{-7}$) and having a first-degree relative with AMD ($p=5.38 \times 10^{-6}$). *CFH* rs10922109 and rs570618 SNPs were associated with drusen area in the ETDRS grid ($p=2.29 \times 10^{-11}$ and $p=3.20 \times 10^{-9}$ respectively) and in the central subfield ($p=1.24 \times 10^{-9}$ and $p=6.68 \times 10^{-8}$ respectively). The *CFH* rs570618 SNP was additionally associated with the presence of calcified drusen ($p=4.24 \times 10^{-6}$). No significant pleiotropic associations were found with phenotypes outside of the AMD spectrum. With the exception of the association between first-degree relative with AMD and rs3750846, all genotype-phenotype correlations were significantly replicated in AREDS (table 2).

ARMS2/HTRA1 locus and subretinal/sub-RPE hemorrhage

A novel genotype-phenotype correlation was detected between the *ARMS2/HTRA1* locus and subretinal/sub-RPE hemorrhage. In the AREDS2 study, subretinal and sub-RPE hemorrhage could not be graded separately on the fundus photography, and thus these two phenotypes were graded as one. In the DeePAS, hemorrhage related to choroidal neovascularization (CNV) as a broader phenotype was associated with *ARMS2/HTRA1* as well. This broader phenotype of 'hemorrhage' comprises subretinal/sub-RPE as well as intraretinal hemorrhage. After correcting for the presence of subretinal/sub-RPE hemorrhage, the association did not persist (data not shown), indicating the relation was driven by subretinal/sub-RPE hemorrhage. *ARMS2/HTRA1* is a locus known to show a preferential association with neovascular AMD;⁹ therefore, we restricted the analysis to only cases with CNV (table 3). In AREDS2, 841 people with genetic testing had CNV at baseline or developed CNV at some time during follow-up. Restricting the analysis to this subset again showed a significant association of the *ARMS2/HTRA1* locus with subretinal/sub-RPE hemorrhage (OR 1.44 (1.18–1.75), $p=2.42 \times 10^{-4}$). We replicated this finding in the AREDS cohort, again restricting the analysis to people with CNV ($n=699$). In AREDS the

ARMS2/HTRA1 variant rs3750846 was significantly associated with subretinal/sub-RPE hemorrhage (OR 1.55 (1.23–1.99), $p=2.29 \times 10^{-4}$).

During the follow-up period of AREDS2, 96 eyes of 94 persons developed subretinal/sub-RPE hemorrhage. Incident subretinal/sub-RPE hemorrhage also showed a significant relation with rs3750846 in a per eye analysis (Hazard ratio (HR) 1.37 (1.05–1.78), $p=0.0207$). After additionally correcting for age, gender, education and smoking the HR was 1.31 (0.99–1.72), $p=0.0597$.

***ARMS2/HTRA1* locus and visual acuity**

People carrying the *ARMS2/HTRA1* rs3750846 risk allele had significantly poorer visual acuity than those without. The beta coefficient for this relation was -4.5 , meaning that the lowest measured visual acuity was reduced by 4.5 ETDRS letters per risk allele. This finding was replicated for participants of AREDS, who had a reduction of 10 letters per risk allele (table 2). In the DeePAS, visual acuity was defined as the lowest visual acuity in ETDRS letters measured over the full course of follow-up. To evaluate whether the decreased visual acuity was due to the occurrence of late AMD, we repeated the analysis restricted to those with CNV, those with subretinal/sub-RPE hemorrhage, those with central GA, and those without any GA or CNV (table 4). Stratification into subgroups showed no significant correlation with visual acuity in the central GA group, or in the group without late AMD. Within the CNV subgroup, we observed an association of *ARMS2/HTRA1* with visual acuity, but this was absent in the subretinal/sub-RPE subgroup. Our results could indicate that the visual acuity loss can be explained mostly by the occurrence of late AMD, and by subretinal/sub-RPE hemorrhage in particular.

***CFH* locus and drusen**

Two SNPs in the *CFH* gene, rs10922109 and rs570618, were associated with drusen area in the ETDRS grid and in the center subfield. Drusen area in the ETDRS grid was measured on an ordinal 8-step scale, scores ranging from 0 to 7, as described in AREDS2 report 2.¹⁶ Drusen area in the center subfield was measured using the same scale, but was truncated at score 5, thus ranging from 0 to 5. The protective minor allele (A) of SNP rs10922109 was associated with reduced drusen area. In contrast, the risk allele rs570618 was associated with increased drusen area. Although drusen area in the ETDRS grid and the center subfield are related, the association with both *CFH* SNPs remained significant after adjusting for both phenotypes. The two *CFH* SNPs are in linkage disequilibrium (LD) ($R^2=0.44$) and are therefore not independent. Linear regression including both SNPs showed that although the rs10922109 variant was driving this association for the most part, there was an independent contribution of the rs570618 variant to drusen area in AREDS2 ($p=0.025$ and $p=0.049$ for drusen area in the ETDRS grid and center subfield, respectively); however, this independent contribution was not observed in AREDS (table 5). The rs570618 *CFH* SNP was additionally associated to the presence of calcified drusen, again providing support for the role of *CFH* in drusen formation. The OR was 1.40 (1.22–1.61), $p=2.00 \times 10^{-6}$ and 1.67 (1.44–1.94), $p=8.62 \times 10^{-12}$ in AREDS2 and AREDS, respectively, after adjusting for covariates.

Discussion

AMD is a multifactorial disease with a highly variable phenotypic presentation and has been linked to phenotypes outside of the AMD-spectrum, such as cardiovascular disease and lipid levels.^{21–23} Recently, Grassmann et al.²⁴ showed genetic overlap between AMD and several complex diseases, including cardiovascular and autoimmune disease, suggesting pleiotropic effects of the genetic variants established by the recent IAMDGC GWAS.⁹ In this study, we aimed to gain further insights into the role of the AMD loci by assessing their effects on phenotypic variability in AMD, and by investigating the pleiotropic effects of these loci. We designed a DeePAS, applying a hypothesis-free approach and correlating the 52 AMD-related genotypes with a large range of well-defined AMD and non-AMD phenotypes. A major strength was the use of deep phenotyping, consisting of detailed grading of fundus photos by a specialized reading center and adjudicated medical history. Our DeePAS revealed several genotype-phenotype associations with AMD phenotypes. A novel finding is the association of the *ARMS2/HTRA1* rs3750846 SNP with subretinal/sub-RPE hemorrhage. This SNP also resulted in poorer visual acuity, due to higher prevalence of late AMD. Additionally, we confirm a relation between variants in *CFH* with drusen area and the presence of calcified drusen. No pleiotropic associations were identified between the AMD-related genotypes and non-AMD phenotypes.

The participants in the current study formed a minority of the AMD cases from the IAMDGC GWAS. However, we should emphasize that although there is an overlap in participants, the study designs are significantly different. Thus, this study is not a replication of the previous GWAS. The GWAS identified SNPs associated with risk of late AMD in cases versus controls, whereas the current study examined potential associations between the same SNPs and specific AMD phenotypes as well as systemic phenotypes within AMD cases. We are not aware of any bias introduced in this way. Indeed, it may be reassuring that the SNPs analyzed in the current study were known to be highly relevant to the participants studied, given that they were identified with genome-wide significance from a population that included this subset.

The *ARMS2/HTRA1* locus was associated with AMD for the first time in 2005 and has since been recognized as one of the major contributors to genetic predisposition of AMD.^{25–28} Carriers of the risk allele demonstrated higher risk of late AMD with a preferential association towards neovascular AMD.^{9, 29, 30} Here, we have confirmed these findings and showed that the risk allele in *ARMS2/HTRA1* exhibits a tendency towards phenotypes related to CNV through a novel finding linking the risk allele to subretinal/sub-RPE hemorrhage. The researchers from the Comparison of Age-Related Macular Degeneration Treatment Trials (CATT) have previously investigated the association of different phenotypes of neovascular AMD with known AMD genotypes and found that the *ARMS2/HTRA1* rs10490924 risk allele (in perfect LD with rs3750846) was associated with the occurrence of retinal arterial proliferation (RAP) and larger area of CNV lesion.³¹ They did not report a relation with hemorrhage or baseline visual acuity. It should be noted, however, that the CATT study was a randomized controlled trial with specific inclusion criteria and hemorrhage was only recorded when it was subfoveal. They do not report whether they make a distinction between subretinal/sub-RPE or intraretinal hemorrhage. Differences in

grading definitions and inclusion criteria could make comparisons between the CATT and AREDS2 findings difficult. Other studies have suggested a relation of the *ARMS2/HTRA1* rs10490924 risk allele with the occurrence of vitreous hemorrhage, specifically in the presence of polypoidal vasculopathy (PCV).^{32–34} We could not confirm this finding, as our study did not include grading of neovascular lesion type on images from fluorescein angiography or indocyanine green angiography. Associations of *ARMS2/HTRA1* to hemorrhage in PCV, and subretinal/sub-RPE hemorrhage in our DeePAS, further strengthen its role in neovascularization. We postulate that the *ARMS2/HTRA1* locus is involved in a vascular component of the retina. Despite exhibiting the strongest association with AMD, no study has so far definitively unraveled the causal gene(s) and variant(s) at this locus and demonstrated their functional impact on retina/RPE/choriocapillaris. In this regard, our observation on the phenotypic involvement of this locus may help direct future investigations.

A link between drusen area and variants in the *CFH* gene has been suggested before. The well-known *CFH* rs1061170 (Y402H) variant, which is in perfect LD with rs570618, was shown to be related to drusen progression³⁵ and was strongly associated with the cuticular drusen subtype of AMD, which is characterized by innumerable small drusen.³⁶ Carriers of the rare variant rs121913059 at the *CFH* locus were shown to have a higher drusen load in a large phenotyping study³⁷; however, this *CFH* variant was not associated with drusen area in our DeePAS ($p>0.05$), probably because of the lack of power ($n=17$ in AREDS2 individuals). A genotype-phenotype association that only barely did not reach Bonferroni corrected significance was rs116503776 in *C2/CFB* with drusen area in the center subfield ($p=8.80 \times 10^{-6}$). We suggest that complement genes, in addition to *CFH*, have a role in drusen formation as well. Interestingly, the *CFH* variant rs570618 was also related to calcified drusen, containing glistening white calcium deposits, which are considered a more advanced drusen stage and a possible precursor for geographic atrophy.^{38, 39} The consistent observation of the involvement of the complement system in drusen formation may have implications for the use of complement inhibitors, which are mostly being applied to reduce the growth of geographic atrophy. These treatments have had limited success so far, although some favorable results have been obtained with the complement factor D inhibitor lampalizumab, especially in carriers of risk alleles in the *CFI* gene.^{40–42} However, accumulating evidence suggests that the effect of the complement system in the development of AMD may occur at an early stage of drusen formation.³⁵ Our results now show that *CFH* variants are strongly related to drusen area and calcified drusen. Thus, complement inhibiting treatments would perhaps be more useful in preventing drusen progression and less effective once geographic atrophy has already developed.

Notably, we did not find any pleiotropic effects of the AMD loci, although previous research suggested genetic overlap with other complex diseases.²⁴ A potential limitation to our study was that although AREDS2 is an elderly population, this group was not specifically at high-risk for other diseases apart from AMD. Therefore, phenotypes not in the AMD-spectrum may be relatively less common, resulting in lower power to detect pleiotropic associations. In contrast, this design was especially well equipped to identify genotype associations with AMD-specific phenotypes. The AREDS2 population consists of a homogeneous group of people at the far end of the AMD-spectrum. Genotype-phenotype associations in AREDS2

are therefore less likely to be caused by misclassification. Remarkably, the replications in AREDS tended to show a stronger association than the original finding in AREDS2. Possibly contributing to this phenomenon is the larger range of AMD severity of the AREDS population, despite limiting the selection of participants with AMD category 3 or 4 at inclusion. Even within the subgroups of participants with CNV, we saw a slightly larger effect of rs3750846 on subretinal/sub-RPE hemorrhage in AREDS than in AREDS2. This could be explained by the introduction of anti-VEGF therapy at the time AREDS2 started enrolment. During AREDS, this treatment option was not yet available. Treatment with anti-VEGFs could have ameliorated some of the effect of the *ARMS2/HTRA1* SNP. Indeed, the prevalence of subretinal/sub-RPE hemorrhage was much higher in AREDS than in AREDS2, indicating successful anti-VEGF treatment. Similarly, the effect on visual acuity was more profound in AREDS, possibly explained by improved treatment options. The only genotype-phenotype association we were not able to replicate in AREDS was that of the *ARMS2/HTRA1* SNP and family history of AMD. The strong effect on AMD risk of this locus makes a role in heritability highly plausible. Noticeably, only 18.3% of people in AREDS reported having a first-degree family member with AMD as opposed to 36.9% in AREDS2. Lack of replication could thus be the result of low power for this phenotype in AREDS (n=273 with complete family history) and increased awareness of AMD over time. A strength, as well as a potential limitation of our study, was the conservative Bonferroni p-value threshold. In the presence of many association tests, this p-value adjustment helps avoid false-positive findings. However, this can be at the expense of increasing false-negatives. Although we cannot claim significant results for associations that do not reach the threshold, we show all genotype-phenotype associations performed in this DeePAS in hopes of encouraging researchers to consider further exploration and to generate hypotheses.

In this DeePAS we performed hypothesis free correlations of AMD-related genotypes and well-characterized phenotypes. We identified specific phenotype associations of well-known AMD susceptibility genotypes, providing further insights into the high phenotypic variability of AMD. We confirm the involvement of *CFH* genotypes with drusen formation and we identified a novel association of the *ARMS2/HTRA1* locus with subretinal/sub-RPE hemorrhage. We did not find evidence for pleiotropy of the AMD-associated SNPs. Cohorts of large sample size with detailed phenotyping may be required to demonstrate pleiotropic effects. Alternatively, recent advancements in text mining techniques utilizing the abundance of genotype-phenotype associations in the literature, can speed up the process of finding candidates for pleiotropy.^{43–45} Improving our understanding of the effects of genetic variants on phenotype, may help us pinpoint the affected gene, the target tissue and their mechanism of action. At the moment, our options to reduce progression of AMD are restricted to lifestyle changes and AREDS supplements. Although the supplements can limit AMD progression only by a modest effect of 25%, this means a large proportion of cases with late AMD cannot yet be prevented. However, because of the large number of persons affected with AMD, this modest effect may reduce as a large number of affected individuals. Knowing how genetic variants contribute to AMD, may help us identify therapeutic targets and design individualized treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Deep Phenotype Association Study analysis

Manhattan plot of the p-values for all tested genotype-phenotype associations. The horizontal red line represents the threshold for Bonferroni-corrected statistical significance at $p=6.9 \times 10^{-6}$.

Table 1

Patient characteristics of those with genotype information in the discovery and replication cohorts

Discovery cohort: AREDS2		Replication cohort: AREDS	
Total n=1776		Total n=1435	
		<i>only includes AMD category 3 or 4</i>	
Age in years, mean (SD)	72.2 (7.7)	Age in years, mean (SD)	69.6 (5.2)
Male gender, n (%)	732 (41.2%)	Male gender, n (%)	627 (43.7%)
Caucasian, n (%)	1776 (100%)	Caucasian, n (%)	1435 (100%)
Smoking status, n (%)		Smoking status, n (%)	
never	769 (43.3%)	never	610 (42.5%)
past	902 (50.8%)	past	716 (49.9%)
current	105 (5.9%)	current	109 (7.6%)
Baseline status, n (%)		Baseline status, n (%)	
Bilateral large drusen	1172(66.0%)	AMD category 3	940 (65.5%)
Unilateral advanced AMD	604 (34.0%)	AMD category 4	495 (34.5%)

Table 2

Associations from the DeepAS analysis in AREDS2 that reached Bonferroni corrected significance and their replication in AREDS

AREDS2 discovery cohort n=1776						
Phenotypes	SNP	Locus	p-value	Beta	SE	Odds ratio (95%-CI)
Drusen area in ETDRS grid	rs10922109	<i>CFH</i>	2.29E-11	-0.245	0.036	
Drusen area in center subfield	rs10922109	<i>CFH</i>	1.24E-09	-0.230	0.038	
Drusen area in ETDRS grid	rs570618	<i>CFH</i>	3.20E-09	0.174	0.029	
Drusen area in center subfield	rs570618	<i>CFH</i>	6.68E-08	0.164	0.030	
Subretinal/Sub-RPE hemorrhage	rs3750846	<i>ARMS2/HTRA1</i>	2.67E-07	0.441	0.086	1.55 (1.31–1.84)
Lowest measured visual acuity in ETDRS letters	rs3750846	<i>ARMS2/HTRA1</i>	6.82E-07	-4.446	0.892	
Hemorrhage characteristic of nAMD	rs3750846	<i>ARMS2/HTRA1</i>	7.59E-07	0.380	0.077	1.46 (1.26–1.70)
Calcified drusen	rs570618	<i>CFH</i>	4.24E-06	0.320	0.070	1.38 (1.20–1.58)
First degree relative with AMD	rs3750846	<i>ARMS2/HTRA1</i>	5.38E-06	0.309	0.068	1.36 (1.19–1.55)
AREDS replication cohort n=1435						
Phenotypes	SNP	Locus	p-value	Beta	SE	Odds ratio (95%-CI)
Drusen area in ETDRS grid	rs10922109	<i>CFH</i>	6.68E-27	-0.656	0.06	
Drusen area in center subfield	rs10922109	<i>CFH</i>	1.93E-26	-0.645	0.059	
Drusen area in ETDRS grid	rs570618	<i>CFH</i>	2.24E-17	0.446	0.053	
Drusen area in center subfield	rs570618	<i>CFH</i>	2.33E-16	0.426	0.053	
Subretinal/Sub-RPE hemorrhage	rs3750846	<i>ARMS2/HTRA1</i>	3.82E-13	0.58	0.08	1.79 (1.53–2.09)
Lowest measured visual acuity in ETDRS letters	rs3750846	<i>ARMS2/HTRA1</i>	3.24E-22	-10.091	1.024	
Hemorrhage characteristic of nAMD *	rs3750846	<i>ARMS2/HTRA1</i>	3.82E-13	0.58	0.08	1.79 (1.53–2.09)
Calcified drusen	rs570618	<i>CFH</i>	7.42E-12	0.512	0.075	1.67 (1.44–1.93)
First degree relative with AMD **	rs3750846	<i>ARMS2/HTRA1</i>	5.72E-01	0.121	0.214	1.13 (0.74–1.72)

ETDRS = Early Treatment for Diabetic Retinopathy Study; nAMD = neovascular age-related macular degeneration; SNP = single nucleotide polymorphism; SE = standard error; CI = confidence interval.

* In AREDS AMD grading, there was no differentiation between subretinal/sub-RPE hemorrhage and all types of hemorrhage related to nAMD;

** Family history was available for 273 AREDS participants

Table 3
Subretinal/Sub-RPE hemorrhage and the rs3750846 SNP at the *ARMS2/HTRA1* locus

AREDS2 Discovery cohort	Subretinal/Sub-RPE hemorrhage, n (%)	OR (95%-CI)	p-value
Total cohort (n=1776)	307 (17.3%)	1.62 (1.36–1.94)	6.65E-08
CNV at any time (n=841)	307 (36.5%)	1.44 (1.18–1.75)	2.42E-04
AREDS Replication cohort	Subretinal/Sub-RPE hemorrhage, n (%)	OR (95%-CI)	p-value
Total cohort (n=1435)	478 (33.3%)	1.80 (1.54–2.12)	5.76E-13
CNV at any time (n=699)	478 (68.4%)	1.55 (1.23–1.99)	2.29E-04

CNV = choroidal neovascularization; RPE = retinal pigment epithelium; OR = odds ratio; CI = confidence interval. From logistic regression models adjusting for age, gender, education and smoking.

Table 4

Visual acuity and *ARMS2/HTRA1*

AREDS2 Discovery cohort	Lowest measured visual acuity in ETDRS letters, median (interquartile range)	Snellen equivalent	Beta (95%-CI)	p-value
Total cohort (n=1776)	64 (34 – 74)	20/50	-4.508 (-6.170 - -2.846)	1.17E-07
CNV only (n=582)	46 (11 – 66)	20/125	-4.134 (-7.127 - -1.141)	0.007
Subretinal/Sub-RPE hemorrhage only (n=211)	22 (0 – 48)	20/400	-3.094 (-8.237 - 2.049)	0.237
Central GA only (n=188)	50 (28 – 66)	20/100	-2.281 (-7.081 - 2.520)	0.350
No advanced AMD (n=660)	74 (68 – 80)	20/32	-0.044 (-1.426 - 1.337)	0.95

CNV=choroidal neovascularization; GA=geographic atrophy; RPE = retinal pigment epithelium; ETDRS = Early Treatment for Diabetic Retinopathy Study; CI = confidence interval. The CNV and hemorrhage subgroups are excluding those with any GA. The central GA subgroup is excluding those with CNV. No advanced AMD excludes any GA and CNV. Adjusted for age, gender, smoking and education.

Table 5

CFH SNPs and drusen area

AREDS2				
Phenotypes	SNP	Beta	SE	p-value
Drusen area in ETDRS grid	rs10922109	-0.179	0.047	1.32E-04
	rs570618	0.084	0.038	0.025
Drusen area in center subfield	rs10922109	-0.171	0.049	4.45E-04
	rs570618	0.077	0.039	0.049
AREDS				
Phenotypes	SNP	Beta	SE	p-value
Drusen area in ETDRS grid	rs10922109	-0.539	0.083	1.25E-10
	rs570618	0.124	0.073	0.089
Drusen area in center subfield	rs10922109	-0.593	0.083	1.57E-12
	rs570618	0.062	0.073	0.396

ETDRS = Early Treatment for Diabetic Retinopathy Study; SNP = single nucleotide polymorphism; SE = standard error.
From linear regression models including both *CFH* SNPs and adjusting for age, gender, smoking and education.