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Association of Hyperreflective Foci Present in Early Forms of Age-Related Macular Degeneration With Known Age-Related Macular Degeneration Risk Polymorphisms

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Submitted: December 14, 2015

Accepted: July 12, 2016

Citation: Altay L, Scholz P, Schick T, et al. Association of hyperreflective foci present in early forms of age-related macular degeneration with known age-related macular degeneration risk polymorphisms. *Invest Ophthalmol Vis Sci.* 2016;57:4315–4320. DOI:10.1167/iops.15-18855

PURPOSE. We evaluated the association of hyperreflective foci (HF) observed in early and intermediate age-related macular degeneration (AMD) with known AMD risk alleles.

METHODS. In this pilot case-control study, HF were defined as lesions with reflectivity equal or higher than the retinal pigment epithelium band in spectral domain optical coherence tomography (SDOCT). Hyperreflective foci in the outer nuclear layer and photoreceptor complex were evaluated in 518 individuals with early and intermediate AMD. Definite presence of HF was defined as at least 10 HF in all SDOCT scans. Genotyping was performed for 22 single nucleotide polymorphisms (SNPs). Associations between AMD severity stages, HF, and SNPs were determined by logistic regression analyses.

RESULTS. Hyperreflective foci ($n \geq 10$) were significantly associated with AMD severity and the association was strongest with intermediate AMD (odds ratio [OR], 8.45; $P = 1.092 \times 10^{-8}$). Independently, HF showed associations with *ARMS2* rs104909/*HTRA1* rs11200638 (OR, 1.64; $P = 0.017$), *CFH* rs1061170 (OR, 1.70; $P = 0.011$), and *APOE4/TOMM40* rs2075650 (OR, 2.26; $P = 0.005$) variants. Within the group of intermediate AMD, associations were similar (*ARMS2* rs104909/*HTRA1* rs11200638 OR, 1.79, $P = 0.010$; *CFH* rs1061170 OR, 1.77, $P = 0.013$; *APOE4/TOMM40* rs2075650 OR, 1.98; $P = 0.034$) and showed additional trending associations with *VEGFA* rs943080 variant (OR, 0.59; $P = 0.024$). After Bonferroni-correction for 22 SNPs, none of the associations was statistically significant ($P \leq 0.0023$).

CONCLUSIONS. The presence of HF is related to AMD severity. Despite limited power of this pilot study, our results suggest an association of HF with polymorphisms in *ARMS2/HTRA1*, *CFH*, *APOE4/TOMM40*, and *VEGFA* genes which could be triggered by modification of the extracellular matrix, altered complement system or lipid metabolism.

Keywords: hyperreflective foci, age-related macular degeneration, *ARMS2*, *HTRA1*, *CFH*, *APOE*, *VEGFA*, microglia

Age-related macular degeneration (AMD) is a multifactorial neurodegenerative disease with a broad spectrum of phenotypic varieties. Early forms are characterized by the presence of drusen and pigmentary changes identified with fundus photographs (FP) while choroidal neovascularization (CNV) and central geographic atrophy (GA) represent late forms.¹ In recent years, AMD diagnosis was facilitated by the wide use of noninvasive high-resolution spectral domain optical coherence tomography (SDOCT) and even different pathologic morphologic features including hyperreflective foci (HF) could be identified.^{2–7}

Hyperreflective foci are defined as “discrete, well-circumscribed lesions with equal or greater reflectivity than the RPE band.”³ Hyperreflective foci have been observed in the outer nuclear layer (ONL), in proximity to the drusen and RPE-atrophic areas.^{2,5} In the longitudinal Age-Related Eye Disease 2

(AREDS2) study, HF proliferation and migration were associated with the development of GA.⁸

Hyperreflective foci were associated with hyperpigmentations observed on FP^{3,5} and showed dynamic changes during anti-VEGF therapy in neovascular AMD. They may present in vivo inflammatory components of the disease and, therefore, may be used as a new clinical biomarker.^{4,6,9} In histologic analyses, intraretinal HF represented cholesterol crystal precipitations of phagocytic origin, which also may originate from microglia.¹⁰

In AMD, heritability accounts for approximately 71% of the condition.¹¹ In recent years, >30 AMD risk loci have been identified and many of the genes at these loci encode components of the complement system, lipid metabolism, and extracellular matrix biology.^{12,13} To our knowledge, it has not yet been investigated whether genetic polymorphisms are



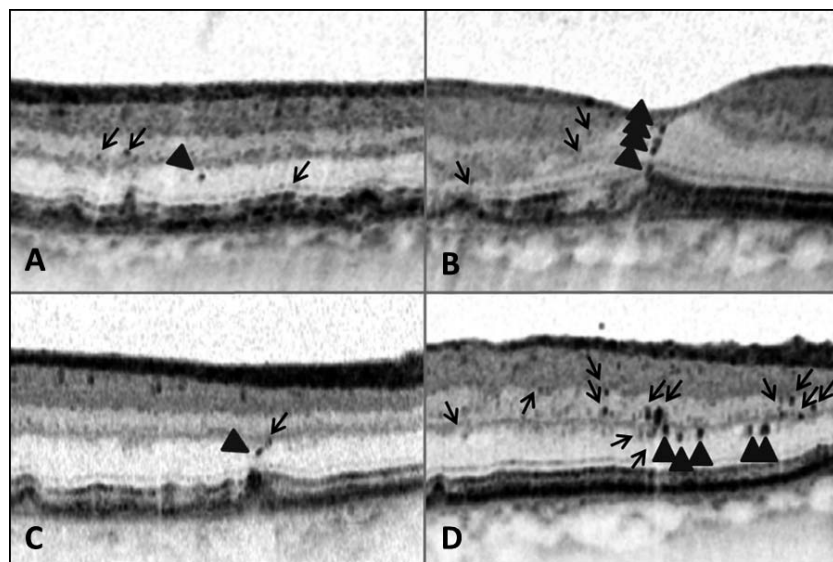


FIGURE 1. Grading examples of HF. Counted HF are marked by triangles. Noncounted dots are marked by arrows. The grading for HF is based on the quantity of HF in all B-scans of the SDOCT volume scan. (A) Solitary HF in the ONL. (B) Hyperreflective foci in photoreceptor complex and in ONL sequentially adjacent to each other. (C) Solitary HF close to a druse in the ONL. (D) Hyperreflective foci in the ONL adjacent to HF in inner retinal layers.

associated with the presence of HF, which may shed light on their etiology.

The purpose of this study was to analyze associations of HF with AMD severity and known AMD-related genetic risk polymorphisms.

METHODS

In our study, basic demographic (age, sex, ocular comorbidity) and ophthalmologic clinical data of all participants of the European Genetic Database (EUGENDA, available in the public domain at www.eugenda.org) with early and intermediate AMD were reviewed. Participants were recruited in the time period between 2007 and 2014 in the University Hospital of Cologne (Cologne, Germany) and the Radboud University Medical Center (Nijmegen, The Netherlands). Data obtained from questionnaires (e.g., smoking) were not included in this study. Subjects with poor image quality, images of only one eye, or other concomitant retinal pathologies (diabetic retinopathy/maculopathy, severe macular pucker, macular hole, macular edema, high myopia) were excluded. Clinical imaging included FP (performed using the Canon Uvi fundus camera at the 40° setting; Canon, Tokyo, Japan), FA (Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany), and SDOCT (Heidelberg Engineering). The study was performed in accordance with the tenets of the Declaration of Helsinki and the Medical Research Involving Human Subjects Act (WMO), and was approved by the local ethics committee of the University Hospitals in Cologne and Nijmegen. Written informed consent was obtained from all participants.

Grading

Severity staging of AMD was performed by grading of stereo FP, FA images, and SDOCT scans according to the standard protocol of the Cologne Image Reading Center (CIRCL) by certified graders (LA, TR). Patients were categorized as having early and intermediate AMD. Early AMD was defined by the presence of at least 10 small drusen (<63 μ m) together with pigmentary changes or by the presence of 1 to 14 intermediate

(63–124 μ m) drusen in the Early Treatment Diabetic Retinopathy Study (ETDRS) grid. Intermediate AMD was classified by the presence of ≥ 15 intermediate or any large drusen (≥ 125 μ m diameter) in the ETDRS grid.

Hyperreflective foci on SDOCT were defined in analog to previous studies as small, well-circumscribed dense particles with equal or higher reflectivity than RPE (Fig. 1).³ In this study, HF from the ONL to the RPE (RPE excluded) were evaluated. These layers were chosen as HF were presumed to reflect accumulated inflammatory material, such as activated microglia that can be found in the ONL.^{14–18} Staging of HF was based on the number of HF detected in the ONL to RPE band. Spectral domain OCT volume scans (4 \times 6 mm, Spectralis SDOCT; Heidelberg Engineering) with at least 37 B-scans centered on the fovea of both eyes were analyzed by two independent graders (LA, PS). Discrepancies between graders were solved by open adjudication. Definite presence of HF was defined as at least 10 HF in all scans. The more severely affected eye was used for HF staging of an individual.

Genotyping

Genomic DNA was extracted from peripheral blood samples using standard procedures. A total of 22 single nucleotide polymorphisms (SNPs) in or near AMD-associated risk genes that represent the majority of loci associated with AMD were chosen.¹³ Genotyping of SNPs in the *ARMS2/HTRA1* (rs10490924/rs11200638), *CFH* (rs800292, rs1061170, rs12144939), *C3* (rs2230199, rs1047286, rs433594), *CFB* (rs4151667, rs641153), *TIMP3* (rs9621532), *APOE4/TOMM40* (rs2075650), *APOE1* (rs4420638), *CETP* (rs3764261), *ADAMTS9* (rs6795735), *SLC16A8* (rs8135665), *VEGFA* (rs943080), *TGFBR1* (rs334353), *SKIV2L* (rs429698), *RAD51B* (rs8017304), *LIPC* (rs493258, rs1048017), and *TNFRSF10A* (rs1327806) genes were done using the KASPar SNP Genotyping System by LGC Genomics (Berlin, Germany) as described previously.¹⁹ The genotyping success rate was very high and varied between 99.0% and 99.8%. Also, minor allele frequencies (MAF) were comparable to those reported by HapMAP-CEU or 1000genomes-EUR.

TABLE 1. Distribution of HF Quantity in Early and Intermediate AMD Stages

	Early AMD	Intermediate AMD	Total
No HF	152	68	220
HF 1-9	117	118	235
HF 10-19	9	36	45
HF ≥ 20	0	18	18
Total	278	240	518

Statistical Analyses

All calculations were performed using SPSS software version 21.0 (IBM Software and Systems, Armonk, NY, USA). Associations between HF (≥10 vs. <10) and SNPs (0, no major allele; 1, major allele; 2, homozygous major allele; additive model) were calculated separately by logistic regression analyses after adjustment for age, sex, site (University of Cologne/University of Nijmegen), and AMD-severity (early/intermediate). Additional subanalysis were conducted for the intermediate AMD cases after adjustments. After Bonferroni correction for 22 SNPs, *P* values ≤0.0023 were accepted as significant. All SNPs were tested for Hardy-Weinberg equilibrium (HWE) and SNPs outside the equilibrium (*P* < 0.05) were not analyzed.

RESULTS

We included 518 subjects with early and intermediate AMD in the analysis. The grading comprised the quantification of HF numbers and AMD severity (early/intermediate AMD). The distribution of HF in early/intermediate AMD is shown in Table

1. Definitive presence of HF with at least 10 HF in all scans was observed in 63 subjects.

Effect of Age, Sex, and AMD Severity Stages on HF

Mean age was 74.92 ± 10.80 years for subjects with definitive presence of HF and 72.27 ± 8.17 years for subjects with less than 10 HF. Our cohort was predominated by female sex (total number of females, 325 [62.74%]) but the distribution of sex in the groups HF ≥ 10 and HF < 10 was similar (number of females 42 [66.67%] vs. 283 [62.20%]).

Multivariate regression analyses of age, sex, and AMD severity stages with HF (*n* ≥ 10 vs. *n* < 10) were performed. Hyperreflective foci ≥ 10 were significantly associated with age (*P* = 0.022, odds ratio [OR] = 1.04, 95% confidence interval [CI], 1.01-1.07), and intermediate AMD (OR, 8.45; *P* = 1.092*10⁻⁸; 95% CI, 4.07-17.58).

Association of SNPs With HF

Table 2 shows MAFs and HWE of all included SNPs, for HF < 10 and HF ≥ 10. For all the SNPs with MAF > 5%, association analyses were performed after adjustment for age, sex, site, and AMD severity (early/intermediate AMD) in an additive model (0, no major allele; 1, major allele; 2, homozygous major allele) and results are shown in Table 3. Analyses revealed an association of HF ≥ 10 with *ARMS2* rs104909/*HTRA1* rs11200638 (OR, 1.64; *P* = 0.017), *CFH* rs1061170 (OR, 1.70, *P* = 0.011), and *APOE4/TOMM40* rs2075650 (OR, 2.26; *P* = 0.005) variants (Table 2).

Association of SNPs With HF in the Subgroup of Intermediate AMD

To rule out an imperfect adjustment for AMD severity, we additionally performed a subgroup analyses only for subjects

TABLE 2. Minor Allele Frequencies and Associations of Each SNP With HF

Locus Name	Variant	Major/Minor Allele	MAF				P Value*		HWE	
			HF < 10	HF ≥ 10	OR*	CI 95%*	P Value HF < 10	P Value HF ≥ 10	P Value HF < 10	P Value HF ≥ 10
<i>ADAMTS9</i>	rs6795735	C/T	0.42	0.39	0.93	0.63-1.39	0.732	0.39	0.19	
<i>APOE/TOMM40</i>	rs2075650	A/G	0.11	0.19	2.26	1.28-4.00	0.005	0.18	0.56	
<i>APOE/APOC1</i>	rs4420638	A/G	0.13	0.18	1.63	0.93-2.86	0.086	0.24	0.35	
<i>ARMS2/HTRA1</i>	rs104909/rs11200638	G/T G/A	0.29	0.42	1.64	1.09-2.46	0.017	0.32	0.94	
<i>C3</i>	rs433594	C/T	0.38	0.40	0.87	0.56-1.34	0.519	0.80	0.94	
<i>C3</i>	rs1047286	G/A	0.23	0.24	1.01	0.63-1.60	0.984	0.79	0.34	
<i>C3</i>	rs2230199	G/C	0.24	0.25	1.01	0.64-1.58	0.969	0.09	0.45	
<i>CETP</i>	rs3764261	G/T	0.37	0.52	0.73	0.49-1.08	0.113	0.01	0.45	
<i>CFB</i>	rs4151667	T/A	0.09	0.10	2.00	0.72-5.54	0.183	0.45	0.45	
<i>CFB</i>	rs641153	G/A	0.06	0.13	1.33	0.56-3.18	0.516	0.64	0.59	
<i>CFH</i>	rs800292	G/A	0.24	0.17	0.67	0.39-1.14	0.141	0.36	0.95	
<i>CFH</i>	rs1061170	T/C	0.43	0.60	1.70	1.13-2.57	0.011	0.87	0.72	
<i>CFH</i>	rs12144939	G/T	0.16	0.21	0.77	0.42-1.41	0.394	0.20	0.65	
<i>LIPC</i>	rs493258	C/T	0.43	0.45	0.97	0.64-1.47	0.870	0.97	0.09	
<i>LIPC</i>	rs10468017	C/T	0.26	0.29	1.05	0.68-1.63	0.813	0.69	0.56	
<i>RAD51B</i>	rs8017304	A/G	0.37	0.38	1.00	0.67-1.49	0.996	0.44	0.32	
<i>SKIV2L</i>	rs429608	G/A	0.11	0.13	1.43	0.79-2.60	0.240	0.31	0.99	
<i>SLC</i>	rs8135665	C/T	0.23	0.27	1.35	0.85-2.12	0.201	0.45	0.14	
<i>TIMP3</i>	rs9621532	A/C	0.08	0.14	1.85	0.78-4.36	0.162	0.14	0.14	
<i>TGFBR1</i>	rs334353	T/G	0.25	0.21	0.90	0.55-1.47	0.678	0.64	0.50	
<i>TNFRSF10A</i>	rs1327806	T/G	0.45	0.50	0.72	0.48-1.09	0.122	0.74	0.52	
<i>VEGFA</i>	rs943080	T/C	0.49	0.41	0.68	0.45-1.02	0.063	0.37	0.89	

* OR, *P* values and 95% CI are based on the multivariate logistic regression for each SNP (0, no major allele; 1, major allele; 2, homozygous major allele, additive model) which included adjustments for age, sex, recruited site (University of Cologne/University of Nijmegen), and AMD-Severity (early/intermediate AMD). After Bonferroni-correction for 22 SNPs *P* values ≤ 0.0023 were accepted as significant.

TABLE 3. Associations of Each SNP With Hyperreflective Foci Within the Subgroup of Intermediate AMD

Locus Name	Variant	Major/Minor Allele	MAF		OR*	CI 95%*	P Value
			HF < 10	HF ≥ 10			
<i>ADAMTS9</i>	rs6795735	C/T	0.25	0.38	0.86	0.55-1.34	0.507
<i>APOE/TOMM40</i>	rs2075650	A/G	0.10	0.18	1.98	1.05-3.73	0.034
<i>APOE/APOC1</i>	rs4420638	A/G	0.12	0.19	1.72	0.93-3.18	0.083
<i>ARMS2/HTRA1</i>	rs104909/rs11200638	G/T G/A	0.31	0.45	1.79	1.15-2.79	0.010
<i>C3</i>	rs433594	C/T	0.40	0.39	0.93	0.58-1.50	0.764
<i>C3</i>	rs1047286	G/A	0.24	0.24	0.93	0.56-1.55	0.792
<i>C3</i>	rs2230199	G/C	0.24	0.25	0.96	0.59-1.57	0.880
<i>CETP</i>	rs3764261	G/T	0.40	0.35	0.81	0.53-1.25	0.338
<i>CFB</i>	rs4151667	T/A	0.02	0.04	2.00	0.55-7.33	0.297
<i>CFB</i>	rs641153	G/A	0.05	0.06	1.62	0.62-4/28	0.328
<i>CFH</i>	rs800292	G/A	0.21	0.18	0.74	0.40-1.35	0.321
<i>CFH</i>	rs1061170	T/C	0.47	0.62	1.77	1.13-2.78	0.013
<i>CFH</i>	rs12144939	G/T	0.13	0.08	0.62	0.30-1.28	0.199
<i>LIPC</i>	rs493258	C/T	0.44	0.44	0.89	0.56-1.41	0.608
<i>LIPC</i>	rs10468017	C/T	0.28	0.29	1.03	0.64-1.65	0.914
<i>RAD51B</i>	rs8017304	A/G	0.35	0.41	1.14	0.74-1.76	0.547
<i>SKIV2L</i>	rs429608	G/A	0.08	0.12	1.53	0.78-3.00	0.213
<i>SLC</i>	rs8135665	C/T	0.21	0.26	1.26	0.77-2.08	0.357
<i>TIMP3</i>	rs9621532	A/C	0.03	0.07	2.23	0.85-5.87	0.105
<i>TGFBR1</i>	rs334353	T/G	0.23	0.19	0.79	0.45-1.36	0.388
<i>TNFRSF10A</i>	rs1327806	T/G	0.43	0.50	1.44	0.91-2.27	0.119
<i>VEGFA</i>	rs943080	T/C	0.53	0.40	0.59	0.37-0.93	0.024

MAF, minor allele frequency; HF, hyperreflective foci; OR, odds ratio; CI, confidence interval; AMD, age-related macular degeneration.

* OR, P values and 95% CI are based on the multivariate logistic regression for each SNP (0: no major allele, 1: major allele, 2: homozygous major allele, additive model) which included adjustments for age, sex, recruited site (University of Cologne/University of Nijmegen) and AMD-severity (early/intermediate AMD). After Bonferroni-correction for 22 SNPs P values ≤ 0.0023 were accepted as significant.

with intermediate AMD, after adjustments mentioned above (age, sex, site). The results of these subgroup analyses were consistent with our previous results (*ARMS2* rs104909/*HTRA1* rs11200638 OR, 1.79; $P = 0.010$; *CFH* rs1061170 OR, 1.77; $P = 0.013$; *APOE4/TOMM40* rs2075650 OR, 1.98; $P = 0.034$) and showed an additional association with *VEGFA* rs943080 variant (OR, 0.59; $P = 0.024$). Detailed results are demonstrated in Table 3.

DISCUSSION

In the present study, we investigated and quantified HF in the ONL and in the photoreceptor complex of early and intermediate AMD patients and found a strong association between HF and intermediate AMD in our cohort. Furthermore, the data suggested an association of genetic variants in the genes *ARMS2/HTRA1*, *CFH*, *APOE*, and *VEGFA* genes with the presence of HF.

Hyperreflective foci are distinctive SDOCT features which are observed repeatedly in association with photoreceptor layer thinning² and RPE atrophy.²⁰ Hyperreflective foci are considered a risk factor for disease progression to GA.⁸ Hyperreflective foci phenotype seems to be tightly connected to large drusen considering its significant association with intermediate AMD and drusen are not only hallmark of AMD, but also are considered as biomarkers of local immune-mediated inflammation.^{21,22}

In this study, we found a trend that the HF phenotype was associated with *CFH* rs1061170. Complement factor H (CFH) is a key regulator of the alternative pathway of the complement system and genetic changes can lead to dysregulation of the complement cascade and trigger drusen formation with subsequent accumulation of macrophages to Bruch's membrane (BrM).^{23,24} Thus, it is possible that HF phenotype is a

result of the proinflammatory responses associated with polymorphism in *CFH*.

Furthermore, the study also suggests an AMD-severity independent association of HF phenotype with *ARMS2/HTRA1* genes. There is debate on whether the *ARMS2*, *HTRA1*, or both genes are associated with AMD and the functions of their gene products are not fully understood. However, the *ARMS2* and *HTRA1* proteins have been reported to interact with extracellular matrix (ECM) proteins and with proteins involved in remodeling of BrM.^{25,26} Alteration of BrM can lead to a disruption of signaling pathways between RPE and BrM²⁵ and aberrant expression of ECM proteins can influence immune cell activation.²⁷ Earlier studies proposed HF as migrating RPE cells^{3,28} and recent histologic findings of a donor eye with vitelliform dystrophy described the intraretinal HF as a complex of lipofuscin granules, melanolipofuscin granules and melanosomes.²⁸ In contrast, histologic findings of AMD-donor eyes suggested that HF in AMD are very likely not solitary RPE granules alone but may consist of lipid droplets accumulated within phagocytes, such as microglia and macrophages.¹⁰ This latter finding is supported by the observation of HF in other retinal diseases with neuro-inflammatory components, such as diabetic maculopathy^{29,30} or retinitis pigmentosa.³¹

Microglial cells are the primary resident immune cells located in the inner retina involved in the pathogenesis of AMD, diabetic retinopathy, or retinitis pigmentosa.^{16,17,32,33} Once activated, microglia can migrate to the outer retina and to the subretinal space^{14-16,18,34} and induce structural and functional alterations of RPE.¹⁷ In human retinas with GA, activated microglia were found in the ONL where they phagocytize cell debris.¹⁶ In mouse and rat studies, VEGF blockade has been shown to inhibit/reduce microglia activation.^{35,36} Moreover, decrease in HF quantity also was observed after anti-VEGF treatment in AMD and diabetic maculopathy

patients.^{4,6,9,30} Interestingly, in this study, HF also might be associated with rs943080 variant at the *VEGFA* gene in intermediate AMD patients, which has been reported previously to have influence on treatment response.³⁷ Besides, *VEGFA* variant rs943080 is known to be inversely associated with the presence of AMD.¹²

Although HF also were associated with a variant of *APOE4/TOMM40* (translocase of outer mitochondrial membrane) in our study, the numbers of the cases regarding *APOE4/TOMM40* variants in our study are very restricted. Nevertheless, this gene also is known to be associated with neurodegenerative Alzheimer disease.³⁸ Human apolipoprotein E (ApoE) not only is essential for lipid transport and metabolism, but also is involved in neuronal repair, remodeling and degeneration.³⁸ The presumed nature of HF to consist partly from lipid droplets¹⁰ could be related to an association with *ApoE* variants. It is known that RPE and microglia can express ApoE.^{39,40} In mouse model, *APOE4* genotype has been shown to be associated with increased microglia activation⁴¹ and an ApoE-dependent microglial migration was demonstrated.⁴² It is assumed that there is an autoregulatory feedback between microglia activation and ApoE expression for neuroprotective purpose.⁴⁰ It is possible that altered lipid metabolism may enhance microglial migration or activation, with induced formation of HF. However, to evaluate the association of *APOE4/TOMM40* variants with HF presence, studies with larger cohorts are needed.

Our study has several limitations. The phenotype-genotype associations were evaluated for 518 subjects but the findings should be confirmed in additional cohorts. Hyperreflective foci quantification also is challenging and not yet standardized. However, the inclusion of two graders reduced the possibility of subjective judgment of the phenotypes. We limited the HF grading to scans from ONL to RPE to increase accuracy. In the future, the distribution of HF in additional retinal layers and also advanced AMD could be evaluated.

Our study has a limited number of patients that showed HF ($n = 63$), which limited multiple testing criteria. Nevertheless, this is a pilot study to evaluate the association of HF with AMD risk alleles. After Bonferroni correction for 22 SNPs, none of the associations was statistically significant as our sample size was not sufficient for the analyses. Thus, our results are suggestive and should be validated in an independent cohort.

In summary, we found that HF are strongly related to AMD severity in patients with early and intermediate AMD. Furthermore, we suggested an association of *ARMS/HTRA1*, *CFH rs1061170* with the HF-phenotype found in outer retinal layers and in the photoreceptor complex. Our findings supported the notion that modification of BrM or an altered complement activation triggered by *ARMS2/HTRA1* and *CFH* polymorphisms, or altered lipid metabolism triggered by *APOE* may have a role on the formation of HF. Furthermore *VEGFA* polymorphism seems to be a protective factor for HF. Assuming that HF are accumulated inflammatory components, such as activated microglia, further histopathologic studies are needed to understand the relationship between these polymorphisms, inflammatory cells, and the HF phenotype.

Acknowledgments

Supported by the Deutsche Forschungsgemeinschaft (DFG-FOR2240).

Disclosure: **L. Altay**, None; **P. Scholz**, None; **T. Schick**, None; **M. Felsch**, None; **C.B. Hoyng**, None; **A.I. den Hollander**, None; **T. Langmann**, None; **S. Fauser**, Novartis (C), Roche (C), Bayer (C), Quantel (C)

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