Distribution of risk alleles in patients with age-related macular degeneration

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ABSTRACT

INTRODUCTION: Age-related macular degeneration (AMD) is a leading cause of vision loss in elderly people. Several single-nucleotide polymorphisms (SNP) have been shown to either increase or reduce the risk of developing AMD. In this study, we investigated the frequency of ten known risk alleles in a Danish cohort across subtypes of late AMD and explored any relationship to accelerated development of bilateral neovascular AMD.

METHODS: A total of 206 participants were included, 73 hereof had neovascular AMD, 57 geographic atrophy (GA), 28 polypoidal choroidal vasculopathy (PCV) and 48 were healthy aged controls. Genotyping was performed using the Kompetitive allele-specific polymerase chain reaction genotyping assay. Participants with neovascular AMD were followed in the clinic for four years and registered as having developed bilateral disease or having persistent unilateral disease.

RESULTS: We found that patients with neovascular AMD and GA, but not PCV, had a higher frequency of the risk allele for rs10490924 in age-related maculopathy susceptibility 2 (ARMS2) as well as several SNPs related to the complement pathway. Patients who developed bilateral disease within the four-year follow-up had an increased frequency of the risk-allele for rs1061170 in complement factor H (CFH).

CONCLUSIONS: Our results support the notion that *ARMS2* and *CFH* are central in neovascular AMD and GA, and that the risk allele for rs1061170 in *CFH* is associated with accelerated onset of bilateral neovascular AMD.

FUNDING: The Velux Foundation, the Danish Eye Research Foundation, Fight for Sight Denmark, the University of Copenhagen, and Region Zealand funded this study. None of the funding bodies had any role in the design, execution or interpretation of the research performed.

TRIAL REGISTRATION: not relevant.

Age-related macular degeneration (AMD) is a very common acquired age-related disease of the retina, characterised by late-onset neurodegeneration of the photoreceptors and the retinal pigment epithelium (RPE). Genetic predisposition and heritability are important risk factors for AMD. Individuals with a sibling or a parent with AMD are 12-27 times more susceptible to developing AMD [1]. In a twin study of small hard drusen, heritability was estimated to 63% [2]. Several single-nucleotide polymorphisms (SNP) in the complement pathway (*CFH*, *CFI*, *C2*, *CFB* and *C3*), particularly Y402H (rs1061170-C allele) in the gene encoding complement factor H (*CFH*), have been identified as central risk factors of AMD. Possession of one histidine at position 402 (CT genotype) increases the risk of AMD by approximately 2.5-fold, and by six-fold in case of two histidines (CC genotype) [3]. Similarly, A69S (rs10490924-T allele) in age-related maculopathy susceptibility 2 (*ARMS2*) is strongly associated with AMD development and progression, even though *ARMS2* does not have an identified gene product, and the underlying mechanism remains unclear [4]. ARMS2 rs10490924 and the CFH rs1061170 are the most firmly established risk-associated SNPs in AMD [3, 4], but also CFH rs1410996, CFH rs3753394 and CFH rs800292 have been described to be associated with AMD [5]. In patients with polypoidal choroidal vasculopathy (PCV), the CFH rs800292 and the CFB rs2072633 have been shown to be associated with disease [6]. A number of SNPs in the C2 (rs9332739, rs547154) and the CFB gene (rs4151667, rs641153) have been shown to be protective of disease, albeit these protective alleles occur rarely [7].

Early and moderate stages of AMD are typically asymptomatic and observed clinically as lipoproteinaceous debris accumulated below the RPE. Late-stage AMD typically manifests in one of two different, distinct clinical forms; geographic atrophy (GA) or neovascular AMD. GA is one or more areas of macular atrophy of the outer retina and RPE. Neovascular AMD is characterised by new vessels protruding from the choroid or retinal vessels leaking blood and fluid into the retinal layers. PCV is a distinct variant of neovascular AMD, which has a large aneurysmal component, as the choroidal neovascularisation forms a polyp below the RPE [8]. PCV is clinically distinguished from neovascular AMD by indocyanine green (ICG) angiography. The clinical presentation of AMD and its course of disease are very heterogeneous, underscoring the need for a more personalised approach to treatment and risk of progression stratification. One method that may potentially be used to develop a personalised approach to patients with AMD is gene stratification. Therefore, we studied the distribution of known risk alleles in a Danish cohort with different subtypes of late AMD and

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Dan Med J 2020;67(3):A05190295

investigated the influence of risk alleles on disease progression.

METHODS

Study design

This was an observational prospective study comparing known risk alleles in subtypes of AMD with healthy controls. The study was approved by the Regional Committee of Ethics in Research in the Region Zealand, Denmark (R. no. SJ-385 and SJ-379). Oral and written informed consent was obtained from each participant prior to inclusion.

Participants, diagnosis and inclusion

All participants were of Caucasian descent and recruited from the retinal outpatient programme at the Zealand University Hospital, Denmark. Patients' spouses were invited to participate as healthy controls. Since participants were also invited to participate in studies of systemic immunological alterations in AMD [9-13], our study population was restricted to those without any cancer, infectious diseases, haematologic or immunological disorders, and patients not receiving any immune-modulating medication. All participants underwent a detailed examination including best-corrected visual acuity, slit-lamp examination, indirect dilated fundoscopy, spectral domain optical coherence tomography (OCT), and fundus autofluorescence (FAF). We performed angiography with fluorescein and ICG in cases with suspected neovascularisation.

Participants in the following categories were included:

- Healthy controls had no ocular disease, none or less than ten small drusen (< 63 µ) and no pigment abnormalities.
- Participants with GA secondary to AMD had funduscopic soft or reticular drusen and one or more well-defined atrophic areas of depigmentation of the RPE in the macula area corresponding to a reduced FAF signal on the FAF image obtained. Atrophic lesion included the RPE and the outer photoreceptor layer evaluated on OCT. The included patients with GA had no current or former neovascularisation in either eye. Patients with any sign of former neovascularisation (such as fibrosis) were not included in this group.
- Participants with neovascular AMD had drusen maculopathy and exudative changes, fibrovascular RPE detachments and choroidal neovascular membranes with subretinal or sub-RPE haemorrhage. Choroidal neovascularisation was evaluated by angiography, and leakage of fluorescein in the retina was interpreted as activity.
- Participants with PCV had one or more polyps seen in early-phase ICG angiography, which were seen as distinct hyperfluorescent areas with a hypofluorescent halo.

Genotyping analysis

Venous blood was sampled from the antecubital vein of each participant into a 5-ml tube coated with ethylendiamine-tetraacetic acid. DNA was extracted using Chemagic Magnetic Seperation Module 1. DNA samples were sent for genotyping at LGC genomics, using the Kompetitive Allele Specific Polymerase chain reaction (KASP) genotyping assay. The KASP assay is a form of allele-specific polymerase chain reaction that enables accurate bi-allelic scoring of SNPs. The following SNPs were analysed: *ARMS2* rs10490924, *CFH* rs1061170, *CFH* rs800292, *CFH* rs1410996, *CFH* rs3753394, *C2* rs547154, *C2* rs9332739, *CFB* rs4151667, *CFB* rs641153 and *CFB* rs2072633.

Follow-up and neovascular age-related macular degeneration in the fellow eye

All 73 included patients with a diagnosis of neovascular AMD were treated with anti-vascular growth factor agents. The treatment was based on a pro re nata protocol, and at control visits patients had retinal examination performed of both eyes, including best-corrected visual acuity, slit-lamp, indirect dilated fundoscopy, and optical-coherence tomography. A total of 56 patients were followed in the clinic for a four-year period. Seven of the patients had the diagnosis neovascular AMD in both eyes at their initial visit. In all, 25 additional patients developed choroidal neovascularisation in the fellow eye during the observation period. The diagnosis was made using the same criteria as at the initial visit.

Data analysis and statistics

Statistics were done using SPSS 24, and figures were prepared using GraphPad Prism 8. Normally distributed continuous data were presented as mean and standard deviation and compared using parametric tests. Categorical data, such as genotype and allele frequencies, were compared between AMD subtypes and healthy controls using Fisher's exact test. $p < 0.05$ was considered statistically significant. We used the Benjamini-Hochberg procedure to correct for familywise error rate when performing numerous statistical analyses. We assessed the difference in trend towards development of bilateral disease using the log-rank test. The Hardy-Weinberg equilibrium was calculated in the group of healthy controls for each genotype to test for any selection of participants.

Trial registration: not relevant.

RESULTS

A total of 206 participants were included: 73 had neovascular AMD, 57 GA, 28 PCV and 48 were healthy aged controls. Participant age and gender at the time of inclusion are presented in **Table 1**. Participants with PCV and healthy controls were younger than patients with GA and neovascular AMD ($p < 0.001$, one-way ANOVA).

Compared with healthy controls, patients with neovascular AMD had a higher frequency of the risk allele rs10490924-T in *ARMS2*, *CFH* (rs1061170, rs800292, rs1410996) and *C2* rs9332739 (**Table 2**).

Similarly, compared with the control group, patients with GA had a higher frequency of risk alleles in *ARMS2* rs10490924, *CFH* (rs800292, rs1410996), *C2* rs9332739 and *CFB* rs4151667 (Table 2). Patients with PCV did not differ from the healthy controls with respect to allele frequency in any of the investigated SNPs (Table 2).

None of the SNPs deviated from the Hardy-Weinberg equilibrium (*ARMS2* rs10490924: $\chi^2 = 2.074$, $p = 0.150$; *CFH* rs1061170: $\chi^2 = 3.495$, $p = 0.062$; *CFH* rs800292: $\chi^2 = 0.979$, $p = 0.322$; *CFH* rs1410996: $\chi^2 = 1.920$, $p = 0.166$; *CFH* rs3753394: $\chi^2 = 1.141$, $p = 0.285$; *C2* rs547154: $\chi^2 = 0.297$, $p = 0.586$; *C2* rs9332739: $\chi^2 = 1.920$, $p = 0.166$; *CFB* rs4151667: $\chi^2 = 0.145$, $p = 0.703$; *CFB* rs641153: $\chi^2 = 0.312$, $p = 0.576$; *CFB* rs2072633: $\chi^2 = 1.241$, $p = 0.265$).

Furthermore, we found that patients with development of bilateral neovascular AMD had a significantly higher frequency of the risk allele in *CFH* rs1061170 than patients with persistent unilateral disease ($p = 0.036$, Fisher's exact test) (**Figure 1**). Additionally, we created Kaplan-Meier curves for each genotype showing the rate of development of bilateral disease over time (**Figure 2**). The difference in rate was different for the various genotype groups ($\chi^2 = 3.786$; $p = 0.05$, log-rank test).

TABLE 1 / Demographic participant information.

	Healthy controls (n = 47)	Geographic atrophy (n = 57)	PCV (n = 28)	Neovascular AMD (n = 73)	p-value
Age, yrs, mean (± SD)	72.9 (± 8.4)	80.1 (± 7.9)	72.8 (± 7.9)	76.7 (± 7.3)	〈 0.001[a]
Female, n (%)	28 (59.6)	34 (59.6)	16 (57.1)	40 (54.8)	0.944[b]

AMD = age-related macular degeneration; PCV = polypoidal choroidal vasculopathy; SD = standard deviation.
a) 1-way ANOVA test. b) χ^2 test.

DISCUSSION

Scandinavian populations have one of the highest prevalences of late AMD [14] and the prevalence of late AMD is expected to double in Denmark due to demographic shifts [14]. This is the first genotyping study of Danish patients with AMD, and it reveals important information about Danes with AMD. Danish patients with late-stage AMD, both GA and neovascular AMD, have a higher frequency of risk-associated alleles in *ARMS2*. In one large genome-wide association study, the ARMS2 locus was associated with a greater risk of CNV compared with GA. This finding was supported by a sibling correlation study showing that the hereditability of GA and CNV could be explained by *ARMS2* [15]. However, ARMS2 increases the risk of both advanced stages; and in our cohort, the frequency was similar between groups. We found the complement-associated at-risk-alleles to be more frequent in patients with neovascular AMD (CFH rs1061170, CFH rs800292, CFH rs1410996) and in patients with GA (CFH rs800292, CFH rs1410996). Furthermore, we observed that patients with GA more frequently lacked the protective alleles in C2 rs9332739 and in CFB rs4151667 than patients with neovascular AMD. Unlike patients with GA and neovascular AMD, Danish patients with PCV did not differ significantly from healthy controls with respect to the frequency of any risk-alleles measured. Importantly, PCV differs from GA and neovascular AMD in that drusen is far less present and only at a rate similar to that observed in healthy aged controls. Hence, our findings may not be surprising. However, meta-analyses of Asian patients suggest that *ARMS2* rs10490924, *CFH* rs1061170, *CFH* rs800292, *CFH* rs3753394 and *C2* rs547154 are associated with PCV, albeit to a significantly lesser degree than neovascular AMD [16].

Patients who develop neovascularisation in the fellow eye have a higher frequency of the C-allele in *CFH* rs1061170. The CFH genotype has previously been shown to have implications for treatment response as the C allele was associated with a poorer visual acuity and central foveal thickness [17]. A large American population study found no association between genotype and incidence of neovascular AMD in the fellow eye after two years [18]. Studies of Asian and Australian populations found that fellow eye involvement was associated with *ARMS2* and the *CFH* genotype [19, 20]. These opposing results indicate that there is a need to investigate the genotype's influence on clinical manifestation on different continents as these likely differ. Detection of patients at a high risk of developing neovascular AMD in the fellow eye has considerable clinical importance, as the involvement of the fellow eye deeply affects the individual, limiting key aspects of daily life, self-maintenance and ultimately quality of life. Early detection and treatment are crucial to reduce the progression of disease and prevent legal blindness. The ability to predict a patient at risk of developing bilateral disease can potentially help ophthalmologists

TABLE 2 / Frequency of risk alleles in subgroups of late age-related macular degeneration.

			Phenotype				p-value (corrected value)[a], vs healthy		
			healthy controls (n = 48)	GA (n = 57)	PCV (n = 28)	nAMD (n = 73)	GA	PCV	nAMD
ARMS2 rs10490924, risk allele T	Genotype frequency, %	T:T	6.5	17.5	3.6	21.1	< 0.001 (0.010)	0.426 (0.852)	0.004 (0.020)
		T:G	23.9	57.9	39.3	39.4			
		G:G	69.9	24.6	57.1	39.4			
	Allele frequency, %	T	18.5	46.5	23.2	40.8	< 0.001 (0.010)	0.487 (0.740)	< 0.001 (0.001)
		G	81.5	53.5	76.8	59.1			
CFH rs1061170, risk allele C	Genotype frequency, %	C:C	12.5	26.8	25.0	40.6	0.228 (0.326)	0.425 (0.852)	0.005 (0.020)
		C:T	62.5	55.4	53.6	42.0			
		T:T	25.0	17.9	21.4	17.4			
	Allele frequency, %	C	44.7	54.5	51.8	61.6	0.164 (0.234)	0.399 (0.740)	0.010 (0.025)
		T	55.3	45.6	48.2	38.4			
CFH rs800292, risk allele C	Genotype frequency, %	C:C	52.2	71.9	64.3	76.4	0.083 (0.166)	0.702 (0.867)	0.015 (0.037)
		T:C	43.5	26.3	32.1	22.2			
		T:T	4.3	1.8	3.6	1.4			
	Allele frequency, %	C	74.0	85.0	80.3	87.5	0.046 (0.092)	0.371 (0.740)	0.001 (0.005)
		T	26.0	15.0	19.7	12.5			
CFH rs1410996, risk allele G	Genotype frequency, %	G:G	29.8	54.4	46.4	58.9	0.039 (0.098)	0.356 (0.852)	0.006 (0.020)
		A:G	59.6	40.4	42.9	34.2			
		A:A	10.6	5.3	10.7	6.8			
	Allele frequency, %	A	59.6	74.6	67.9	76.0	0.021 (0.053)	0.310 (0.740)	0.007 (0.023)
		G	40.4	25.5	32.1	24.0			
CFH rs3753394, risk allele T	Genotype frequency, %	T:T	8.5	10.5	7.1	2.8	0.107 (0.178)	0.858 (0.867)	0.265 (0.294)
		T:C	21.3	38.6	28.6	29.2			
		C:C	70.2	50.9	64.3	68.1			
	Allele frequency, %	T	19.1	29.8	21.4	17.4	0.077 (0.128)	0.736 (0.920)	0.726 (0.807)
		C	80.9	70.2	78.6	82.6			

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individualise retinal examination frequency in an attempt to reduce the debilitating consequences of bilateral vision loss.

Important limitations must be mentioned. The average age differs between patients with neovascular AMD or GA and the healthy age-matched controls. Especially patients with GA had reached a higher age at the time of inclusion. The younger patients were more frequently accompanied by a spouse, but the older patients were more frequently widowed. This difference has no influence on patient genotype, but it poses a risk since these individuals may develop AMD at a later age. On the other hand, the healthy individuals had an average age of 73 years, < 10 small drusen and no pigment abnormalities. Hence, it is unlikely that these individuals would develop late AMD if we had recruited them a few years later. The relatively small sample size of the patient group results in a lower power level, and a larger sample size would have been desirable, especially for determination of genotype differences in unilateral and bilateral neovascular AMD. Furthermore, it is important that we restricted patients to those without any infectious or immune disease and without any cancer. We are unaware of any association between these conditions and the selected SNPs, but if such a relation exists it would affect our results. To test the influence of the potential selection bias, we tested each SNP and found that none of them deviated from Hardy-Weinberg.

CONCLUSIONS

This prospective study confirmed ARMS2 and complement pathway as part of the AMD pathogenesis. Furthermore, our data underline the clinical relevance of genotyping and show that special attention should be paid to patients with a high-risk CFH genotype as they have an increased risk of developing bilateral disease. A larger survey mapping the genotypes observed in retinal degeneration might pave the way for personalised treatment of this highly heterogeneous disease.

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TABLE 2 CONTINUED / Frequency of risk alleles in subgroups of late age-related macular degeneration.

			Phenotype				p-value (corrected value)[a], vs healthy		
			healthy controls (n = 48)	GA (n = 57)	PCV (n = 28)	nAMD (n = 73)	GA	PCV	nAMD
C2 rs547154, risk allele C	Genotype frequency, %	C:C	85.1	87.7	85.7	94.4	0.777 (0.863)	0.612 (0.867)	0.110 (0.138)
		C:A	14.9	12.3	14.3	5.6			
		A:A	0.0	0.0	0.0	0.0			
	Allele frequency, %	C	92.5	93.9	92.9	97.2	0.708 (0.709)	0.945 (0.945)	0.089 (0.148)
		A	7.5	6.1	7.1	2.8			
C2 rs9332739, risk allele G	Genotype frequency, %	G:G	89.1	100.0	96.4	97.2	0.016 (0.057)	0.399 (0.852)	0.033 (0.066)
		C:G	10.9	0.0	3.6	1.4			
		C:C	0.0	0.0	0.0	1.4			
	Allele frequency, %	C	94.5	100.0	98.2	97.9	0.017 (0.053)	0.275 (0.740)	0.159 (0.209)
		G	5.5	0.0	1.8	2.1			
CFB rs4151667, risk allele T	Genotype frequency, %	T:T	89.4	100.0	96.4	97.2	0.017 (0.057)	0.401 (0.852)	0.055 (0.092)
		A:T	10.6	0.0	3.6	1.4			
		A:A	0.0	0.0	0.0	1.4			
	Allele frequency, %	T	94.7	100.0	98.2	97.9	0.018 (0.053)	0.285 (0.740)	0.167 (0.209)
		A	5.3	0.0	1.8	2.1			
CFB rs641153, risk allele C	Genotype frequency, %	C:C	84.4	89.5	85.7	94.4	0.322 (0.403)	0.582 (0.867)	0.075 (0.107)
		T:C	15.6	10.5	14.3	5.6			
		T:T	0.0	0.0	0.0	0.0			
	Allele frequency, %	C	92.2	94.7	92.9	97.2	0.465 (0.581)	0.888 (0.945)	0.083 (0.148)
		T	7.8	5.3	7.2	2.8			
CFB rs2072633, risk allele C	Genotype frequency, %	C:C	34.0	35.1	39.3	35.7	0.905 (0.905)	0.867 (0.867)	0.983 (0.983)
		T:C	42.6	45.6	42.9	41.4			
		T:T	23.4	19.3	17.9	22.9			
	Allele frequency, %	C	55.3	57.9	60.7	56.4	0.709 (0.709)	0.518 (0.740)	0.877 (0.877)
		T	44.7	42.1	39.3	43.6			

ARMS2 = gene encoding age-related maculopathy susceptibility 2; *C2* = gene encoding complement 2; *CFB* = gene encoding complement factor B; *CFH* = gene encoding complement factor H; GA = geographic atrophy; nAMD = neovascular age-related macular degeneration; PCV = polypoidal choroidal vasculopathy.
a) Fisher's exact test (based on Benjamini-Hochberg procedure).

FIGURE 1 / Patients with bilateral disease (**A**) during a four-year follow-up period were associated with a higher frequency of the C allele in CFH Y402H than patients with unilateral disease (**B**) (p = 0.036, Fisher's exact test).

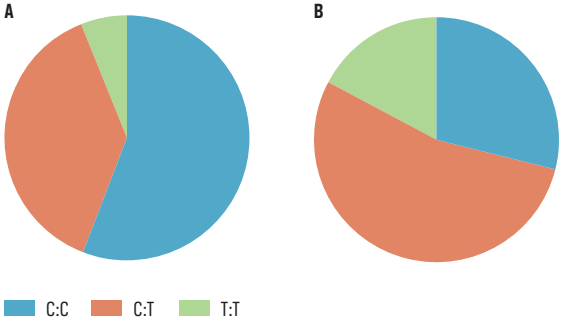
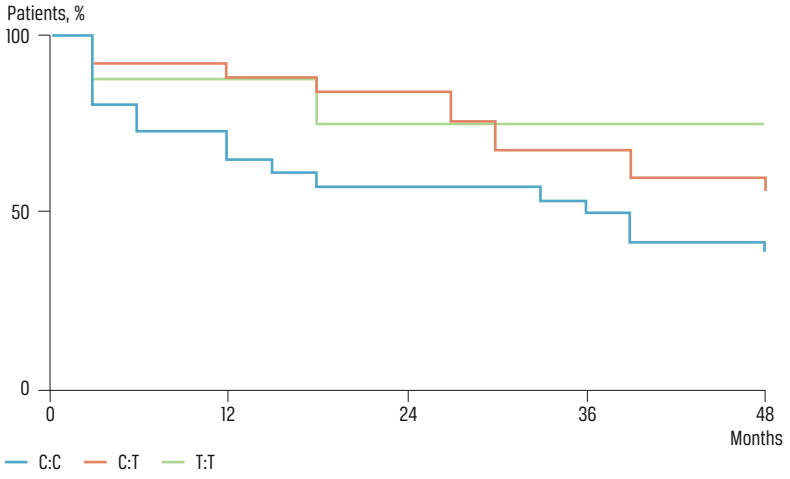


FIGURE 2 / Kaplan-Meier curves demonstrating the development of bilateral disease during 48 months of follow-up based on the *CFH* Y402H genotype.



ACCEPTED: 11 December 2019

CONFLICTS OF INTEREST: none. Disclosure forms provided by the authors are available with the full text of this article at Ugeskriftet.dk/dmj

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