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Association between polymorphism of the *NQO1, NOS3* and *NFE2L2* genes and AMD

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1. ABSTRACT

Oxidative stress may play a role in the pathogenesis of age-related macular degeneration (AMD). In this study we examined the association between AMD risk and polymorphisms of genes encoding enzymes involved in the generation and removal of iron-mediated oxidation: *NQO1* (609C> T, rs1800566), *NOS3* (894G>T, rs1799983) and *NFE2L2* (28312647A>G, rs6726395). We found that the G/G genotype of the rs6726395 polymorphism was associated with a decreased risk of AMD wet form (OR 0.44) and on the other hand the T allele of the rs1799983 polymorphism increased such risk (OR 1.63). We also observed that the C/C-G/T combined genotype of the rs1800566 and rs1799983 polymorphisms was positively correlated with a reduced risk of AMD as well as of its dry form (OR 0.40 and 0.35). The presence of the G/T-G/G combined genotype of the rs1799983 and rs6726395 polymorphisms decreased the risk of this disease (OR 0.35). The results obtained in our study suggest a potential role of the rs1800566, rs1799983 and rs6726395 polymorphisms in the AMD pathogenesis.

2. INTRODUCTION

Age-related macular degeneration (AMD) is one of the most common causes of vision loss in the elderly. Despite the increase in the awareness of this condition, its pathogenesis still remains unclear. About 11 million people across the globe are affected by AMD, which incidence increases with age (1-2). Two manifestations of AMD can be distinguished, dry form (also referred to as atrophic, nonexudative, or geographic atrophy) and wet form (also referred to as neovascular, exudative, choroidal neovascularisation, or disciform AMD) (3).

The identification of risk factors is important for the understanding of the genesis and for establishing strategies to prevent and cure AMD. Both environmental and genetic risk factors may play a role in the pathogenesis of AMD: age, gender, Caucasian ethnicity, hypertension, obesity, cataract and cataract surgery, high–fat diet and chronic sunlight exposure, tobacco smoking (4-16). Over the past few years, several single nucleotide

polymorphisms (SNPs) have been associated with AMD (17-24).

High oxygen concentrations, prolonged exposure to light, and the presence of photosensitizers are factors favoring the generation of reactive oxygen species (ROS) in the macular region. Oxidative stress may play a role for age-related accumulation of lipofuscin in pigmented epithelium cells located near the macula (25). Increased exposure to free oxide and nitrile radicals results in enhanced activity of enzymes causing degradation of free radicals.

While bioavailability of iron is generally limited, its pathological accumulation within tissues aggravates the generation of ROS and elicits toxic effects, which are mainly related to oxidative stress. Iron could play a role in the pathogenesis of AMD by catalyzing Haber-Weiss and Fenton reactions that convert hydrogen peroxide (H_2O_2) to free oxide radicals (26). AMD-affected eyes were found to have an excess of both chelatable and nonchelatable iron in the retinal pigmented epithelium (RPE) and Bruch's membrane including drusen (27). Macular iron levels were found to increase with age (28). Deficiency of the iron ferroxidases ceruloplasmin and hephestin, which interferes with export of iron, led to a retinal degeneration by iron overload with some features of AMD (29, 30).

Several enzymes are important in the formation and reduction of iron-generated ROS. NAD(P)H:quinine oxidoreductase 1 (NQO1) plays a role in the reduction of endogenous catechol estrogens generated in the metabolism of estrogen. By catalyzing the two-electron reduction of catechol estrogens and other quinones, including the reactive semiquinone intermediate that drives the Fenton reaction is bypassed, and superoxide-mediated release of iron from ferritin stores is prevented (31). The rs1800566 polymorphism of the *NQO1* gene was reported to be linked to the loss of NQO1 enzyme activity (32, 33). The expression of the *NQO1* gene is regulated by nuclear factor erythroid2-related factor 2 (NFE2L2) (27). Oxidative stress promotes nuclear accumulation of NFE2L2 and activates transcription of *NQO1* (34). The polymorphisms of the *NFE2L2* gene may play a role in the etiology of different kind of diseases including age-related cataract (35-39).

The constitutive endothelial nitric oxide synthase (NOS3) is expressed in the endothelium and generates low amounts of short-lived nitric oxide (NO) by converting L-arginine to citrulline. NO is considered to be cytoprotective and can act as an antioxidant by scavenging ROS and can bind to iron to reduce redox cycling (40, 41). The 894G>T polymorphism of the *NOS3* gene was reported to lead to reduced NO levels (42).

The relationship between the genetic variability of NQO1, NOS3 and NFE2L2 and the development of AMD remains unknown. In the present work we searched for the association between the rs1800566 polymorphism of the *NQO1* gene, the rs1799983 polymorphism of the *NOS3* gene as well as the rs6726395 polymorphism of the *NFE2L2* gene and the risk of AMD in a Polish population. Moreover, we looked for the modulation of this association by some environmental and social factors including age, gender, living environment (urban/rural) and AMD in family.

The polymorphisms we have chosen cover a small part of genetic variation of the *NQO1*, *NOS3* and *NFE2L2* genes – so far the number of polymorphisms of these genes has been exceeded 800, according to the NCBI base.

The first two polymorphisms are located in the coding region of the *NQO1* and *NOS3* genes, respectively, whereas the third one is placed in the intron of the *NFE2L2* gene. Polymorphisms located in the coding region of the gene may affect the structure and/or function of its protein product(s), while polymorphism located in intron regions may directly affect the splicing, which may eventually result in the structure/function changes in the final product. These polymorphisms have a relatively high population frequency and they have not been studied in AMD.

3. MATERIALS AND METHODS

3.1. Clinical subjects

Blood samples were obtained from 281 patients with AMD and from 105 individuals without AMD (controls). Among AMD patients, 101 had dry AMD and the remaining 180 – wet form of the disease. The control subjects searched for medical advice in Department of Ophthalmology, University of Warsaw, Poland in 2010 due to various ophthalmological disturbances, mainly cataract. They had no clinical evidence of AMD after undergoing the same ophthalmic examination that was performed to confirm AMD in the patient group. Medical history was obtained from all subjects, and no one reported current or previous genetic disease. The characteristics of the subjects enrolled in this study are presented in Table 1. All patients and controls were examined in the Department of Ophthalmology, Medical University of Warsaw. They underwent ophthalmic examination, including best-corrected visual acuity, intraocular pressure, slit lamp examination, and fundus examination, performed with a slit lamp equipped with either non-contact or contact fundus lenses. Diagnosis of AMD was confirmed by optical coherence tomography (OCT) and, in some cases, by fluorescein angiography (FA) and indocyanin green angiography (ICG). OCT evaluated retinal thickness, the presence of RPE atrophy, drusen, or subretinal fluid and intraretinal edema; angiography assessed the anatomical status of the retinal vessels, the presence of choroidal neovascularization and leakage. The OCT examinations were performed with Stratus OCT model 3000, software version 4.0 (Oberkochen, Germany). The FA and ICG examinations were completed with a Topcon TRC-50I IX fundus camera equipped with the digital Image Net image system, version 2.14 (Topcon, Tokyo, Japan). Structured questionnaire was used to get information about smoking habit, living environment and the history of AMD among first-degree relatives. The genetic analyses did not interfere with diagnostic or therapeutic procedures. The study was approved by the Bioethics Committee of the Medical

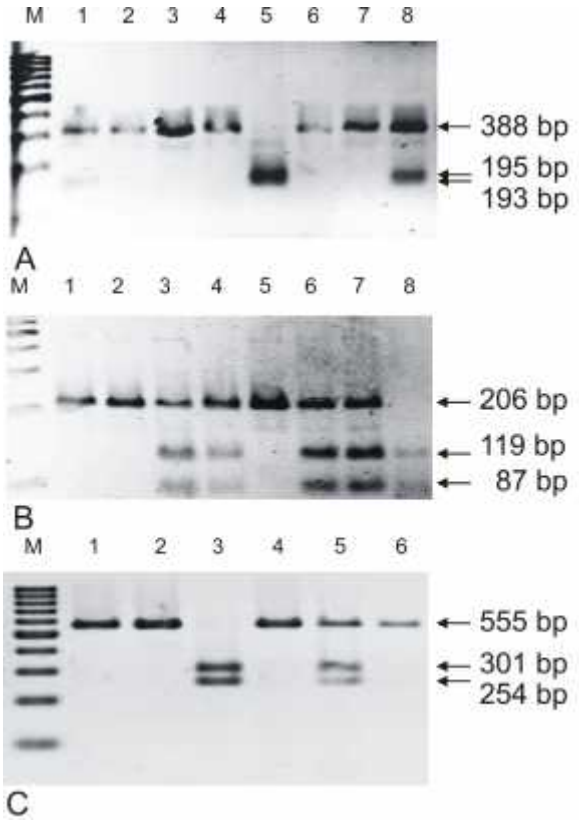

Figure 1. A representative picture of the rs1800566, rs1799983 and rs6726395 polymorphisms analysis. Band sizes are indicated on the right of the panel. A) PCR-RFLP of the rs1800566 polymorphism of the *NQO1* gene. Lane M, DNA marker 100 bp, lane 2-4, 6 and 7 the C/C homozygote is not cleaved by *Hin*fI enzyme and remains the single 388 bp band, lane 5 the T/T homozygote is cleaved by *Hin*fI and yields 195 bp and 193 bp bands, lane 1 and 8 the C/T heterozygote contains all 3 bands (388, 195 and 193 bp) following restriction digestion. B) PCR-RFLP of the rs1799983 polymorphism of the *NOS3* gene. Lane M, DNA marker 100 bp, lane 1, 2 and 5 the G/G homozygote is not cleaved by *Mbo*I enzyme and remains the single 206 bp band, lane 8 the T/T homozygote is cleaved by *Mbo*I and yields 119 bp and 87 bp bands, lane 3, 4, 6 and 7 the G/T heterozygote contains all 3 bands (206, 119 and 87 bp) following restriction digestion. C) PCR-RFLP of the rs6726395 polymorphism of *NFE2L2* gene. Lane M, DNA marker 100 bp (Fermentas), lane 1, 2, 4 and 6 the A/A homozygote is not cleaved by *Cvi*QI enzyme and remains the single 555 bp band, lane 3 the G/G homozygote is cleaved by *Cvi*QI and yields 301 bp and 254 bp bands, lane 5 the A/G heterozygote contains all 3 bands (555, 301 and 254 bp) following restriction digestion.

University of Warsaw, Poland, and each patient gave a written informed consent.

3.2. DNA preparation

Peripheral blood lymphocytes (PBLs) were isolated by centrifugation in a density gradient of Histopaque-1077 (15 min, 280×g). The pellet containing the PBLs was resuspended in Tris–EDTA buffer, pH 8, to yield about 1-3×10^5 cells/ml. Genomic DNA was extracted from the PBLs by DNA Blood Mini Kit (A & A Biotechnology, Gdansk, Poland). The final samples were kept in Tris-EDTA buffer, pH 8, at −20°C until use.

3.3. Genotype determination

Restriction fragments length polymorphism PCR (RFLP-PCR) was employed to determine the genotypes of the rs1800566, rs1799983 and rs6726395 polymorphisms. Each PCR tube contained an aliquot of 20 µl of mix consisting of 10 ng genomic DNA, 1.25 U *Taq* polymerase (Epicentre, Madison, WI, USA) in 1×PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 11 mM MgCl2, 0.1% gelatine), 1.5 mM $MgCl_2$, 50 mM dNTPs, and 250 nM each primer. Thermal cycling conditions were as follows: initial denaturation step at 95°C for 5 min, 30 cycles at 95°C for 30 sec and 30 sec at the 55°C annealing temperature, and at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The PCR was carried out in a MJ Research, INC thermal cycler, model PTC-100 (Waltham, MA, USA).

The *NQO1* rs1800566 polymorphism was determined using the following primers (Sigma-Aldrich, St. Louis, MO, USA): sense, 5'-GTAAGAGAGAGACGCTAGCTCTGAA-3; antisense, 5'- TCTTGTTTTTCTCCTCATCCTG-3'. The 388 bp PCR product was digested 3 hours with 2 units of the restriction enzyme *Hin*fI (New England Biolabs, Ipswich, UK). The C/C homozygote is not cleaved by *Hin*fI enzyme and remains as the single 388 bp band, but the T/T homozygote is cleaved by *Hin*fI and yields a 195 bp and 193 bp bands. The PCR products were separated onto a 3% agarose gel. Figure 1 A presents a representative gel obtained after genotyping of this polymorphism.

The rs1799983 polymorphism of *NOS3* gene was determined using the following primers (Sigma-Aldrich, St. Louis, MO, USA): sense, 5'-CATGAGGCTCAGCCCCAGAAC-3; antisense, 5'-AGTCAATCCCTTTGGTGCTCAC-3'. The 206 bp PCR product was digested 3 hours with 2 units of the restriction enzyme *Mbo*I (New England Biolabs, Ipswich, UK). The T/T homozygote was digested into 119 and 87 bp fragments whereas the G/G variant is not cleaved by *Mbo*I enzyme and remains as the single 206 bp band. The PCR products were separated onto a 3% agarose gel. The PCR products were separated onto a 3% agarose gel. Figure 1 B presents a representative gel obtained after genotyping of this polymorphism.

The rs6726395 polymorphism of the *NFE2L2* gene was determined using the following primers (Sigma-Aldrich, St. Louis, MO, USA): sense, 5'-AAGGAGATCCCAGGATAAAAATC-3'; antisense, 5'-ACCAAGCAATGAAGCTGTCC-3'. The 555 bp PCR product was digested 3 hours with 2 units of the restriction enzyme *Cvi*QI (New England Biolabs, Ipswich, UK). The G/G homozygote was digested into 301 and 254 bp fragments whereas the A/A variant is not cleaved by *Cvi*QI enzyme and remains as the single 555 bp band. The PCR

Table 1. Characteristics of patients with age-related macular degeneration (AMD) and individuals without visual disturbances (controls; mean ± SD)

Individuals	Number	Age (years)	Gender (females+males)
All	386	72.8 ± 9.5	255F + 131M
AMD	281	72.8 ± 8.4	185F + 96M
Dry AMD	101	74.3 ± 8.7	67F + 34M
Wet AMD	180	72.5 ± 8.0	118F + 62M
Controls	105	71.7 ± 10.2	70F + 35M

Table 2. The occurrence of AMD with age, gender, smoking habit, environment of life and familiar status of AMD

Characteristics	Controls, number (%)	AMD patients, number (%)	OR (95% CI)	*p* value
Age				
Up to 75 years	77 (73)	150 (53)	Ref.	
Over 75 years	28 (27)	131 (47)	**2.40 (1.46 – 3.92)**	**< 0.001**
Mean ± SD	68.29 ±10.95	72.46 ± 8.51		
Range	50-88	52-93		
Gender				
Female	82 (78)	185 (66)	Ref.	
Male	23 (22)	96 (34)	**1.85 (1.09 – 3.12)**	**0.021**
Smoking				
Never	59 (66)	135 (59)	Ref.	
Yes (ever, moderate, heavy)	30 (34)	94 (41)	1.36 (0.82 – 2.28)	0.229
Enviroment of life				
Rural	33 (37)	50 (30)	Ref.	
Urban	56 (63)	115 (70)	1.35 (0.78 – 2.33)	0.272
AMD in family				
No	86 (97)	132 (80)	Ref.	
Yes	3 (3)	33 (20)	**7.16 (2.13 – 24.09)**	**< 0.001**

Data in boldface are statistically significant ($p < 0.05$), Ref. denotes reference group, ie. group relative which ORs were calculated.

products were separated onto a 3% agarose gel. Figure 1 C presents a representative gel obtained after genotyping of this polymorphism. We sequenced 15% randomly chosen samples and the results obtained by sequencing were 100% concordant with those obtained by PCR-RFLP. Figure 2 shows some representative chromatograms from the sequencing.

3.4. Statistical analysis

The allelic frequencies were estimated by gene counting and the genotypes were scored. The χ^2 analysis was used to compare the observed number of genotypes with that expected for a population in Hardy–Weinberg equilibrium. The χ^2 analysis was also used to test the significance of the differences of observed alleles and genotypes between groups. A logistic regression model was used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). In all tests p values of less than 0.05 were considered statistically significant. The genotype-associated risk was given by the crude ORs and the *p* value. Odd ratios were then adjusted for possible interfering factors. To verify a potential gene-environment interaction, the patients and control groups were stratified depending on age, gender and the occurrence of AMD among first-degree relatives. Multiple unconditioned logistic regression analyses were run to test the association of genotypes and environmental and social factors with AMD. We adjusted the estimated *p*-values for a multiple testing, and hence corrected them for the occurrence of false positives, with the use of the Bonferroni's correction, which is considered very stringent statistical procedure and leads to possibly the least number of false positives. Statistical analysis was performed using STATISTICA 9.0 package (Statsoft, Tulusa, OK, USA).

4. RESULTS

Clinical characteristics of patients and controls enrolled in the study are presented in Table 1.

The genotype and allele distributions of the rs1800566-*NQO1*, rs1799983-*NOS3* and rs6726395-*NFE2L2* polymorphisms in AMD patients and controls are presented in Tables 3, 4 and 5. The distribution of the genotypes of the polymorphisms of the *NQO1* and *NFE2L2* genes were in Hardy-Weinberg equilibrium ($p > 0.05$, data not shown), but this was not the case for the genotypes of the *NOS3* gene for both AMD patients and controls ($p < 0.05$; data not shown). This effect may be underlined by a very low frequency of the T/T genotype in Polish population.

The distribution of genotypes of the rs1799983 polymorphism in cases (AMD and wet form of AMD) was significantly ($p = 0.008$ and $p = 0.001$, respectively) different from controls (Tables 3 and 5). Moreover, the difference between distribution of genotypes of the rs6726395-*NFE2L2* polymorphism in the wet form of AMD and controls was statistically significant ($p = 0.043$, Table 5).

We performed analysis of the genotypes of the rs1800566, rs1799983 and rs6726395 polymorphisms in the control group and in AMD patients (Table 3) as well as in groups with dry (Table 4) and wet (Table 5) form of the disease. The T allele of the rs1799983 polymorphism was associated with the occurrence of wet AMD (Table 5). We observed an association of AMD wet form with the G/G genotype of the rs6726395 polymorphism of the *NFE2L2* gene (Table 5). This genotype decreased the risk of AMD

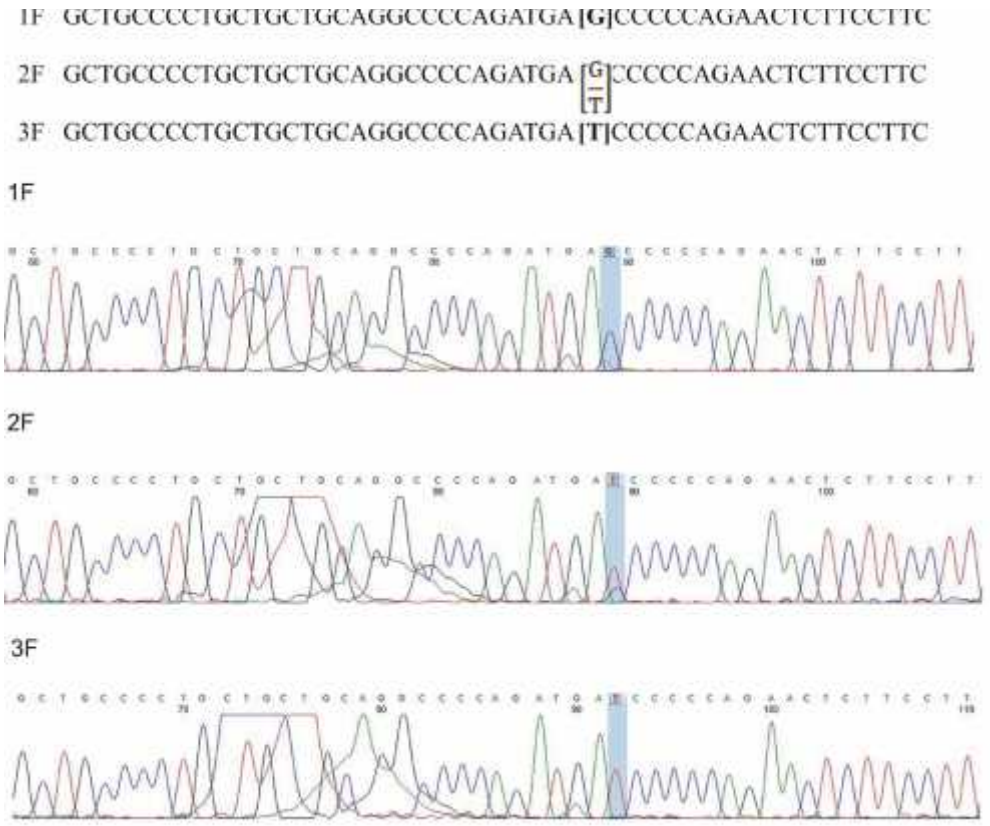

Figure 2. A representative chromatograms from sequencing of the fragment of DNA containing the rs1799983 polymorphism site in the *NOS3* gene. 1F denotes the G/G genotype, 2F – the G/T genotype, 3F – the T/T genotype.

Table 3. Distribution of genotypes, frequency of alleles of the rs1800566 of the *NQO1* gene, the rs1799983 polymorphisms of the *NOS3* gene and the rs6726395 polymorphisms of the *NFE2L2* gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with age-related macular degeneration (AMD) and individuals without visual disturbances (controls).

Genotype or allele (polymorphism in bold)	**Controls (n=105)**		**AMD (n=281)**		**Crude OR (95% CI)**	**Adjusted[a] OR (95% CI)**
	Number	**Frequency**	**Number**	**Frequency**		
rs1800566						
C/C	87	0.83	215	0.76	Ref.	Ref.
C/T	14	0.13	53	0.19	0.95 (0.27 – 3.28)	0.97 (0.22 – 4.27)
T/T	4	0.04	13	0.05	0.81 (0.23 – 2.88)	1.03 (0.23 – 4..54)
C	188	0.90	483	0.86	Ref.	
T	22	0.10	79	0.14	1.57 (0.83 – 2.97)	1.13 (0.54 – 2.40)
$\chi^2 = 1.855$, $p > 0.05$[b]						
rs1799983						
G/G	46	0.44	159	0.57	Ref.	Ref.
G/T	57	0.54	106	0.38	0.23 (0.05 – 1.07)	0.26 (0.05 – 1.28)
T/T	2	0.02	16	0.05	4.22 (0.93 – 19.10)	3.84 (0.78 – 18.85)
G	149	0.71	424	0.75	Ref.	Ref.
T	61	0.29	138	0.25	1.64 (1.01 – 2.68)	1.64 (1.03 – 2.69)
$\chi^2 = 9.668$, $\mathbf{p = 0.008}$[b]						
rs6726395						
A/A	24	0.18	49	0.17	Ref.	Ref.
A/G	56	0.42	175	0.62	1.44 (0.82 – 2.53)	1.46 (0.79 – 2.71)
G/G	25	0.52	57	0.21	0.69 (0.39 – 1.22)	0.68 (0.37 – 1.26)
A	104	0.49	273	0.49	Ref.	Ref.
G	106	0.51	289	0.51	1.47 (0.85 – 2.57)	1.58 (0.87 – 2.86)
$\chi^2 = 2.656$, $p > 0.05$[b]						

Data in boldface are statistically significant ($p < 0.05$), Ref. denotes reference group, i.e. group relative which ORs were calculated. aAdjusted for age, gender and familiar status of AMD; bThe difference between distributions for cases and controls

Table 4. Distribution of genotypes, frequency of alleles of the rs1800566 of the *NQO1* gene, the rs1799983 polymorphisms of the *NOS3* gene and the rs6726395 polymorphisms of the *NFE2L2* gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with dry age-related macular degeneration (AMD) and individuals without visual disturbances (controls)

Genotype or allele (polymorphism in bold)	Controls (n=105)		Dry AMD (n=101)		Crude OR (95% CI)	Adjusted[a] OR (95% CI)
	Number	Frequency	Number	Frequency		
rs1800566						
C/C	87	0.83	74	0.64	Ref.	Ref.
C/T	14	0.13	23	0.31	0.91 (0.21 – 3.82)	0.90 (0.12 – 6.76)
T/T	4	0.04	4	0.05	1.11 (0.26 – 4.76)	1.11 (0.15 – 8.34)
C	188	0.90	171	0.79	Ref.	
T	22	0.10	31	0.21	1.69 (0.78 – 3.65)	0.88 (0.33 –2.34)
$\chi^2 = 3.162$, $p > 0.05$[b]						
rs1799983						
G/G	46	0.44	57	0.56	Ref.	Ref.
G/T	57	0.54	40	0.40	0.19 (0.03 – 0.98) $p = 0.046$	0.17 (0.03 – 0.95) $p = 0.040$
T/T	2	0.02	4	0.04	5.25 (1.02 – 26.09)	5.77 (1.05 – 31.82)
G	149	0.71	154	0.76	Ref.	Ref.
T	61	0.29	48	0.24	1.59 (0.91 – 2.77)	1.66 (0.91 – 3.02)
$\chi^2 = 4.745$, $p > 0.05$[b]						
rs6726395						
A/A	24	0.18	15	0.15	Ref.	Ref.
A/G	56	0.42	54	0.53	0.77 (0.40 – 1.47)	0.73 (0.36 – 1.50)
G/G	25	0.52	32	0.32	1.30 (0.68 – 2.49)	1.36 (0.66 – 2.77)
A	104	0.49	84	0.42	Ref.	Ref.
G	106	0.51	118	0.58	1.63 (0.77 – 3.45)	1.72 (0.77 – 3.85)
$\chi^2 = 2.896$, $p > 0.05$[b]						

Data in boldface are statistically significant ($p < 0.05$), Ref. denotes reference group, ie. group relative which ORs were calculated. [a]Adjusted for age, gender and familiar status of AMD. [b]The difference between distributions for cases and controls

wet form (Table 5). We did not observe any association between the occurrence of AMD and the rs1800566 polymorphism.

The C/C-G/T genotype of the rs1800566 and rs1799983 polymorphisms exerted a protective effect against AMD and its dry form. (Table 6). The presence of the combined G/T-G/G genotype of the rs1799983 and rs6726395 polymorphisms decreased the risk of AMD (Table 6). We also investigated relationship between the risk of AMD and age, gender, smoking habit, living environment (urban or rural) and family status of AMD independently of genotype. We compared controls and AMD patients according to these parameters. Male gender, age over 75 years and familiar history of AMD significantly increased the risk of AMD (Table 2).

Next, we examined, whether associations between the polymorphisms and AMD were modulated by environmental factors. To assess the interaction between gene and environment in relation to AMD risk we performed analysis with stratification of controls and AMD patients in separate groups depending on age, gender and family status of AMD. Only matching variables and factors that altered the ORs by ≥ 10% were considered as risk factor in the final multivariate model. Adjustment for the potential confounders did not alter the previously observed estimates of an association between the G/G genotype of the rs1799983 polymorphism of the *NFE2L2* gene and wet AMD risk (Table 5).

Further, we made a multiple genes analysis, using the data from single gene models and all genes model of logistic regression. We did not find any correlation between these analysis and AMD risk (data not shown).

5. DISCUSSION

The pathogenesis of AMD is not completely known and this hampers the development of rational therapies in this disease. Genetic factors have received attention from the aspect of susceptibility to the development of AMD (43). Among a number of issues, it remains to be elucidated why the disease develops in only a limited number of aged individuals who share similar environment and quality of life. Identifying the genetic factors would contribute to understanding the pathogenesis. Recent studies report a significant association between the genetic polymorphism of genes encoding antioxidant enzymes and development of the age-related macular degeneration (44).

Iron can be involved in the pathogenesis of AMD through the oxidative stress as a source of free radical damage but this hypothesis has not been verified experimentally and further studies are needed to establish the relationship between disturbance in iron homeostasis and AMD. It is well documented that genetic polymorphism of genes encoding enzymes involved in the generation and removal of iron-mediated reactive oxygen species including NQO1, NOS3 and NFE2L2 may be associated with breast cancer risk, after supplementation of iron (45). Enzyme products of *NQO1*, *NOS3* and *NFE2L2* genes have antioxidant properties, and therefore, genetic alternations in these genes may lead to their lower activities, presumably leading to a limited ability to reduce levels of iron-generated and non-iron-generated oxidative

Table 5. Distribution of genotypes, frequency of alleles of the rs1800566 of the *NQO1* gene, the rs1799983 polymorphisms of the *NOS3* gene and the rs6726395 polymorphisms of the *NFE2L2* gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with wet age-related macular degeneration (AMD) and individuals without visual disturbances (controls)

Genotype or allele (polymorphism in bold)	Controls (n=105)		Wet AMD (n=180)		Crude OR (95% CI)	Adjusted[a] OR (95% CI)
	Number	Frequency	Number	Frequency		
rs1800566						
C/C	87	0.83	141	0.78	Ref.	Ref.
C/T	14	0.13	30	0.17	0.94 (0.25 – 3.52)	0.76 (0.17 – 3.47)
T/T	4	0.04	9	0.05	0.68 (0.17 – 2.69)	0.79 (0.15 – 4.11)
C	188	0.90	312	0.87	Ref.	Ref.
T	22	0.10	48	0.13	1.49 (0.75 – 2.95)	1.26 (0.54 – 2.94)
$\chi^2 = 0.853$, $p > 0.05$[b]						
rs1799983						
G/G	46	0.44	102	0.57	Ref.	Ref.
G/T	57	0.54	66	0.37	0.27 (0.05 – 1.32)	0.27 (0.05 – 1.51)
T/T	2	0.02	12	0.06	3.68 (0.75 – 17.76)	3.57 (0.66 – 19.43)
G	149	0.71	270	0.75	Ref.	Ref.
T	61	0.29	90	0.25	**1.63 (1.00 – 2.67)** $p^* = 0.014$	1.54 (0.91 – 2.62)
$\chi^2 = 13.518$, $\boldsymbol{p = 0.001}$[b]						
rs6726395						
A/A	24	0.18	34	0.19	Ref.	Ref.
A/G	56	0.42	121	0.67	2.55 (1.16 – 4.34)	2.26 (1.10 – 4.63)
G/G	25	0.52	25	0.14	**0.44 (0.23 – 0.85)** $p^* = 0.039$	0.44 (0.21 – 0.90)
A	104	0.49	189	0.53	Ref.	Ref.
G	106	0.51	171	0.47	1.32 (0.73 – 2.40)	1.92 (0.89 – 4.17)
$\chi^2 = 6.293$, $\boldsymbol{p = 0.043}$[b]						

Data in boldface are statistically significant ($p < 0.05$), *- denote p-values with the Bonferroni's correction, Ref. denotes reference group, ie. group relative which ORs were calculated. [a]Adjusted for age, gender and familiar status of AMD. [b]The difference between distributions for cases and controls

stress (32, 33, 42, 46–48). That is why we decided to study the genetic variability in the *NQO1*, *NOS3* and *NFE2L2* genes in both forms of AMD. This study showed that polymorphisms associated with iron-generated oxidative stress might be important in AMD etiology.

We found that the G/G genotype of the rs6726395 polymorphism of the *NFE2L2* gene decreases the risk of wet AMD. We can speculate that this might be caused by the change in splicing pattern of the gene resulting in an increased ability to reduce levels of iron-generated oxidative stress in AMD patients.

Oxidative stress promotes nuclear accumulation of NFE2L2 and activates transcription of *NQO1*. In animals, NQO1 suppression increases estradiol-dependent tumor formation (49, 50). We did not find any association between the rs1800566 polymorphism of the *NQO1* gene and AMD risk. However, this polymorphism was reported to be associated with other diseases. Zhu *et al.* suggested that it might serve as a functional genetic marker for gastric cancer in the Singapore-Chinese population (51). Hubackova *et al.* showed that the role of *NQO1* in human mammary gland carcinogenesis does not seem to be directly associated with clinico-pathological factors (52). Also Zai *et al.*did not observe a significant association of the rs1800566 polymorphism with tardive dyskinesia occurrence scores in Caucasian and African American populations (53).

NOS3 generates a short-living nitric oxide which is considered to be cytoprotective and can act as an antioxidant by ROS scavenging (40, 41, 54). We found an association between the rs1799983 polymorphism of the *NOS3* gene and AMD risk. The T allele variant of this polymorphism decreased the risk of wet AMD. This polymorphism was also associated with other diseases, including cancer, migraine, type 2 diabetes mellitus and coronary artery disease (55-58).

The *NQO1* C/C genotype was not associated with the occurrence of AMD, but it was associated with a decreased risk in combination with the *NOS3* G/T genotype. Moreover, the *NOS3* G/T genotype was also not associated with the occurrence of AMD, but in combination with the *NFE2L2* G/G genotype decreased the risk of the disease. This was probably due to a complex interaction between these polymorphisms.

Because AMD is caused by environmental factors triggering disease in genetically susceptible subjects (59-63), we investigated the relationship between biological and environmental parameters and risk of AMD independently of genotype. Our studies suggest that age, gender and familiar history of AMD are risk factors in AMD. This is in general agreement with results obtained by others (64-67). We did not find any association between tobacco smoking and AMD risk in contrary to some other studies (68). It may follow from different criteria for classification of controls and patients into particular groups, because we stratified controls and AMD patients into two groups: never smokers and smokers (former and current). We also correlated in multivariable model parameters such as age, gender and family status of AMD and *NQO1*, *NOS3*

Table 6. Distribution of genotypes of the rs1800566 of the *NQO1* gene, the rs1799983 polymorphisms of the *NOS3* gene and the rs6726395 polymorphisms of the *NFE2L2* gene and odds ratio (OR) with 95% confidence interval (95% CI) in patients with dry or wet age-related macular degeneration (AMD) and in individuals without visual disturbances (controls)

Genotype (polymorphisms in bold)	Controls (n=105)	AMD (n=281)	Crude OR (95% CI)	Dry AMD (n=101)	Crude OR (95% CI)	Wet AMD (n=180)	Crude OR (95% CI)
	Number (frequency)	Number (frequency)		Number (frequency)		Number (frequency)	
rs1800566-rs1799983							
C/C-G/G	40 (0.38)	124 (0.44)	Ref.	44 (0.43)	Ref.	79 (0.44)	Ref.
C/C-G/T	46 (0.44)	79 (0.28)	**0.40 (0.22 – 0.75)** p^* **= 0.012**	26 (0.26)	**0.35 (0.16 – 0.73)** p^* **= 0.015**	54 (0.30)	0.48 (0.25 – 0.92)
C/C-T/T	1 (0.01)	12 (0.04)	5.12 (0.65-40.21)	6 (0.06)	7.53 (0.88-64.56)	6 (0.03)	4.04 (0.48-34.38)
C/T-G/G	3 (0.03)	24 (0.09)	3.59 (1.04 – 2.23)	8 (0.08)	3.37 (0.85 – 13.40)	16 (0.09)	3.89 (1.10 – 13.93)
C/T-G/T	10 (0.09)	25 (0.09)	1.00 (0.45 – 2.23)	10 (0.10)	1.17 (0.45 – 3.05)	15 (0.08)	0.96 (0.40 – 2.29)
C/T-T/T	1 (0.01)	4 (0.01)	1.62 (0.18 – 14.78)	1 (0.01)	1.14 (0.07 – 18.70)	3 (0.02)	1.96 (0.20 – 19.25)
T/T-G/G	3 (0.03)	10 (0.04)	1.36 (0.36 – 5.11)	4 (0.04)	1.56 (0.33 – 7.28)	6 (0.03)	1.30 (0.31 – 5.41)
T/T-G/T	1 (0.01)	3 (0.01)	1.21 (0.21 – 11.83)	2 (0.02)	2.33 (0.21 – 26.37)	1 (0.01)	0.64 (0.04 – 10.42)
T/T-T/T	-	-	-	-	-	-	-
rs1800566-rs6726395							
C/C-A/A	18 (0.17)	39 (0.13)	Ref.	13 (0.13)	Ref.	26 (0.14)	Ref.
C/C-A/G	46 (0.43)	133 (0.47)	1.05 (0.64 – 1.71)	40 (0.39)	0.74 (0.41 – 1.35)	93 (0.52)	1.26 (0.75 – 2.14)
C/C-G/G	23 (0.22)	43 (0.15)	0.59 (0.33 – 1.05)	23 (0.23)	0.99 (0.50 – 1.93)	20 (0.11)	0.45 (0.24 – 0.87)
C/T-A/A	6 (0.06)	8 (0.03)	0.45 (0.15 – 1.35)	2 (0.02)	0.31 (0.06 – 1.60)	6 (0.03)	0.63 (0.21 – 1.94)
C/T-A/G	6 (0.06)	34 (0.12)	2.17 (0.88 – 5.35)	10 (0.10)	1.73 (0.60 – 4.99)	24 (0.13)	2.44 (0.96 – 6.21)
C/T-G/G	2 (0.02)	11 (0.04)	1.99 (0.43 – 9.16)	7 (0.07)	3.67 (0.74 – 18.20)	4 (0.02)	0.55 (0.08 – 3.96)
T/T-A/A	1(0.01)	1 (0.01)	0.35 (0.02 – 5.67)	-	-	1 (0.01)	1.68 (0.17 – 16.36)
T/T-A/G	3 (0.03)	10 (0.04)	1.19 (0.32 – 4.42)	4 (0.04)	1.33 (0.29 – 6.14)	6 (0.03)	0.55 (0.11- 2.76)
T/T-G/G	-	2 (0.01)	-	2 (0.02)	-	-	-
rs1799983-rs6726395							
G/G-A/A	13 (0.12)	25 (0.09)	Ref.	6 (0.06)	Ref.	19 (0.11)	Ref.
G/G-A/G	25 (0.24)	98 (0.35)	1.62 (0.96 – 2.74)	29 (0.29)	1.38 (0.78 – 2.43)	69 (0.38)	1.33 (0.81 – 2.18)
G/G-G/G	8 (0.08)	34 (0.12)	1.58 (0.70 – 3.56)	21 (0.21)	0.82 (0.46 – 1.44)	13 (0.07)	0.79 (0.49 – 1.30)
G/T-A/A	12 (0.11)	22 (0.08)	0.62 (0.29 – 1.30)	9 (0.09)	1.10 (0.15 – 1.11)	13 (0.07)	1.10 (0.22 – 1.22)
G/T-A/G	29 (0.28)	68 (0.24)	0.77 (0.46 – 1.29)	20 (0.19)	1.48 (0.32 – 6.85)	48 (0.27)	1.20 (0.29 – 4.94)
G/T-G/G	16 (0.15)	18 (0.06)	**0.35 (0.17 – 0.73)** p^* **= 0.021**	9 (0.09)	1.09 (0.21 – 5.63)	9 (0.05)	0.59 (0.11 – 3.00)
T/T-A/A	-	1 (0.01)	-	-	-	1 (0.01)	-
T/T-A/G	1 (0.01)	11 (0.03)	4.02 (0.51 – 31.78)	5 (0.05)	1.10 (0.47 – 39.04)	6 (0.03)	1.11 (0.18 – 17.17)
T/T-G/G	1 (0.01)	4 (0.02)	1.42 (0.15 – 12.97)	2 (0.02)	-	2 (0.01)	0.76 (0.43 – 1.32)

Data in boldface are statistically significant ($p < 0.05$), *- denote p-values with the Bonferroni's correction, Ref. denotes reference group, i.e. group relative which ORs were calculated.

and *NFE2L2* polymorphisms with AMD risk. We have not observed any association between these factors and polymorphisms under study. It is possible that genetic factors may be associated with environmental and other risk factors that contribute to AMD. If those could be identified it may be possible to modify lifestyle or develop novel therapies to prevent AMD or decrease its severity.

6. CONCLUSION

The G/G genotype of the rs6726395 polymorphism of the *NFE2L2* gene may be associated with an impaired risk of wet AMD. Moreover, the T allele variant of the rs1799983 polymorphism of the *NOS3* gene may exert a protective effect against wet AMD. Our findings suggest that the rs6726395 and rs1799983 polymorphisms may be linked with the risk of AMD. However, expression profiles and biological activities of polymorphic variants *in vivo* will provide a better understanding of their role in AMD risk at the molecular level.

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