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Retinal function and *CFH-ARMS2* polymorphisms analysis: a pilot study in Italian AMD patients

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Abstract

Two major susceptibility genes, complement factor H (*CFH*) and age-related maculopathy susceptibility 2 (*ARMS2*), have been implicated in age-related macular degeneration (AMD) pathogenesis. We analyzed the association between *CFH* rs1061170 and/or *ARMS2* rs10490924 polymorphisms with central retinal function properties, as evaluated by focal electroretinogram (fERG). Forty early AMD patients, with preserved visual acuity and typical macular lesions, underwent fERG recording (in response to 41 Hz flicker stimuli presented to the central 18 degrees) and *CFH/ARMS2* genotyping. Mean fERG amplitude and sensitivity decreased in patients carrying *CFH* rs1061170 polymorphism ($p < 0.01$), compared with wild type ones, although visual acuity and funduscopic features were similar across the 2 groups. No significant fERG phase changes were observed. No association was detected between *ARMS2* (rs10490924) polymorphism and fERG parameters. Our findings indicate that *CFH* (rs1061170) polymorphism impacts significantly on retinal function in early AMD patients, and support the hypothesis that dysfunctional *CFH* might result in early retinal function loss due to a reduction in the immune antioxidant defense mechanism.

Keywords: Age-related macular degeneration; Complement factor H; Early diagnosis; Electroretinography; Flicker sensitivity; Gene polymorphisms

1. Introduction

Age-related macular degeneration (AMD) is a degenerative disorder of the macula involving the retinal pigment epithelium (RPE), Bruch's membrane, and photoreceptors (Bird et al., 1995). Early AMD is characterized by the presence of large, soft drusen, hyperpigmentation and/or hypopigmentation of the RPE. Late AMD is the potentially blinding stage of disease. It includes both "dry" (with geographic atrophy of the RPE) and "wet" (with subretinal neovascularization) forms (Bird et al., 1995). Many studies support the evidence that AMD is a genetic multifactorial disorder, although the specific genetic mechanisms have not been fully clarified (Klein, 2007).

Several genes have now been identified as strongly and significantly associated with AMD (Patel et al., 2008). Among these, the complement factor H gene (*CFH*), located in chromosome 1q32 (GenBank NG_007259), was the first major AMD-associated gene (Edwards et al., 2005). *CFH* encodes the major negative regulator of the complement alternative pathway and this protein is implicated in all stages of AMD from early hallmarks such as drusen to vision-disabling late AMD (Hageman et al., 2005; Haines et al., 2005). The first strongly AMD-associated single nucleotide genetic variant (SNP) of this gene is the coding variant p.Y402H (rs1061170, c.1277T>C) in exon 9 (Klein et al.,

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2005; Neale et al., 2010; Yu et al., 2011). The average odds ratios (ORs) for the *CFH* p.Y402H variant are 2- to 7-fold, depending on the number of risk alleles. Furthermore, homozygous individuals for the risk allele C at the *CFH* p.Y402H polymorphism have a 48% risk of developing late AMD by age 95 years, whereas this risk does not exceed 22% for noncarriers (Scholl et al., 2007).

Besides the complement pathway, the major genetic contributor to AMD susceptibility lies in the *ARMS2/HTRA1* region (Jakobsdottir et al., 2005; Jones et al., 2011; Rivera et al., 2005). Recent studies support the involvement of *ARMS2* gene (LOC387715) as the possible susceptibility candidate (Kanda et al., 2007; Rivera et al., 2005). The *ARMS2* gene encodes a mitochondrial protein. Although age-related maculopathy susceptibility 2 (ARMS2) function is still unknown, this protein seems involved in mitochondrial homeostasis by modulating oxidative stress and resulting apoptosis (Allikmets and Dean, 2008; Haines et al., 2007). A strong association between c.205G>T (p.A69S) *ARMS2* polymorphism and AMD was reported (Kanda et al., 2007; Neale et al., 2010; Yu et al., 2011).

Macular cone function in early AMD can be evaluated by using the focal electroretinogram (fERG), a signal that is generated from the macular region in response to flicker stimulation (Seiple et al., 1986a, 1993). The fERG reflects the activity of cone photoreceptors and bipolar cells, and provides a well-established index of the function of the outer retina (Bush and Sieving, 1996; Seiple and Holopigian, 1996; Seiple et al., 1992, 1993). Using a relatively large stimulus size (e.g., 18° diameter, 9° of eccentricity from the fovea) we were able to detect in individuals with early AMD significant retinal function deficits involving both central and peripheral regions, indicating that abnormalities may extend well outside the macula, where the early funduscopic lesion typically appears (Piccardi et al., 2009). fERG-derived flicker modulation thresholds can be estimated by different techniques (Falsini et al., 2000; Nelson et al., 1984; Seiple and Holopigian, 1996; Seiple et al., 1986a, 1986b, 1993; Wu et al., 1995). Direct assessment of outer retinal sensitivity by using the fERG might help to evaluate early cone dysfunction in early AMD, as shown by several clinical studies (Falsini et al., 2000, 2010; Klein et al., 1991; Nelson et al., 1984; Seiple et al., 1986b; Wu et al., 1995). In addition, testing fERG modulation sensitivity is of potential interest to evaluate the progression of dysfunction and the effects of treatments aimed at preserving and/or rescuing cone photoreceptors.

In the present study, fERGs were recorded in early AMD patients. fERG response characteristics were quantified and statistically compared between groups of patients, based on the results of genetic analysis for the 2 major susceptibility loci, *CFH* (c.1277T>C; p.Y402H; rs1061170) and *ARMS2* (c.205G>T; p.A69S; rs10490924). The aim of the study was to evaluate whether, and to what extent, the fERG response features were related to these 2 relevant genotypes in early AMD patients.

2. Methods

2.1. Subjects

Forty patients (17 males and 23 females, mean age: 70 ± 8.5 range: 55–85 years) of Caucasian origin with a diagnosis of bilateral early AMD were included in the study. They were from the central and southern part of Italy (from regions Lazio, Abruzzo, Marche, and Campania); the study was approved by the Institutional Review Board. Written, informed consent was obtained from each patient after the procedures and methods of the study were fully explained. The research followed the tenets of the Declaration of Helsinki. Demographic, clinical, and genetic data of patients are reported in Table 1. All patients were nonsmokers. Each patient underwent standard general and ophthalmic examination. Clinical diagnosis of AMD was established as previously reported (Falsini et al., 2010).

All patients met the following inclusion criteria: best corrected visual acuity of 0.4 or better in the study eye, central fixation (assessed by direct ophthalmoscopy), normal color vision with Farnsworth D-15 testing, no signs of other retinal or optic nerve disease, and clear optical media. None of the patients had significant systemic disease (such as diabetes, systemic hypertension) or were taking medication (e.g., chloroquine) that are known to affect macular function. The eye with the best visual acuity underwent the full fERG protocol. If both eyes had equal acuity, 1 eye was randomly selected. In all patients, clinical evaluations (including fundus grading; see below) and fERG testing were performed within 2 to 3 weeks of each other.

2.2. Fundus grading

AMD lesions of the studied eyes were graded on stereoscopic fundus photographs of the central 30° of the posterior pole (centered on the fovea). A macular grading scale based on the international classification and grading system was used (Bird et al., 1995). The presence of drusen and focal RPE hypo-and/or hyperpigmentation were noted within each of the 9 subfields delimited by the scoring grid (Klein et al., 1991). Drusen were graded with respect to their size, type, area, and confluence. Focal RPE hyper/hypo-pigmentation was graded from none to 50% or more of the examined area. In order to rule out more advanced AMD, all patients included in the study also underwent fluorescein angiography according to standard techniques (Schatz, 1989). According to the results of grading, all studied eyes were diagnosed as having AMD and, following the classification also employed by Jackson et al. (1998), as having stage 2, i.e., 1 or more large drusen (>63 μm) and/or focal hyperpigmentation.

Table 1
Demographic, clinical, genetic, and electrophysiological findings of 40 AMD patients

Patient number, age, sex	Study eye	Acuity	*CFH* c.1277T>C	*ARMS2* c.205G>T	fERG threshold	fERG slope
1, 55, F	Left	1.0	T/T	G/G	1.70	1.40
2, 71, M	Left	0.7	C/C	G/G	2.98	1.64
3, 73, M	Right	0.8	T/C	G/G	1.80	1.96
4, 61, F	Left	0.9	T/C	G/T	1.09	1.06
5, 83, M	Right	0.4	C/C	G/G	2.00	1.92
6, 73, M	Left	1.0	T/C	T/T	1.14	1.23
7, 73, M	Right	1.0	T/C	G/T	1.79	1.55
8, 73, M	Left	1.0	T/C	G/T	2.37	1.66
9, 72, F	Right	1.0	C/C	G/G	2.99	1.68
10, 74, F	Left	1.0	C/C	G/G	1.25	1.68
11, 75, M	Right	1.0	T/T	G/T	2.31	1.59
12, 65, F	Right	1.0	T/C	T/T	2.00	1.88
13, 78, F	Left	0.9	T/T	G/G	1.40	1.39
14, 71, F	Right	0.8	C/C	G/G	1.43	1.37
15, 64, F	Left	1.0	T/T	G/G	1.12	1.01
16, 83, F	Right	0.4	T/T	G/G	0.99	1.16
17, 68, M	Right	0.9	T/C	T/T	3.66	1.80
18, 61, M	Right	0.9	C/C	T/T	2.0	1.88
19, 71, M	Left	0.9	T/C	T/T	4.54	1.81
20, 69, M	Left	1.0	T/T	G/G	2.11	1.50
21, 56, F	Left	1.0	T/T	G/T	1.45	1.33
22, 82, F	Left	0.4	T/C	G/T	2.84	1.72
23, 68, F	Left	0.7	T/C	G/T	2.00	1.96
24, 70, M	Left	0.7	T/C	G/G	1.51	1.50
25, 81, F	Left	0.4	T/C	G/G	2.29	1.63
26, 65, F	Left	1.0	T/T	G/G	0.57	0.37
27, 58, M	Right	1.0	T/C	G/G	2.00	1.97
28, 85, M	Right	1.0	T/T	G/G	1.81	1.48
29, 55, F	Left	1.0	C/C	G/T	1.62	1.3
30, 63, M	Right	1.0	T/C	G/G	2.54	1.70
31, 55, M	Left	1.0	T/C	T/T	1.86	1.44
32, 71, F	Right	0.7	T/C	G/T	2.00	1.92
33, 79, M	Right	0.4	T/C	G/G	2.00	1.97
34, 68, F	Right	0.8	C/C	G/G	0.97	0.98
35, 62, F	Left	1.0	C/C	G/G	0.82	0.80
36, 63, F	Left	1.0	T/C	T/T	1.12	0.96
37, 80, F	Left	0.8	T/T	G/T	2	1.92
38, 77, F	Right	0.9	T/T	G/T	2	1.92
39, 81, F	Left	0.9	T/C	G/T	0.93	1.46
40, 64, F	Right	0.8	T/C	G/T	2	1.92

Key: AMD, age-related macular degeneration; fERG, focal electroretinogram.

2.3. Electrophysiological methods

fERGs were recorded from the macular region according to a published technique (Falsini et al., 2000, 2003). Retinal responses were elicited by the LED-generated sinusoidal luminance modulation of a circular uniform field (diameter, 18°; mean luminance, 80 cd/m^2; dominant wavelength, 630 nm), presented at the frequency of 41 Hz on the rear of a Ganzfeld bowl, illuminated at the same mean luminance as the stimulus. This technique was developed according to the indications of published clinical studies, in which the fERG response to sinusoidal flicker stimulation was used to test retinal flicker sensitivity in comparison with psychophysical flicker sensitivity in normal and pathologic conditions (Seiple et al., 1992, 1993).

fERGs were recorded monocularly by means of Ag-AgCl superficial cup electrodes taped over the skin of the lower eyelid. A similar electrode, placed over the eyelid of the contralateral, patched eye, was used as the reference (interocular electroretinogram; ERG) (Fiorentini et al., 1981). fERG signals were amplified, band-pass filtered between 1 and 250 Hz (−6 dB per octave), sampled with 12-bit resolution (2 kHz sampling rate), and averaged. A discrete Fourier analysis was performed off-line to isolate the fERG fundamental harmonic and estimate its amplitude (in μV) and phase (in degrees). In all subjects, the fERG testing protocol was started after a 20-minute period of preadaptation to the stimulus mean illuminance. Pupils were pharmacologically (tropicamide, 1%) dilated to 8–9 mm. In AMD patients having a response signal-to-noise (S/N) ratio $\geq$ 8, fERG signals were also acquired in sequence for 6 values of modulation depth between 16.5% and 93.5%, presented in an increasing order. For each stimulus modulation depth, fERG responses were accepted only if their S/N ratio was $\geq$2. As described elsewhere (Falsini et al.,

2000), fERG log amplitudes were plotted for each patient as a function of log modulation depth. The resulting function slope was determined by linear regression. From the same regression line, fERG threshold was estimated from the value of log modulation depth yielding a criterion amplitude, corresponding to an S/N ratio of 3 (Falsini et al., 2000).

2.4. Genetic analysis

All patients signed the informed consent approving general/anonymous research use of respective specimens. Genomic DNA was extracted from blood using High Pure PCR Template Preparation Kits (Roche Diagnostic, Basel, Switzerland) and quantified by NanoPhotometer (Implen, Australia).

Polymerase chain reaction (PCR) amplification and high resolution melting (HRM) acquisition for *CFH rs*1061170 and *ARMS2 rs*10490924 detection was performed in a 96-well plate for the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Switzerland, http://www.roche.com/index.htm) as previously described (Mello et al., 2012).

2.5. Statistical analysis

From each patient included in the study, 1 eye, typically the eye with the best visual acuity, was selected and designated as the study eye. The data from these studied eyes were included in the statistical analysis. Main outcome variables were fERG amplitude, phase, fERG function slope, and modulation threshold. fERG amplitude data underwent logarithmic transformation to better approximate normal distribution. fERG slope and threshold are reported as log10 values.

Sample size estimates were based on those in previous investigations (Falsini et al., 2000, 2003) in which the between- and within-subjects variability (expressed as the standard deviation) of fERG parameters was determined in patients with early AMD. Assuming between- and within-subject SDs in fERG amplitude and phase of 0.1 log μV and 20°, respectively, the sample sizes of patients belonging to the different genotype subgroups provided a power of 80%, at an $\alpha = 0.05$, for detecting a between group difference of 0.1 log μV and 30° in amplitude and phase, respectively. Given the absolute mean amplitude and phase values of the patients' fERGs, these differences were considered to be clinically meaningful, because they corresponded approximately to a 25%–30% change in either amplitude or phase.

Electrophysiological results were analyzed by multivariate statistics (multivariate analysis of variance for repeated measures, MANOVA). Dependent variables in the MANOVA design were fERG log amplitude and phase. Stimulus modulation depth and groups (wild type vs. gene polymorphism positive groups) were the independent variables (within- and between-subjects factors, respectively). fERG log thresholds and slopes, estimated from the corresponding functions, were individually determined by considering only responses with an S/N $\geq$ 3.

In all the analyses, results with a $p < 0.05$ were statistically significant.

3. Results

Results regarding *CFH* and *ARMS2* genotyping are reported in Table 1. We found 10 wild type homozygous T/T, 20 heterozygous C/T, and 10 homozygous C/C for *CFH* c.1277T>C polymorphism. Regarding *ARMS2* c.205G>T variant, we genotyped 20 wild type homozygous G/G, 13 heterozygous G/T, and 7 homozygous T/T.

Based on the different genotypes, visual acuity and funduscopic features did not differ significantly among patients. The results of fERG modulation functions recorded in patients with different *CFH* genotypes (T/T, C/T, and C/C) are reported in Fig. 1A. Mean fERG amplitudes (± standard error of the mean [SEM]) are plotted as a function

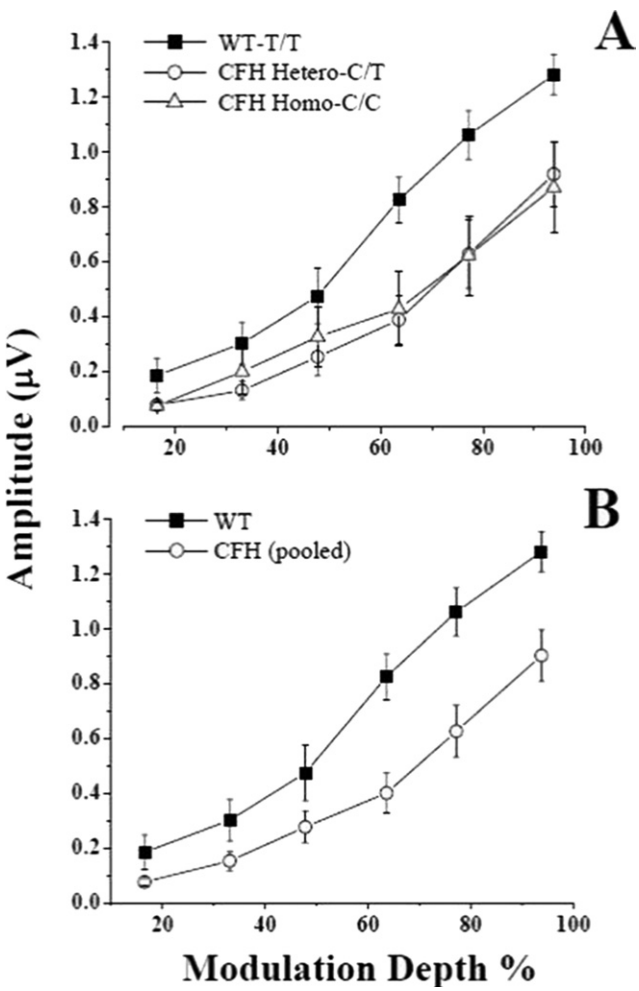

Fig. 1. (A) Mean focal electroretinogram (fERG) amplitudes (± standard error of the mean [SEM]) plotted as a function of stimulus modulation depth for age-related macular degeneration (AMD) patients with different *CFH* c.1277T>C genotypes (T/T, T/C, C/C). (B) Pooled mean fERG amplitude data for patients T/C and C/C for *CFH* c.1277T>C polymorphism compared with wild type T/T patients.

of stimulus modulation depth. A significant difference across groups was observed ($p < 0.01$), due to the difference between the T/T genotype mean fERG function and the fERG functions of the other 2 genotypes (C/T and C/C). Mean fERG amplitudes of T/T patients were larger than those recorded from patients having the other 2 genotypes. These latter groups did not differ significantly for their fERG functions, and therefore their data were pooled together (Fig. 1B). Figure 1B shows pooled mean fERG amplitude data for patients with C/C and C/T genotype compared with wild type T/T patients. fERG amplitudes were, on average, lower in patients carrying the risk allele C, compared with wild type patients (MANOVA, $F(2,36) = 7.7$; $p < 0.01$). In addition, MANOVA showed that the differences between fERG amplitudes recorded from wild type patients and those carrying the risk allele C were dependent upon the stimulus modulation depth (MANOVA, $F(5,34) = 3.1$; $p < 0.05$). The observed fERG amplitude loss was associated with an amplitude shift versus modulation depth function, indicating an increase in mean fERG threshold.

Figure 2 shows box plots of the mean fERG thresholds distribution (±interquartile and 99 percentile ranges) recorded in the different *CFH* c.1277T>C genotypes. The mean fERG threshold increase indicates a loss of sensitivity in the fERG response. This variation was statistically significant (univariate $F(2,36) = 4.9$; $p < 0.01$).

Figure 3 shows mean fERG amplitudes (± SEM) plotted as a function of stimulus modulation depth for the 3 *ARMS2* c.205G>T different genotypes (G/G, G/T, and T/T). No significant differences were revealed by MANOVA analysis.

The fERG phase did not show any significant association with either *CFH* or *ARMS2* gene polymorphism subtypes.

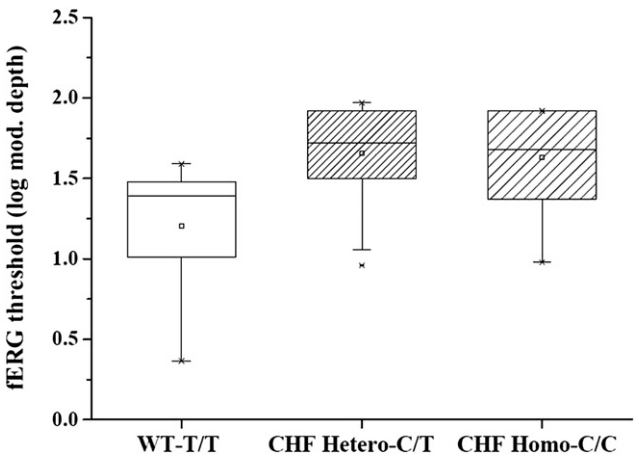

Fig. 2. Box plots of the distribution of mean focal electroretinogram (fERG) thresholds (± interquartile and 99 percentile ranges) recorded in *CFH* c.1277T>C wild type (T/T), hetero- (T/C), and homozygous (C/C) patients.

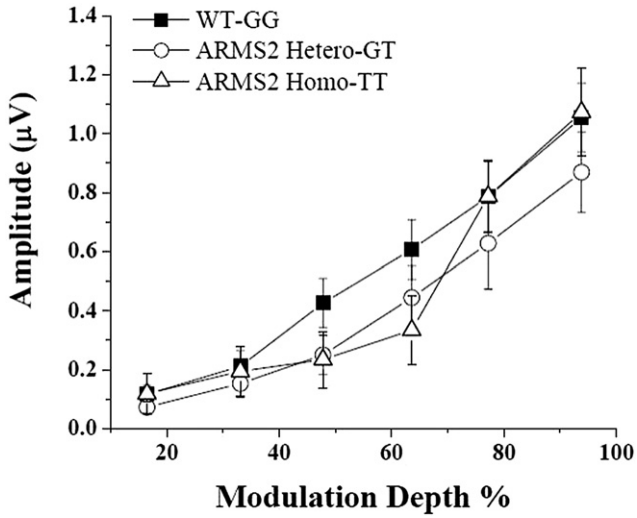

Fig. 3. Mean focal electroretinogram (fERG) amplitudes (± standard error of the mean [SEM]) plotted as a function of stimulus modulation depth for age-related macular degeneration (AMD) patients with different *ARMS2* c.205G>T genotypes (G/G, G/T, T/T).

The combined effect of both polymorphisms on retinal function was evaluated by comparing results of patients homozygous for either *CFH* or *ARMS2* wild type genotypes (T/T and G/G, respectively) with those of patients carrying at least 1 risk allele (in hetero- or homozygous status), and with those of patients carrying either polymorphisms in homozygosis (C/C and T/T, respectively). No significant differences were found, indicating an absence of synergistic effects of both polymorphisms on the macular cone function in our study population.

4. Discussion

The aim of this pilot study was to evaluate the association between *CFH* rs1061170 and/or *ARMS2* rs10490924 polymorphisms with retinal function properties in a small, selected sample of Italian patients with early AMD. We selected, based on clinical characteristics and ethnicity, a homogenous group of patients. This approach allowed us to perform a comparison across groups for their electrophysiological macular function, as the main outcome variable. The results showed that our early AMD patients carrying *CFH* c.1277T>C polymorphism differed substantially for their retinal function results compared with those with the wild type allele. This functional difference reflected a loss of retinal sensitivity in the macular region, undetectable by visual acuity, a method testing more selectively the function of the fovea.

Complement factor H (CFH) is the major fluid phase regulator of the alternative complement pathway and any change in its regulation leads to an uncontrolled activation and/or regulation of the alternative complement pathway (Donoso et al., 2010). A strong association between a broad

range of AMD phenotypes and *CFH* polymorphisms has been reported (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). The pathological relevance of these genetic studies is supported by the demonstration that drusen, the pathogenic hallmark of AMD, contain complement proteins, including CFH (Johnson et al., 2001). Experimental evidence in transgenic mouse models indicate that the deregulation of the complement alternative pathway due to CFH deficiency causes retinal abnormalities and visual dysfunction (Coffey et al., 2007). In aged CFH deficient mice, an increase in autofluorescent subretinal deposits, as well as abnormalities in Bruch's membrane, retinal pigment epithelial cells and rod outer segments were documented, indicating that CFH is critically required for the long-term functional health of the retina (Coffey et al., 2007). Recently, Weismann et al. (2011) demonstrated that CFH plays a major role in the innate immune defense mechanism against oxidative damage. It was indeed demonstrated (Weismann et al., 2011) that CFH binds to malondialdehyde (MDA), a reactive decomposition product generated from oxidative damage to lipids in the cell membrane. The p.Y402H CFH variant from AMD patients showed a marked reduction in malondialdehyde binding compared with normal CFH. The Weismann et al. (2011) findings clarify the exact role of CFH in the anti-inflammatory defense against oxidative damage, and support the hypothesis that dysfunctional CFH might result in an abnormality of outer retina function because of a reduction in this defense mechanism. The current findings, linking retinal function and *CFH* genotype in absence of any other apparent difference in clinical parameters as visual acuity and fundus lesion characteristics, provide a clinical support to such hypothesis.

It is unknown how the p.Y402H *CFH* variant affects central retinal function (as evaluated by fERG) in nonaffected individuals. It could be possible that retinal sensitivity, as a result of chronic inflammatory/oxidative insult, is diminished in these *CFH* variant carriers, as compared with noncarriers. Such an analysis, however, was beyond the scope of this pilot study, whose aim was limited to assess, in early AMD patients, the potential relationship between *CFH* genotypes and retinal function. Further ongoing studies in our laboratory will address the question of whether an abnormality of the alternative complement pathway involving *CFH* may lead to an early retinal dysfunction even preceding any manifest funduscopic sign of AMD.

The major noncomplement genetic contributor to AMD susceptibility lies in the *ARMS2/HTRA1* region (Jakobsdottir et al., 2005; Jones et al., 2011; Kanda et al., 2007; Rivera et al., 2005). *ARMS2* messenger RNA (mRNA) transcripts can be detected in the retina (Kanda et al., 2010). Although the function of ARMS2 is still unknown, and the predicted protein shows little homology with other proteins, the protein was shown to localize to the outer membrane of the mitochondria (Fritsche et al., 2008; Kanda et al., 2007). It was hypothesized that the p.A69S (rs10490924) variant might affect the conformation/interaction of the protein that might further affect the functions of mitochondria (Kanda et al., 2007). Recent studies in transgenic mice showed that overexpression of *HTRA1* induced typical features of neovascular AMD, including branching networks of choroidal vessels, polypoidal lesions, severe degeneration of the elastic laminae, and tunica media of choroidal vessels. *HTRA1* mice also displayed retinal pigment epithelium atrophy and photoreceptor degeneration (Jones et al., 2011). Unlike the results obtained for the *CFH* polymorphism, we did not find any significant relationship between retinal function and the rs10490924 polymorphism in the *ARMS2* gene. In fact, there were no significant differences in the fERG response parameters between patients carrying different c.205G>T *ARMS2* genotypes. However, a weak, but statistically significant association, resulting from a study in a cohort of patients larger than that of the current pilot study, cannot be excluded.

In conclusion, our study establishes a direct link between *CFH* genotype and retinal function in AMD patients. It is likely that standard clinical examination and the morphologic appearance of AMD lesions may not provide the full picture of the retinal pathologic status in early disease. Retinal function studies might offer a useful complement to clinical data, while molecular genetic screening offers a personalized approach to the assessment, management, and treatment of AMD. The recent results by Feigl et al. (2011) are also in line with this suggestion. It should be also pointed out that, if a retinal dysfunction is due to the interaction between chronic oxidative damage and abnormal complement cascade regulation function (Weismann et al., 2011), then an early antioxidant/anti-inflammatory therapeutic strategy should be particularly beneficial in AMD patients with the *CFH* risk genotype, resulting even in a functional improvement (Falsini et al., 2010). Clinical trials based on such personalized therapeutic strategy should be able to address this point.

Disclosure statement

None of the authors have any actual or potential conflicts of interest related to this study.

The study was approved by the Institutional Review Board. Written, informed consent was obtained from each patient. The research followed the tenets of the Declaration of Helsinki.

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