Typing of ARMS2 and CFH in Age-Related Macular Degeneration

Case-Control Study and Assessment of Frequency in the Italian Population

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Objectives: To determine the effects of the polymorphisms CFH Tyr402His and ARMS2 del443ins54 on susceptibility to age-related macular degeneration (AMD) and to find the frequencies of these single-nucleotide polymorphisms in an Italian population that was not examined clinically.

Methods: A total of 286 control subjects (126 men and 160 women) and 159 white patients (73 men and 86 women) harboring exudative AMD in 1 eye were recruited. A third group of 182 DNA samples from blood donors of the same geographical areas were also typed to assess the frequency of CFH Tyr402His and ARMS2 del443ins54 polymorphisms in the general population. The data were analyzed statistically by a standard 2 × 2 table, Fisher exact tests, and odds ratios.

Results: The deletion-insertion at chromosome 10q26 (del443ins54) showed the strongest association with AMD (P = 2.7 × 10−13; odds ratio = 3.25), and a highly significant association was also confirmed for Tyr402His at the CFH locus (P = 9.9 × 10−13; odds ratio = 2.86). We found no differences in allele and genotype association between classic and occult choroidal neovascularization. We also observed that 39% of the samples in the general Italian population were at least 5.4 times more likely than control subjects to develop AMD.

Conclusions: To our knowledge, this is the first confirmation of the association of del443ins54 in Italian patients with AMD, and we also confirmed the association of Tyr402His with CFH. Genetic analysis of the general population suggested that analysis of the ARMS2 and CFH risk alleles alone may be helpful in differentiating high-risk individuals (odds ratio > 5.00) from low-risk individuals (odds ratio < 0.45).

Clinical Relevance: Individuals at high risk for developing AMD could be identified and selected for specific prevention programs. In this context, the development of prevention programs based on dietary antioxidants or on close monitoring of at-risk individuals should be considered or suggested.

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AGE-RELATED MACULAR DEGENERATION (AMD) is the most common irreversible cause of severe vision loss throughout the world in people aged 50 years or older. 1 Thirty percent of people older than 75 years show early signs of the disease. 2,3 Laser surgery, photodynamic therapy, and intraocular injections offer some chance of visual improvement, but they require invasive delivery methods and cannot always prevent the progress of the disease. Although several risk factors for AMD such as smoking, being white, and having a family history of AMD have been reported, the risk of developing advanced AMD is assessed on the basis of ocular findings in those who already have the early stages. 2,4,6 Accordingly, early identification of individuals at greatest risk for vision loss due to AMD prior to the development of any signs of the disease can be clinically important. In this respect, the identification and validation of genetic predisposing variants to be used as biomarkers would help to identify people at increased risk for more advanced stages of AMD. Hence, heredity is a primary contributor to AMD susceptibility as suggested by family and twin studies. 7,8 First-degree relatives of patients with AMD are at increased risk (odds ratio [OR] = 2.4) for the condition compared with first-degree relatives in families without the disorder. They are also affected at a younger age and have an increased lifetime risk of late AMD (risk ratio = 4.2). 5,11
In 2005, several groups12-16 independently reported that AMD is strongly associated with the polymorphism Tyr402His in the complement factor H gene (CFH) (GenBank NG_007259), located in chromosome 1q31. CFH is implicated in all stages of AMD from early hallmarks such as drusen to vision-disabling late AMD.11,17

The average ORs for the CFH Tyr402His variant are 2- to 7-fold depending on the number of risk alleles. Also, it was calculated that individuals homozygous for the risk allele C at the CFH Tyr402His polymorphism have a 48% risk of developing late AMD by age 95 years, whereas this risk does not exceed 22% for noncarriers.9 A second polymorphism (Ala69Ser) in the age-related maculopathy susceptibility 2 gene (ARMS2, also known as LOC387715 (GenBank NG_011725) located on chromosome 10q26 was reported as another major locus contributing to the pathogenesis of AMD.18-20 Rivera et al19 found the strong association with LOC387715, reporting a 7.6-fold increased risk for individuals homozygous for the risk allele T of rs10490924. Association at chromosome 10q26 is located between 2 nearby genes, ARMS2 and HTRA1 (high-temperature requirement factor A1; GenBank NG_011554), suggesting 2 equally probable candidates. Recently it was reported that a deletion-insertion polymorphism in ARMS2 (del443ins54) was strongly associated with AMD, directly affecting the transcript by removing the polyadenylation signal and inserting a 54-base pair (bp) element known to mediate rapid messenger RNA turnover.21 As a consequence, ARMS2 shows no detectable expression in homozygous carriers of the deletion-insertion variant. In this article, we present the first confirmation to our knowledge of a deletion-insertion at chromosome 10q26 (del443ins54) in the Italian population, we confirm the association of CFH single-nucleotide polymorphism rs1061170 (Tyr402His), we assess the joint effects of both variations on AMD, and we assess the frequencies of the single and complex genotypes in the general Italian population.

### METHODS

#### STUDY SUBJETS

One hundred fifty-nine consecutive white patients (73 men and 86 women) harboring exudative AMD in 1 eye were prospectively recruited at the Medical Retina Centre (Table 1). All study subjects underwent a detailed eye examination including best-corrected visual acuity and slitlamp biomicroscopy of the fundus. Color fundus photography and fluorescein angiography were performed. Exudative AMD was diagnosed by the investigators (F.R., F.D., and F.M.) according to the international classification guidelines.32

Inclusion criteria were being older than 55 years and having unilateral or bilateral exudative AMD. Exclusion criteria were presence of other retinal disease (eg, diabetic retinopathy, high myopia, or retinal dystrophies), having the atrophic form of AMD, association of atrophic and exudative forms of AMD, and having fibrovascular scarring that did not allow the choroidal neovascularization (CNV) angiographic type to be precisely graded.

To classify the initial lesions before any treatment, exudative AMD was graded on the earliest fluorescein angiography examination results available. The grading was performed based on the presence and extent of fluorescein leakage and the leakage profile. Leakage was defined as bright blue areas that were present for at least 5 minutes in the presence of other major eye disease except mild senile cataracts and low refractive defects. In this group, fundus examination excluded the presence of drusen, focal atrophy, and abnormal retinal pigment epithelium change.

A third group of samples was also obtained from 182 blood donors from the same geographical areas as the patients and control subjects. All of the patients and control subjects were sex matched. The study was conducted according to the Declaration of Helsinki, and informed consent was obtained from the participating subjects after the nature of the study had been explained. The study protocol was approved by the ethics committee of the University of Rome “Tor Vergata,” Rome, Italy.

### GENETIC ANALYSIS

Genotyping of rs1061170 was performed by TaqMan assays (Applied Biosystems, Foster City, California). Reactions were run in an AB7300 (Applied Biosystems) and interpreted using Sequence Detection System 2.1 software (Applied Biosystems). Each plate contained 3 positive control samples (samples previously confirmed by direct sequencing as heterozygous and both homozygous) and 2 negative control samples. No departure from Hardy-Weinberg equilibrium was detected. Results from genotype assessment of rs1061170 and del443ins54 were confirmed by postgenotyping direct resequencing of 10 random samples. Typing of del443ins54 was performed by polymerase chain reaction and agarose gel electrophoresis. Polymerase chain reaction was performed in a 25-μL volume containing 5mM magnesium chloride, 2 μL of 10X buffer (Applied Biosystems), 1 U of Taq Gold polymerase (Applied Biosystems), 1.25mM deoxynucleoside triphosphate (Invitrogen Corp, Carlsbad, California), 80 ng of template DNA, and 10 pmol of each primer (forward: 5’-TCTAAGATCTGATGATCTCC-3’; reverse: 5’-TGAATTCAGCCTTACCC-3’). Polymerase chain reaction amplification included a 10-minute hot start at 94°C, 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute, and a final exten-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients With AMD (n = 159)</th>
<th>Healthy Control Subjects (n = 286)</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>50-91</td>
<td>50-86</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>75.0 (7.8)</td>
<td>69.2 (11.1)</td>
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<tr>
<td>Sex, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>73 (46)</td>
<td>126 (44)</td>
</tr>
<tr>
<td>Female</td>
<td>86 (54)</td>
<td>160 (56)</td>
</tr>
<tr>
<td>Family history of AMD, No. (%)</td>
<td>82 (52)</td>
<td>NA</td>
</tr>
<tr>
<td>CNV, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classic</td>
<td>59 (37)</td>
<td>NA</td>
</tr>
<tr>
<td>Occult</td>
<td>100 (63)</td>
<td>NA</td>
</tr>
<tr>
<td>Bilateral neovascular AMD, No. (%)</td>
<td>15 (9)</td>
<td>NA</td>
</tr>
<tr>
<td>Age at diagnosis, mean (SD)</td>
<td>72.8 (7.8)</td>
<td>NA</td>
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Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; NA, not applicable.
Allele and genotype frequencies of rs1061170 and del443ins54 were characterized in a cohort of 159 patients with AMD and 286 healthy control subjects. In our Italian case-control cohort consisting of patients and healthy control subjects who underwent clinical examinations, the deletion-insertion at chromosome 10q26 (del443ins54) showed the strongest association with AMD in terms of both P value and OR (P = 2.7 × 10^{-15}, OR = 3.25) (Table 2). Genotype association revealed OR values of 3.18 and 20.61 if 1 or 2 risk alleles were present, respectively, with 124 patients (78%) having at least 1 risk allele with respect to 130 control subjects (45%). A highly significant association was also confirmed for the risk allele C of rs1061170 at the CFH locus, generating a P value of 9.9 × 10^{-13} and an OR of 2.86. Genotype association revealed OR values of 2.88 and 13.06 if 1 or 2 risk alleles were present, respectively, with 122 patients (77%) having at least 1 risk allele with respect to 144 control subjects (50%) (Table 2). We found no differences in allele and genotype association between classic CNV and occult CNV.

Table 2. Allele and Genotype Frequency of del443ins54 and rs1061170 in Italian Patients vs Control Subjects and vs the General Population

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Risk Allele</th>
<th>Control Subjects, %</th>
<th>P Value (95% CI)</th>
<th>Genotype</th>
<th>Control Subjects, %</th>
<th>P Value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARMS2</td>
<td>del443ins54</td>
<td>Del</td>
<td>51</td>
<td>24</td>
<td>2.7 × 10^{-15}</td>
<td>3.25 (2.36-4.41)</td>
<td>Del/del</td>
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</tr>
<tr>
<td>CFH</td>
<td>rs1061170</td>
<td>C</td>
<td>54</td>
<td>29</td>
<td>9.9 × 10^{-13}</td>
<td>2.86 (2.14-3.84)</td>
<td>C/C</td>
</tr>
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</table>

Table 3. Joint Odds Ratios for the ARMS2 and CFH Variations in the Patient Population

<table>
<thead>
<tr>
<th>ARMS2 Genotype</th>
<th>C/C</th>
<th>C/T</th>
<th>T/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del/del</td>
<td>59.50 (9.29-384.98)</td>
<td>65.17 (16.88-251.53)</td>
<td>0.44 (0.12-1.59)</td>
</tr>
<tr>
<td>Het</td>
<td>13.05 (4.42-38.59)</td>
<td>2.0 × 10^{-4}</td>
<td>0.20 (0.10-0.37)</td>
</tr>
<tr>
<td>Wt/wt</td>
<td>60.50 (9.38-384.98)</td>
<td>65.17 (16.88-251.53)</td>
<td>0.44 (0.12-1.59)</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CI, confidence interval; del, deletion; het, heterozygous; OR, odds ratio; wt, wild type.

RESULTS

Table 3 shows the joint OR for the ARMS2 and CFH variations. Joint analyses revealed 5 risk diplotype classes with ORs ranging from 5.46 (in samples homozygous for the T allele of rs1061170 and heterozygous for del443ins54) to 59.50 and 65.17 (in samples homozygous for both risk alleles and in samples homozygous for the C allele of rs1061170 and heterozygous for del443ins54, respectively). Diploidy analyses in control samples failed to observe samples homozygous for del443ins54 and heterozygous for rs1061170, making it unfeasible to assess the OR for this diplotype class. The only protective diplotype classes

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were observed for samples homozygous for the nondele-
ted allele of del443ins54 independently from the geno-
type of rs1061170. According to the genotype of rs1061170, we
observed ORs ranging from 0.15 (for individuals ho-
mozygous for the nonrisk allele T of rs1061170) to 0.44
(for individuals homozygous for the risk allele C of
rs1061170).

After this determination of contributing genetic fac-
tors in Italian patients with AMD, we decided to assess
the frequency of combined genotypes at the CFH and
ARMS2 loci in the general population, ie, samples that
were not clinically examined. We typed 182 DNA samples
from blood donors of the same geographical areas as the
patients and control subjects. We observed risk allele fre-
quencies of 36% and 20% for rs1061170 (risk allele C)
and del443ins54, respectively (Table 2). The risk allele
frequency of rs1061170 differed somewhat from that re-
ported in the HapMap database of white individuals (28%),
but the frequency of del443ins54 did not differ from that
reported in the HapMap database calculated using a
marker of complete linkage disequilibrium (rs3750848)
(22%). Table 4 shows the frequency of diplotypes at the
CFH and ARMS2 loci in this general population. Inter-
estingly, 39% of the blood donors are at least 5.4 times
more likely than control subjects to develop AMD.

In particular, on the basis of our screening, 18% and
6% of the population have risks 13 and 60 times greater
than control subjects, respectively, for developing the
disease. On the other hand, 61% of people do not have in-
creased risks for AMD related to the rs1061170 and
del443ins54 genotypes, with most of them showing re-
duced risk with respect to the general population.

COMMENT

To our knowledge, this is the first confirmation of the as-
sociation of del443ins54 with AMD in the Italian popula-
tion. We also confirmed the association with rs1061170
in CFH and assessed the frequency of these 2 variations in
a control Italian population. With respect to the allele fre-
quency of rs1061170 (C allele) previously reported in the
Italian population,23 we observed similar frequencies in
the cohort of patients (57% vs 54%, respectively), whereas lower
frequencies were observed in the healthy control samples
(39% vs 29%, respectively). As a consequence, we
observed higher ORs with respect to those previously re-
ported (13.06 vs 3.90, respectively).23 We believe that such
discrepancies may be due to the selection of healthy con-
trol subjects and/or to the size of the case-control cohort
analyzed. Similarly, we observed higher frequencies for
the risk allele of del443ins54 in Italian patients with respect to
German patients.21 These 2 variations appear to in-
dependently contribute to the susceptibility to AMD in the
Italian patients. Such highly penetrant genetic susceptibil-
ity factors in AMD might be used as diagnostic or prog-
nostic molecular markers and/or for drug response. While
genetic screening has become a routine practice for many
 mendelian diseases, population screening for risk assess-
ment for a complex disease is still unexploited. The rea-
son for this is that susceptibility to common diseases re-
sults from complex and unpredictable interactions of

Table 4. Frequency of Diplotypes at the CFH and ARMS2
Loci in the Italian General Population

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CFH Genotype</th>
</tr>
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<tbody>
<tr>
<td>Del/del</td>
<td></td>
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<tr>
<td>Het/Het</td>
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</tr>
<tr>
<td>Wt/wt</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: del, deletion; het, heterozygous; wt, wild type.

1. Congdon N, O’Colmain B, Klaver CC, et al; Eye Diseases Prevalence Research
Group. Causes and prevalence of visual impairment among adults in the United

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Author Contributions: Dr Giardina had full access to all of
the data in the study and takes responsibility for the in-
tegrity of the data and the accuracy of the data analysis.

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REFERENCES