Apolipoprotein E Gene Associations in Age-related Macular Degeneration
The Melbourne Collaborative Cohort Study


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Initially submitted June 20, 2011; accepted for publication August 26, 2011.

The apolipoprotein E gene (APOE) has been found to be associated with age-related macular degeneration (AMD). Reported associations have been questioned, as they are opposite those for Alzheimer’s disease and cardiovascular disease. The authors examined associations between APOE genotype and AMD using a case-control study (2,287 cases and 2,287 controls individually matched on age, sex, and country of origin) nested within Melbourne Collaborative Cohort Study participants aged 48–86 years at AMD detection. The odds ratio for early AMD among participants with e2-containing genotypes (e2/e2/e3/e3, e2/e2) was 1.32 (95% confidence interval (CI): 1.11, 1.58; P = 0.002) versus persons with genotype e3/e3. Associations with early AMD varied by smoking status; e2-containing genotypes were positively associated with early AMD for never and previous smokers (never smokers: odds ratio (OR) = 1.40, 95% CI: 1.12, 1.76 (P = 0.003); previous smokers: OR = 1.39, 95% CI: 1.00, 1.93 (P = 0.05)) but not for current smokers (OR = 0.66, 95% CI: 0.34, 1.30 (P = 0.2; interaction P = 0.05). The e4-containing genotype group (e3/e4/e4/e4) had an inverse association with early AMD among current smokers only (OR = 0.41, 95% CI: 0.22, 0.77 (P = 0.005)). These results highlight the importance of stratifying by smoking status in elderly populations. Smokers who survive to old age may be more likely to possess unknown genotypes which modify exposure-disease associations.

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Aging; apolipoprotein E; genetics; macular degeneration

Abbreviations: AMD, age-related macular degeneration; CI, confidence interval; OR, odds ratio.

The human apolipoprotein E gene (APOE; OMIM 107741), located on chromosome 19q13.2, was one of the first candidate genes found to be associated with age-related macular degeneration (AMD) (1, 2). It encodes a small, multifunctional lipid transport glycoprotein (299 amino acids) with important functions in systemic and cerebrospinal lipid transport (3). APOE is also involved in regulating immunocompetent cells, metal ion binding, and the remodeling and repair of neural tissue and possesses antioxidative properties (3). APOE is expressed in retinal tissue, particularly the retinal pigment epithelium and Bruch’s membrane (4).

Variation in 2 single nucleotide polymorphisms within the APOE gene, rs429358 and rs7412, results in allelic variants commonly referred to as e2, e3, and e4, which are differentiated on the basis of cysteine and arginine residue interchanges at positions 112 and 158 in the amino acid sequence. Six biallelic genotypes exist: e3/e3, e3/e4, e2/e3, e4/e4, e2/e4, and e2/e2, ranked from most common in European populations to least common (5).

Most previous studies that have explored the relation between APOE and AMD—including our own (6, 7)—have found a protective association for possession of one or more e4 alleles, with the risk of late AMD among persons of Caucasian descent being 20%–50% lower than that for persons who are homozygous for the e3 allele (1, 2, 6–11). Some investigators have also reported an increased risk of disease...
for persons with the ε2 allele compared with those who are homozygous for the ε3 allele (1, 6–10). A meta-analysis (12) and a pooled analysis (13) have both indicated a strong inverse association with AMD for the ε4 allele and a moderate positive association with the ε2 allele (13).

APOE is well established as a longevity gene (14), and known associations include Alzheimer’s dementia and cardiovascular disease (15, 16), where the ε4 allele is associated with increased risk. APOE allele frequencies therefore differ across age groups (17, 18). Some studies have found the ε2 allele to be associated with decreased mortality (15, 18), and others demonstrated an increased risk of age-related disease and death (16, 19) in comparison with homozygosity for ε3. Furthermore, APOE allele frequencies are known to vary with latitude; the proportion of ε4 carriers increases from 10%–15% in Southern Europe to 40%–50% in the North (20, 21). Studies that fail to account for the potentially strong confounding of age and geographic location might produce biased estimates of genetic associations. We examined associations between APOE and the prevalence of AMD using a large case-control study in which cases and controls were individually matched on age, country of origin, and sex.

MATERIALS AND METHODS

Study sample

Cases and controls were selected from the Melbourne Collaborative Cohort Study (Melbourne, Australia), a prospective cohort study of 41,514 people (22). Almost all participants (99.3%) were aged 40–69 years at baseline (1990–1994), with approximately equal proportions of participants across each of the 3 age decades. Participants in the Melbourne Collaborative Cohort Study are descendants of Northern Europeans, predominantly of Anglo-Celtic origin, as well as Southern Europeans who migrated to Australia from Italy and Greece in the 1950s. The latter were deliberately recruited (approximately 25% at baseline) to extend the range of lifestyle exposures and to increase genetic variation.

Photography for detection of AMD and other fundus pathology was conducted during a follow-up study carried out from 2003 to 2007, when participants were aged 48–86 years. For one of several reasons (death, loss to follow-up, confounding macular pathology precluding AMD grading, poor-quality or absent photographs), 20,214 participants (49%) were not included in the final AMD risk factor analysis, which left 21,287 participants with macular photographs centered on the macula.

Statistical analysis

The numbers of each APOE allele were compared with those expected for a population in Hardy-Weinberg equilibrium. Chi-squared tests were used to compare genotype frequencies across the 3 age groups (<60, 60–69, or ≥70 years). Genotype frequencies were calculated separately for people of Northern and Southern European origin. Unconditional polynotomous logistic regression, adjusting for age group, sex, grouped country of origin, and smoking status (never, previous, or current smoker), was performed to estimate odds ratios for each individual genotype compared with ε3 homozygosity (ε3ε3) as the referent group, separately for early AMD and late AMD in either eye.

DNA extraction and APOE genotyping

Genomic DNA was isolated from venous blood leukocytes using a standard phenol/chloroform extraction procedure (26). APOE genotyping was performed by means of multiplex high-resolution ampiclon melting (TrendBio Pty. Ltd., Melbourne, Australia) (27). The primers used were forward 5'-ACGCAGCAGTGTGGCAGGCGGTTG-C3' and reverse 5'-GGCGTCGTCGGATGCGGCTGA-3'. Two primer pairs were designed to encompass 2 sites at amino acid positions 112 (site A) and 158 (site B) of the APOE gene. With regard to the probes used, the APOE 112 probe sequence was 5'-ACATGGAGGAGACGTGTTGCGCGCGCTT-GC3' and the APOE 158 probe sequence was 5'-GGGGCTCTCGCCG-GATGCCGATGACCTGCGAGACGCGCTGGC-C3'. The single nucleotide polymorphism position is underlined and printed in boldface. A sequence variant of c.526C>T for the ε2 allele is present at site A (GenBank reference sequence NM_000041.2) and a sequence variant of c.388T>C for the ε4 allele is present at site B (reference sequence NM_000041.2), resulting in either a cysteine residue or an arginine residue, respectively.
late AMD as compared with no AMD. Constraints were constructed to assess whether the odds ratios for smoking and APOE genotype differed for early and late AMD; models were fitted with and without constraints and compared using the likelihood ratio test.

A 3-way interaction term for age, sex, and grouped country of origin (the variables used for matching) was initially included in the model. Because the odds ratios for the association between genotype and AMD for models with and without the 3-way interaction term were the same, the 3-way interaction term was dropped from the final model. Additionally, estimates were compared with those produced by conditional logistic regression, where each case was compared only with the individually matched control. The estimates were observed to be very similar; therefore, unconditional estimates were compared with those produced by conditional logistic regression, where each case was compared only with the individually matched control. The estimates were observed to be very similar; therefore, unconditional estimates were compared with those produced by conditional logistic regression, where each case was compared only with the individually matched control.

Differences in associations across age groups (<60, 60–69, or ≥70 years), smoking status (never, previous, or current smoker), and grouped country of origin were investigated by fitting interaction terms between each variable and genotype (grouped into the following categories: homozygous e3 (referent group), e2e2/e2e3/e2e4, and e4e4/e4e3/e4) and tested by means of the likelihood ratio test. Other lifestyle factors on which data were collected at baseline—including measured waist-to-hip ratio, alcohol intake, physical activity, red meat intake, saturated fat intake, serum cholesterol level, total energy intake, glycemic load, educational level, and medical history of heart attack, stroke, or diabetes—were tested as potential confounders; only those for which the odds ratios for genotypes changed by 5% or more when the factor was fitted were retained in the models.

Linearity of the relation between age and AMD on the log scale was assessed using the likelihood ratio test, comparing fitted models with age as a categorical variable (<60, 60–69, or ≥70 years) with those with age as a continuous variable.

All statistical analyses were performed using Stata, version 10 (StataCorp LP, College Station, Texas).

RESULTS

Baseline demographic characteristics

Persons participating in the follow-up study and therefore eligible for selection for the nested case-control sample were at baseline younger, less likely to be current smokers, less likely to be obese, and more likely to be of Northern European descent (23).

There were a total of 4,574 persons with complete data on AMD grading, APOE genotyping, and potential confounders, comprising 2,287 cases and 2,287 controls. Of the cases, 109 were classified as having late AMD.

Allele frequencies did not deviate from Hardy-Weinberg equilibrium (P = 0.8). For persons of Northern European descent, the allele frequencies for e2, e3, and e4 were 8.4%, 78.6%, and 12.9%, respectively. For persons of Southern European descent, the corresponding frequencies were 6.0%, 87.3%, and 6.8%.

Table 1 shows that the e3e3 genotype was the most common (64.6%) and the e2e2 genotype the least common (0.9%). Approximately three-quarters (78.5%) of Southern Europeans and 62.7% of Northern Europeans had the e3e3 genotype (Table 2). Few participants were homozygous for the e2 or e4 allele (0.9% and 1.8%, respectively). Table 2 shows genotype frequencies by age for controls; the e2e2 and e4e4 genotypes were more prevalent in the older age group. Genotype frequencies for men and women were very similar (data not shown).

Smoking and AMD

When compared with never smoking, the odds ratios for former smoking were 1.17 (95% confidence interval (CI): 1.0, 1.03; P = 0.03) for early AMD and 1.32 (95% CI: 0.84, 2.07; P = 0.23) for late AMD; however, the likelihood ratio test indicated there was no evidence that the association with former smoking differed for early and late AMD (P value = 0.6). Current smoking was strongly associated with late AMD (odds ratio (OR) = 3.33, 95% CI: 1.83, 5.90; P < 0.001), whereas no relation was observed for early AMD (OR = 1.04, 95% CI: 0.82, 1.34; P = 0.8); this difference was found to be statistically significant (P < 0.001).

APOE and AMD

When genotypes were analyzed, only e2e3 had a significant association with early AMD (Table 3); the odds ratio was 1.33 (95% CI: 1.10, 1.62; P = 0.004) in comparison with the referent group, e3e3. For late AMD, the corresponding odds ratio was 1.72 (95% CI: 1.00, 2.98; P = 0.05) (Table 3). No association was observed for any e4 genotypes (Table 3). The estimated odds ratios for the association with early AMD for the less prevalent genotype groups containing...
Table 2. Apolipoprotein E (APOE) Genotype Frequencies Among Controls Matched to Age-related Macular Degeneration Cases, by Age Group and Country of Origin, Melbourne Collaborative Cohort Study, 2003–2007

<table>
<thead>
<tr>
<th>APOE Genotype</th>
<th>No.</th>
<th>Row %</th>
<th>No.</th>
<th>Row %</th>
<th>No.</th>
<th>Row %</th>
<th>No.</th>
<th>Row %</th>
<th>No.</th>
<th>Row %</th>
<th>No.</th>
<th>Row %</th>
<th>Total No.</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age group, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>267</td>
<td>64.8</td>
<td>2</td>
<td>0.5</td>
<td>54</td>
<td>13.1</td>
<td>11</td>
<td>2.7</td>
<td>72</td>
<td>17.5</td>
<td>6</td>
<td>1.5</td>
<td>412</td>
<td>0.06</td>
</tr>
<tr>
<td>60–69</td>
<td>334</td>
<td>65.8</td>
<td>1</td>
<td>0.2</td>
<td>54</td>
<td>10.6</td>
<td>2</td>
<td>0.4</td>
<td>110</td>
<td>21.7</td>
<td>7</td>
<td>1.4</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td>≥70</td>
<td>887</td>
<td>64.9</td>
<td>14</td>
<td>1.0</td>
<td>138</td>
<td>10.1</td>
<td>28</td>
<td>2.1</td>
<td>271</td>
<td>19.8</td>
<td>29</td>
<td>2.1</td>
<td>1,367</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1,488</td>
<td>65.1</td>
<td>17</td>
<td>0.7</td>
<td>246</td>
<td>10.8</td>
<td>41</td>
<td>1.8</td>
<td>453</td>
<td>19.8</td>
<td>42</td>
<td>1.8</td>
<td>2,287</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Country of origin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northern European</td>
<td>1,221</td>
<td>62.7</td>
<td>17</td>
<td>0.9</td>
<td>218</td>
<td>11.2</td>
<td>38</td>
<td>2.0</td>
<td>412</td>
<td>21.2</td>
<td>41</td>
<td>2.1</td>
<td>1,947</td>
<td></td>
</tr>
<tr>
<td>Southern European</td>
<td>267</td>
<td>78.5</td>
<td>0</td>
<td>0.0</td>
<td>28</td>
<td>8.2</td>
<td>3</td>
<td>0.9</td>
<td>41</td>
<td>12.1</td>
<td>1</td>
<td>0.3</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1,488</td>
<td>65.1</td>
<td>17</td>
<td>0.7</td>
<td>246</td>
<td>10.8</td>
<td>41</td>
<td>1.8</td>
<td>453</td>
<td>19.8</td>
<td>42</td>
<td>1.8</td>
<td>2,287</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a Chi-squared test.

Effect modification of the association between early AMD and APOE genotype

To investigate potential effect modification by smoking, age, and country of origin, we formed 2 genotype groups—the e2 group (e2e2/e2e3/e2e4) and the e4 group (e4e4/e3e4)—since associations with early AMD for the individual genotypes in each of these groups were very similar. No differences in the association between genotype group and early AMD were observed by grouped country of origin (interaction P = 0.35) or age group (interaction P = 0.16). There was evidence that the association between genotype group and early AMD differed by smoking status (interaction P = 0.05); therefore, the estimated odds ratios are presented separately by smoking status (Table 4; for genotype frequencies, see Appendix Table 1).

Across smoking status groups, there was an inversion of the direction of the odds ratios for early AMD for both the e2 and e4 groups, from a positive or null association for never smokers to a negative association for current smokers. The odds ratios for early AMD in the e2 group were 1.40 for never smokers and 0.66 for current smokers; the respective estimates for the e4 group were 1.08 and 0.41. For late AMD, the estimates were imprecise, reflecting the small sample size. As for early AMD, the positive association between the e2 group and late AMD was strongest for participants who had never smoked.

DISCUSSION

In this study, the e2e3 genotype was associated with an increased risk of early AMD, with an odds ratio of 1.33 compared with the most common genotype, e3e3. This association differed by smoking status, with a stronger direct association for never and former smokers than for current smokers, where the point estimate of the association was in the opposite direction, though it was not formally significant. The e4 genotype group had a negative association with early AMD, where the association was evident only for current smokers.

The positive association with the e2-containing genotypes of APOE confirms the results of smaller studies that found weak positive associations of AMD with the e2 allele of APOE in comparison with the e3e3 genotype (1, 6–10);
because $e2$ is the minor allele, with variation in frequency between populations (28), the estimates from these previous studies were imprecise. However, we did not replicate the more often reported inverse association of the $e4$-containing APOE genotypes with AMD (1, 2, 6–11) in the sample overall, finding such an association only for current smokers. For never smokers, the association between the $e4$ genotype group and AMD pointed in a positive direction for early AMD.

We have previously reported that smoking modified the association between abdominal obesity and AMD (23). It is clear that there are interindividual differences in susceptibility to the adverse effects of smoking, both in terms of mortality (29) and in terms of AMD risk, and these differences distort associations with other exposures. Risk factors in older people can differ from those in younger populations, with a reversal of the direction of association for some risk factors (30–32); therefore, studies in elderly populations must take into account the possible genetic enrichment with selection of “healthy agers,” which may skew associations and will go unnoticed if sample sizes are too small to detect effect modification (33). Smoking exerts considerable survival pressure on a population, as it is strongly associated with increased morbidity and mortality; some AMD studies have found attenuated or inverse associations with smoking as a result (34, 35).

Current smokers in the older age groups who are able to participate in an AMD study, particularly controls (who, in addition to being in systemic good health, have no signs of macular aging), are “healthy agers” who are evidently less susceptible to the adverse effects of smoking. Such persons are representative of only a small proportion of agers in their birth cohort. In the current cohort from which the cases and controls were derived, the prevalence of persons who were eligible to be controls—with no drusen of any type—decreased from approximately 55% in the younger age groups to approximately 40% among those above age 75 years; in current smokers, the latter figure was 37%. Therefore, the concept of what constitutes a “normal control” changes with age.

When putative AMD genes are also reported to be associated with other diseases, such as the $APOE$ $e4$ allele’s being associated with Alzheimer’s dementia and cardiovascular disease, the strength of the association with AMD is also related to the strength of the association with the other diseases (36) and is therefore related to the likelihood of nonparticipation. In this study, $e4$ allele frequency did not decline with age in controls as would be predicted, which may reflect a survivorship effect in the controls: That is, there are likely to be other factors for these persons that have protected them from the adverse effect of the $e4$ allele.

Current smokers who survive into older age and who are unaffected by Alzheimer’s disease, cardiovascular disease, or AMD might have genetic modifiers or other factors that render them less susceptible to adverse effects of $e4$. Therefore, although the frequency of the $APOE$ $e4$ allele does not diminish with age, there may be an enrichment—an increase in allele frequency—of such protective genetic modifiers. These modifiers, although currently unknown, are the subject of intense focus in aging research (36). It is widely held that determination of the human life span is complex, with a number of loci interacting to influence longevity (36, 37).

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that determination of the human life span is complex, with
a number of loci interacting to influence longevity (36, 37).
For instance, in persons who survive to extreme old age, the
prevalence of “risk genes” for cancer and cardiovascular
disease is not reduced (38); rather, it appears that enrichment
of modifier genes may protect such persons from the risk
genes’ adverse effects (36). It appears logical that these
protective modifier genes will be most concentrated among
people who survive despite having the greatest exposure to
environmental and genetic risk factors, such as smoking and
$APOE$ $e4$.

A possible explanation for differences between our results
and those of previous studies may be our tighter matching,
where each case was individually matched to a control of the
same age. The possibility of survivor bias was raised in a
meta-analysis of $APOE$ and AMD studies, where it was
noted that many of the reported studies had cases with older
mean ages than controls (1, 8, 11, 12, 37–40).

### Table 4. Odds Ratiosa for the Association of Apolipoprotein E ($APOE$) Genotype With Early and Late Age-related Macular Degeneration, by Smoking Status, Melbourne Collaborative Cohort Study, 2003–2007

<table>
<thead>
<tr>
<th>Genotype Group</th>
<th>Totalb</th>
<th>Never Smokers</th>
<th>Former Smokers</th>
<th>Current Smokersc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>Value</td>
<td>OR</td>
</tr>
<tr>
<td>Early AMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$e3/e3$</td>
<td>1</td>
<td>Referent</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$e2/e2/e3/e2/e3$</td>
<td>1.32</td>
<td>1.11, 1.58</td>
<td>0.002</td>
<td>1.40</td>
</tr>
<tr>
<td>$e4/e4/e3/e4$</td>
<td>0.96</td>
<td>0.82, 1.12</td>
<td>0.6</td>
<td>1.08</td>
</tr>
<tr>
<td>Late AMD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$e3/e3$</td>
<td>1</td>
<td>Referent</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$e2/e2/e3/e2/e3$</td>
<td>1.77</td>
<td>1.08, 2.92</td>
<td>0.03</td>
<td>2.11</td>
</tr>
<tr>
<td>$e4/e4/e3/e4$</td>
<td>1.02</td>
<td>0.62, 1.69</td>
<td>0.9</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CI, confidence interval; OR, odds ratio.

* Odds ratio estimate from unconditional polytomous logistic regression, with adjustment for age group, grouped country of origin, sex, and waist-to-hip ratio.

b Test of the null hypothesis of no association with any genotype from a likelihood ratio test: $P = 0.007$.

c Likelihood ratio test for interaction: $P = 0.05$. 

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**Am J Epidemiol.** 2012;175(6):511–518
Strengths of this study include its size—to our knowledge, it is by far the largest case-control study of AMD to date—and the reliability and validity of the classification of AMD status. Careful matching on age avoided confounding by age, which has been raised as a problem in previous studies (12), and matching on country of origin addressed the problem of population stratification raised by Lewis and Brunner (41), which is of particular relevance in APOE studies. There were limitations of this study as well. An important limitation was the loss to follow-up of 49% in the cohort in which this study was nested, particularly the differential loss according to smoking status (23). Although participants were matched on country of origin, because markers informative for ancestry were not genotyped, we cannot rule out the possibility that population stratification could have made some contribution to our findings.

In conclusion, this large study confirms the ε2 allele as a “risk allele” for early AMD; however, the relation of the APOE genotypes with AMD varied by smoking status. These results highlight the inherent problems involved in reporting isolated genetic associations with disease and the importance of assessing effect modification by smoking status in an elderly population. A survivorship effect from smoking may lead to enrichment of as-yet-unknown genetic or other modifiers in elderly smokers which distorts or inverts true associations. It is likely that smoking distorts associations of other genes with AMD; small studies may not elicit this, and therefore findings may be misleading. We cannot ignore the fact that relations between environmental exposures and disease are different in older populations, and this is likely to reflect a survivorship effect; the same effect might be skewing genetic associations.

ACKNOWLEDGMENTS

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This work was supported by VicHealth; the Cancer Council Victoria (initial cohort recruitment); and the National Health and Medical Research Council of Australia (NHMRC) (program grant 209057, capacity-building grant 251533, and enabling grant 396414). The ophthalmic component of the study was funded by the Ophthalmic Research Institute of Australia, the American Health Assistance Foundation, the John Reid Charitable Trust, the Perpetual Trustees, and the Royal Victorian Eye and Ear Hospital. Support was also provided through the awarding of an NHMRC Practitioner Fellowship to R. H. G., a Wagstaff Fellowship to L. D. R., and an NHMRC PhD Scholarship and Hugh Noel Puckle Scholarship to M. K. M. A. The Centre for Eye Research Australia receives operational infrastructure support from the Victorian government.

The funding organizations did not have any involvement in the study design or in data collection, analysis, or interpretation.

Conflict of interest: none declared.

REFERENCES

**Appendix Table 1.** Apolipoprotein E (APOE) Genotype Frequencies Among Cases With Early and Late Age-related Macular Degeneration and Controls, by Smoking Status, Melbourne Collaborative Cohort Study, 2003–2007

<table>
<thead>
<tr>
<th>Genotype Group</th>
<th>Total</th>
<th>Never Smokers</th>
<th>Former Smokers</th>
<th>Current Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
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Abbreviation: AMD, age-related macular degeneration.