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The association between macular pigment optical density and *CFH*, *ARMS2*, *C2*/*BF*, and *C3* genotype☆

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ABSTRACT

Age-related macular degeneration (AMD) is the most common cause of blindness in older people in developed countries, and risk for this condition may be classified as genetic or environmental, with an interaction between such factors predisposing to this disease. This study investigated the relationship between AMD risk genes, macular pigment optical density (MPOD), which may protect against AMD, and serum concentrations of the macular carotenoids, lutein (L) and zeaxanthin (Z). This was a cross-sectional study of 302 healthy adult subjects. Dietary intake of L and Z was assessed by food frequency questionnaire, and MPOD was measured by customized heterochromatic flicker photometry. We also calculated MPOD Area as the area of MP under the spatial profile curve, to reflect MP across the macula. Serum L and Z were measured by HPLC. Genotyping of tag SNPs in the genes *CFH*, *ARMS2*, *C3*, *C2* and *BF* was undertaken with multiplex polymerase chain reaction (PCR) and primer extension methodology (ABI Snapshot, ABI Warrington UK) on DNA extracted from peripheral blood. The mean ± SD (range) age of the subjects in this study was 48 ± 11 (21–66) years. There was a statistically significant association between *CFH* genotype and family history of AMD, with subjects having two non-risk *CFH* haplotypes ($n = 35$), or one non-risk and one protective *CFH* haplotype ($n = 33$), being significantly more likely to have a negative family history of AMD (Pearson Chi square: $p = 0.001$). There was no significant association between the AMD risk genes investigated and either MPOD (One way ANOVA: $p > 0.05$) or serum concentrations of L or Z (One way ANOVA: $p > 0.05$, for both). Subjects who were homozygous for risk alleles of both *CFH* and *ARMS2* ($n = 4$) had significantly lower MPOD at 0.5° and 1° retinal eccentricity (Independent samples *t* test: $p < 0.05$) and lower MPOD Area which approached statistical significance (Independent samples *t* test: $p = 0.058$), compared to other subjects ($n = 291$). In conclusion, this study did not detect an association between individual AMD risk genotypes and the putatively protective MP, or serum concentrations of its constituent carotenoids. However, the combination of homozygous risk alleles at both *CFH* and *ARMS2* loci was associated with significantly lower MPOD centrally, despite comparable serum concentrations of the macular carotenoids. These findings suggest that the maculae of subjects at very high genetic risk of AMD represent a hostile environment for accumulation and/or stabilization of MP.

1. Introduction

Age-related macular degeneration (AMD) is the most common cause of blindness in people over 50 years of age in the developed world (Bressler, 2004; Congdon et al., 2003; Klein et al., 1995). This degenerative disease results in a loss of central and colour vision, leading to difficulty with reading, recognizing faces, and driving, thus impacting profoundly on the independence of those affected. The two forms of advanced AMD resulting in loss of vision are geographic atrophy (GA) and choroidal neovascularization (CNV) (Bird et al., 1995; Jager et al., 2008). The prevalence of this condition is likely to increase dramatically in the future, as a result of increasing life-expectancy and the consequential increasing senescence of society (van Leeuwen et al., 2003). The pathogenesis of AMD is incompletely understood, but it is believed to involve

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a complex interaction between an individual's genetic background and environmental/lifestyle factors (Nolan et al., 2007; Tomany et al., 2004).

Our understanding of the important role that genetic background plays in the pathogenesis of AMD was greatly enhanced in 2005, with several independent reports associating the complement factor H (*CFH*) gene, located on chromosome 1q31, with this disease (Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Klein et al., 2005). The strong association of the *CFH Y402H* variant allele with increased risk for AMD suggested an important role for the alternative complement pathway and the involvement of inflammation in the pathogenesis of AMD (Anderson et al., 2002; Hageman et al., 2005). The role of the complement system was further emphasized with the discovery of amended risk of AMD associated with the paralogous complement component 2 (*C2*) and complement factor B (*BF*) genes, and the complement component 3 (*C3*) gene (Gold et al., 2006; Maller et al., 2007; Yates et al., 2007). In late 2005, a second major risk allele was reported, the hypothetical *LOC387715*, within the locus of the age-related maculopathy susceptibility 2 (*ARMS2*) gene, localized to chromosome 10q26 (Rivera et al., 2005).

Macular pigment (MP) is composed of the hydroxycarotenoids, lutein (L), zeaxanthin (Z), and *meso*-zeaxanthin (*meso*-Z). L and Z are of dietary origin and are not synthesized *de novo* in humans, whereas *meso*-Z is not found in a conventional western diet, but is understood to be primarily formed in the retina following conversion from L (Bone et al., 1993; Johnson et al., 2005). MP is found in highest concentration at the central macula, where it functions as a powerful antioxidant and acts as a filter of actinic short-wavelength blue light, thus limiting (photo-) oxidative damage to retinal cells (Snodderly, 1995). These properties of MP are believed to confer protection against the development, and/or progression, of AMD.

Although MP is entirely of dietary origin, its concentration at the macula is subject to heritability, as reported in a classic twin study (Liew et al., 2005). In that study of 76 monozygotic and 74 dizygotic female twin pairs, they estimated that heritability accounted for between 67% and 85% of an individual's MP level. Taken together with the recent finding of a relative lack of MP in association with a clinically confirmed family history of AMD (Nolan et al., 2007), we hypothesized that genes associated with AMD risk may contribute to variation of this putatively protective pigment, and we designed this study to test our hypothesis.

2. Methods

2.1. Subjects

Three hundred and two subjects were recruited for this study, which was carried out in the Macular Pigment Research Group (MPRG) laboratory at Waterford Institute of Technology, Ireland. Subjects were recruited following local advertisement in various media. This study was approved by the Research Ethics Committee of Waterford Institute of Technology, and subjects were required to sign an informed consent document prior to participation. All experimental procedures adhered to the tenets of the Declaration of Helsinki.

Inclusion criteria for participation in this study were: age between 20 and 70 years; no clinical evidence of ocular pathology; no dietary supplementation with the MP carotenoids; visual acuity 20/40 or better. The following information was recorded for each subject: demographic details; known family history of AMD (confirmed in writing by the diagnosing ophthalmologist); personal smoking history; dietary intake of L and Z, assessed using a validated 170-item food frequency questionnaire (FFQ). Examination included: visual acuity (Snellen and LogMAR); body mass index [BMI (calculated as kg/m^2)]; MP optical density measurement by customized heterochromatic flicker photometry (cHFP) using the Macular Densitometer™; non-mydriatic fundus photography, using a NIDEK AFC-210 non-mydriatic auto fundus camera to screen for ocular pathology; 12-hour fasting blood samples were taken to quantify serum concentrations of L and Z using high performance liquid chromatography (HPLC), and for genotyping.

2.2. Food frequency questionnaire

Dietary intake of L and Z was assessed by a self-administered, semi-quantitative FFQ developed by the Scottish Collaborative Group (SCG) at the University of Aberdeen, Scotland, UK. This semi-quantitative FFQ is described in detail in a separate study by Loane et al. (Loane et al., 2010).

2.3. Measurement of macular pigment optical density

MP optical density was measured psychophysically by cHFP, a technique that has been validated against the absorption spectrum of MP *in vitro* (Bone et al., 1992). HFP is based on the fact that MP absorbs short-wavelength blue light, with peak absorption occurring at a wavelength of 458 nm. The subject is required to make iso-luminance matches between two flickering lights, a green light (not absorbed by MP) and a blue light (maximally absorbed by MP). The log ratio of the amount of blue light absorbed centrally, where MP peaks, to that absorbed at a peripheral retinal locus (the 'reference point', where MP optical density is assumed to be zero), gives a measure of the subject's MP optical density.

In this study, we used the Macular Densitometer™, a cHFP instrument that is slightly modified from a device described by Wooten, Hammond, Land, and Snodderly (Wooten et al., 1999). The subject is required to observe a flickering target, alternating in square-wave counterphase between a green light (with a wavelength of 564 nm) and a blue light (with a wavelength of 460 nm), and to make iso-luminance matches between these flickering lights. The luminance of the green and blue lights is varied in a yoked manner, which avoids a change in the overall luminance of the test target. When an iso-luminant ('null-flicker') match has been made between these flickering lights, flicker is no longer perceived, and this is the desired endpoint of the test. Different sized targets enable measurement of MP optical density at 0.25°, 0.5°, 1°, and 1.75° retinal eccentricity, relative to a reference point at 7° retinal eccentricity (where MP optical density is assumed to be zero). The targets used to measure MP optical density at 0.25°, 0.5°, 1°, and 1.75° retinal eccentricity are each centrally located circular stimuli with a radius equal to the eccentricity being measured. The subject fixates on a central fixation spot in the middle of each target that is 5 min in diameter. The 7° reference target uses an eccentrically located red LED, 5 min in diameter, as the fixation spot. This is presented to the left-hand side of a blue/green flickering circular disk, which has a diameter of 2° and is centered at an eccentricity of 7° from the red fixation LED. Targets are presented on a blue background test field (wavelength 468 nm) that saturates the S-cone pathway. A minimum of three null-flicker readings, with a coefficient of variance $\leq$ 10%, were recorded for each subject at each of the test loci (0.25°, 0.5°, 1°, 1.75°, and 7° retinal eccentricity). Measurement of MP optical density at these points of retinal eccentricity enabled us to plot the spatial profile of MP across the macula. For each subject, we then calculated the area of MP optical density under the spatial profile, using the Trapezoid Rule, as follows: MP optical density Area = [(((MP optical density at 0.25° + MP optical density at 0.5°)/2)*0.25°) + (((MP optical density at 0.5° + MP optical density at 1°)/2)*0.5°) + (((MP optical density at 1° + MP optical density at 1.75°)/2)*0.75°) + (((MP optical

density at 1.75° + MP optical density at 7°)/2)*5.25°)]; assuming an MP optical density of zero at 7° retinal eccentricity, and also assuming a linear 'fit' between each successive point of measurement of MP optical density, but a non-linear MP spatial profile overall, as illustrated for the entire study group in Fig. 1. This method of calculating MP optical density Area is better suited to take account of the various different MP spatial profiles that may present (e.g., an MP spatial profile exhibiting a 'central dip'), since it does not involve making any prior assumption regarding the configuration of each individual spatial profile, as is the case with the 'Method of Integration' (Kirby et al., 2008; Nolan et al., 2008). This MP optical density Area gives a better measurement of the quantity of MP across the macula than an individual measurement at a single point of retinal eccentricity. MP optical density measurement was performed under conditions of dimmed light (ambient illuminance: 4 lux, as measured with an Iso-Tech ILM 350 Lux Meter) at a viewing distance of 18.5 inches (47 cm).

The major advantage of cHFP over standard HFP instruments is that the flicker frequency of each test target is customized for each individual subject, minimizing the variance between consecutive measurements and, thus, increasing the accuracy and ease of use of the test. A more detailed description of this instrument has been published previously (Wooten et al., 1999). Further information on the technique and advantages of cHFP have also previously been published (Loane et al., 2007; Nolan et al., 2008).

2.4. Blood sample collection

This is described in detail in a separate publication (Loane et al., 2010).

2.5. Serum L and Z analysis

Serum L and Z were quantified using reverse phase HPLC. We used an Agilent 1200 series LC system (Agilent Technologies Ireland Ltd., Dublin, Ireland), with photodiode array detection at 295 nm (detection of the internal standard: alpha tocopherol acetate) and 450 nm (detection of L and Z). A 5 μm analytical/preparative 4.6 × 250 mm 201 TP specialty reverse phase column (Vydac, Hesperia, CA, USA) was used with an in-line guard column. The mobile phase consisted of 97% methanol and 3% tetrahydrofuran, and was degassed using an in-line degasser. The flow rate was 1 ml/min, and the total run time was 15 min. All carotenoid peaks were integrated and quantified using Agilent Chem Station software. Further detail on the methodology used for this analysis is provided in a separate publication (Loane et al., 2010).

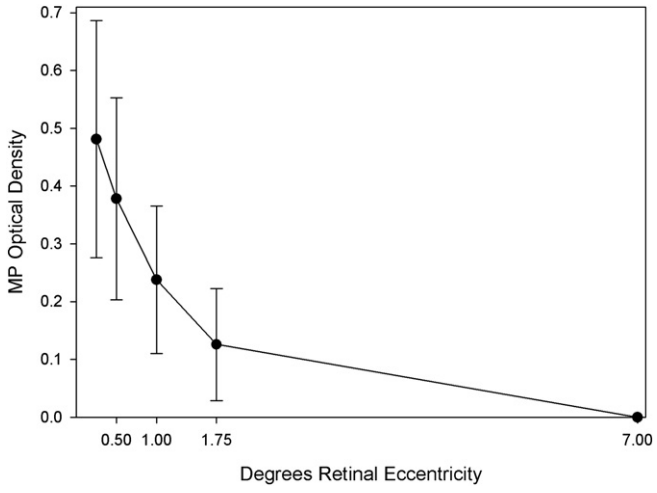

Fig. 1. Mean (±SD error bars) macular pigment (MP) optical density spatial profile for the entire study group.

2.6. Genotyping

DNA was extracted from peripheral blood leucocytes or frozen buffy coat samples using standard protocols. We genotyped six single nucleotide polymorphisms (SNPs) in *CFH* (rs419137, rs6677604, rs2284664, rs3753396, rs1061170, rs800292) and three in *ARMS2* (rs10490924, rs10490923, rs2736911) enabling determination of risk and protective haplotypes across both loci in the current cohort, as described previously using multiplex PCR and primer extension methodology (ABI Snapshot, ABI Warrington, UK) (Hughes et al., 2006, 2007). In a separate Snapshot assay, two tag SNPs (rs1042663, rs2072632) identifying protective and risk haplotypes respectively across the *C2*/*BF* locus, (McKay et al., 2009) and rs2230199 identifying increased risk at *C3*, (Maller et al., 2007; Yates et al., 2007) were also genotyped. All primer and probe sequences are available upon request.

2.7. Statistical analysis

The commercially available statistical software package SPSS version 17 (SPSS, Chicago, IL) was used for analysis of results. The graphical software package SigmaPlot version 8.0 (Systat, San Jose, CA) was used for graphical presentation of results. One way ANOVA was used to investigate differences in variables between the various genotype groups. Further associations were investigated using a general linear model approach, with Pearson Chi square testing and independent samples *t* testing, as appropriate. All SNPs were verified, validated and assessed for Hardy–Weinberg equilibrium. Allele frequency differences were assessed with Pearson Chi square testing. Assessment of the associations of genetic markers with AMD family history, and of interactions between genetic markers and other co-variates were obtained using likelihood ratio Chi squared tests in a logistic regression model. The level of statistical significance was set at the standard $p < 0.05$.

3. Results

Genotype data were available for *CFH*, *ARMS2* and *C3* on 296 (98%) of the 302 subjects, and for *C2*/*BF* on 277 (91.7%) of the 302 subjects in this study. The anthropometric and lifestyle data of all genotyped subjects are detailed in Table 1. The mean ± SD (range) age of all subjects genotyped for *CFH*, *ARMS2* and *C3* in this study was 48 ± 11 (21–66) years. 69.9% of the subjects included in this study were female, and 40.1% had a known positive family history of AMD.

The mean ± SD (range) MP optical density at 0.5° retinal eccentricity for all subjects genotyped for *CFH*, *ARMS2* and *C3* in this study was 0.37 ± 0.17 (0–1.02) optical density units. The mean ± SD (range) MP optical density Area for all subjects genotyped for *CFH*, *ARMS2* and *C3* in this study was 0.71 ± 0.43 (0–3.00) optical density units. The mean ± SD (range) MP optical densities measured at each point of retinal eccentricity, for each genotype group, are detailed in Table 2.

3.1. Assessment of CFH association

The tagged SNPs genotyped across the *CFH* gene enabled the identification of five common haplotypes (two risk, two non-risk, and one protective: haplotypes 1 and 2, which increase risk, are in complete linkage disequilibrium (LD) with the C risk allele at Y402H; haplotypes 3 and 4 are non-risk, and are in complete LD

Table 1
Anthropometric and lifestyle data for all genotyped subjects.

Characteristic	*CFH*, *ARMS2*, *C3* ($n = 296$)	*C2*/*BF* ($n = 277$)
Age (years)	48 ± 11	48 ± 11
Sex		
Male	89 (30.1%)	86 (31%)
Female	207 (69.9%)	191 (69%)
BMI[a]	26.75 ± 4.56	26.56 ± 4.45
MPOD[b] 0.25°	0.48 ± 0.20	0.48 ± 0.21
MPOD 0.5°	0.37 ± 0.17	0.38 ± 0.18
MPOD 1°	0.24 ± 0.13	0.24 ± 0.13
MPOD 1.75°	0.12 ± 0.10	0.13 ± 0.10
MPOD Area	0.71 ± 0.43	0.72 ± 0.43
Smoking (pack years)[c]	5.79 ± 11.39	5.85 ± 11.44
Dietary L (mg)	1.373 ± 1.161	1.389 ± 1.175
Dietary Z (mg)	0.187 ± 0.111	0.188 ± 0.111
Serum L (μg/ml)	0.088 ± 0.047	0.091 ± 0.048
Serum Z (μg/ml)	0.016 ± 0.011	0.016 ± 0.011
Positive family history of AMD (%)	39.5	37.9

L: Lutein.
Z: Zeaxanthin.
AMD: Age-related macular degeneration.
[a] BMI (Body mass index) is defined as: body weight in kilograms divided by height in metres squared (kg/m^2).
[b] MPOD: MP optical density.
[c] Pack year calculation = (number of cigarettes smoked per day multiplied by number of years smoking) divided by 20.

with the T allele at Y402H; haplotype 5 (the most protective haplotype) is in complete LD with the deletion at *CFHR1* and *CFHR3*), as described previously (Hughes et al., 2006), allowing for categorization of subjects into the following *CFH* groups: Group 1: two risk haplotypes; Group 2: one risk haplotype, one non-risk haplotype; Group 3: one risk haplotype, one protective haplotype; Group 4: two non-risk haplotypes; Group 5: one protective haplotype, one non-risk haplotype; Group 6: two protective haplotypes. There was a statistically significant association between these *CFH* groups and family history of AMD in this study sample, with subjects in *CFH* Group 4 and 5 being significantly more likely to have no known family history of AMD (Pearson Chi square: $p = 0.001$; illustrated in Fig. 2). There was no significant difference in the distribution of subjects with and without a known positive family history of AMD in the other *CFH* groups ($p > 0.05$, for all). There was no significant association between *CFH* group and sex (Pearson chi square: $p > 0.05$), or between *CFH* group and age, BMI, MP optical density at any degree of retinal eccentricity or MP optical density Area, cigarette smoking, dietary intake of L or Z, or serum concentrations of L or Z (One way ANOVA: $p > 0.05$, for all).

3.2. Assessment of ARMS2 association

The tagged SNPs genotyped across the *ARMS2* gene allowed categorization of subjects into the following *ARMS2* groups: Group 1: two risk haplotypes; Group 2: one risk haplotype, one non-risk haplotype; Group 3: two non-risk haplotypes. The risk haplotype corresponded to the T allele at rs10490924. There was no statistically significant association between *ARMS2* group and family history of AMD, or sex in this study sample (Pearson Chi square: $p > 0.05$, for both). There was no significant association between *ARMS2* group and either age, BMI, MP optical density at any degree of retinal eccentricity or MP optical density Area, cigarette smoking, dietary intake of L or Z, or serum concentrations of L or Z (One way ANOVA: $p > 0.05$, for all).

3.3. Assessment of C2/BF association

The tagged SNPs genotyped across the *C2*/*BF* genes allowed categorization of subjects into the following *C2*/*BF* groups: Group 1: one or two risk haplotypes; Group 2: two non-risk haplotypes; Group 3: one or two protective haplotypes, no risk haplotypes. Risk haplotypes were identified through the T allele at rs2072632, and protective haplotypes were identified through the A allele at rs1042663 (McKay et al., 2009). There was no statistically significant association between *C2*/*BF* group and family history of AMD or sex in this study sample (Pearson Chi square: $p > 0.05$, for both). There was no significant association between *C2*/*BF* group and either age, BMI, MP optical density at any degree of retinal eccentricity or MP optical density Area, cigarette smoking, dietary intake of L or Z, or serum concentrations of L or Z (One way ANOVA: $p > 0.05$, for all).

3.4. Assessment of C3 association

The tag SNP (rs2230199), associated with increased risk at *C3*, allowed categorization of subjects into the following *C3* groups: Group 1: two non-risk alleles; Group 2: one risk allele, one non-risk

Table 2
Mean ± SD (range) macular pigment optical density (MPOD) values at each degree of retinal eccentricity with respect to genotype group.

Genotype	MPOD 0.25°	MPOD 0.5°	MPOD 1°	MPOD 1.75°	MPOD area
CFH Group 1 ($n = 64$)	0.49 ± 0.20 (0.22–1.03)	0.38 ± 0.16 (0.03–0.81)	0.23 ± 0.11 (0.01–0.48)	0.12 ± 0.07 (0.00–0.30)	0.70 ± 0.34 (0.13–1.39)
CFH Group 2 ($n = 113$)	0.49 ± 0.20 (0.12–0.98)	0.39 ± 0.18 (0.05–0.86)	0.24 ± 0.14 (0.00–0.87)	0.13 ± 0.11 (0.00–0.67)	0.75 ± 0.47 (0.04–3.00)
CFH Group 3 ($n = 39$)	0.41 ± 0.18 (0.03–0.81)	0.32 ± 0.15 (0.00–0.56)	0.19 ± 0.10 (0.00–0.35)	0.10 ± 0.09 (0.00–0.26)	0.59 ± 0.36 (0.00–1.15)
CFH Group 4 ($n = 34$)	0.44 ± 0.19 (0.06–0.88)	0.36 ± 0.17 (0.05–0.73)	0.24 ± 0.13 (0.00–0.48)	0.13 ± 0.08 (0.00–0.31)	0.72 ± 0.38 (0.04–1.32)
CFH Group 5 ($n = 33$)	0.55 ± 0.25 (0.22–1.32)	0.39 ± 0.19 (0.16–1.02)	0.26 ± 0.13 (0.04–0.65)	0.15 ± 0.12 (0.00–0.57)	0.81 ± 0.49 (0.14–2.55)
CFH Group 6 ($n = 8$)	0.56 ± 0.22 (0.20–0.85)	0.40 ± 0.22 (0.00–0.70)	0.27 ± 0.15 (0.00–0.53)	0.14 ± 0.11 (0.03–0.31)	0.81 ± 0.51 (0.11–1.63)
ARMS2 Group 1 ($n = 14$)	0.43 ± 0.20 (0.16–0.84)	0.34 ± 0.12 (0.14–0.53)	0.20 ± 0.11 (0.02–0.37)	0.12 ± 0.09 (0.00–0.27)	0.66 ± 0.40 (0.17–1.33)
ARMS2 Group 2 ($n = 119$)	0.49 ± 0.22 (0.13–1.32)	0.39 ± 0.18 (0.03–1.02)	0.25 ± 0.14 (0.00–0.87)	0.13 ± 0.10 (0.00–0.67)	0.75 ± 0.45 (0.04–3.00)
ARMS2 Group 3 ($n = 158$)	0.47 ± 0.19 (0.03–1.01)	0.36 ± 0.17 (0.00–0.81)	0.23 ± 0.12 (0.00–0.65)	0.12 ± 0.09 (0.00–0.57)	0.70 ± 0.40 (0.00–2.55)
C2/*BF* Group 1 ($n = 121$)	0.50 ± 0.21 (0.06–1.32)	0.39 ± 0.17 (0.03–1.02)	0.25 ± 0.12 (0.01–0.58)	0.13 ± 0.09 (0.00–0.38)	0.74 ± 0.38 (0.05–1.69)
C2/*BF* Group 2 ($n = 121$)	0.46 ± 0.21 (0.14–1.01)	0.37 ± 0.18 (0.00–0.86)	0.23 ± 0.14 (0.00–0.87)	0.12 ± 0.11 (0.00–0.67)	0.71 ± 0.47 (0.04–3.00)
C2/*BF* Group 3 ($n = 31$)	0.51 ± 0.20 (0.16–1.03)	0.39 ± 0.18 (0.05–0.81)	0.25 ± 0.13 (0.00–0.53)	0.14 ± 0.09 (0.00–0.31)	0.78 ± 0.40 (0.04–1.63)
C3 Group 1 ($n = 178$)	0.47 ± 0.21 (0.06–1.32)	0.38 ± 0.18 (0.00–1.02)	0.23 ± 0.13 (0.00–0.65)	0.13 ± 0.09 (0.00–0.57)	0.73 ± 0.42 (0.04–2.55)
C3 Group 2 ($n = 101$)	0.50 ± 0.20 (0.03–1.03)	0.38 ± 0.17 (0.00–0.86)	0.24 ± 0.13 (0.00–0.87)	0.12 ± 0.10 (0.00–0.67)	0.75 ± 0.44 (0.00–3.00)
C3 Group 3 ($n = 13$)	0.45 ± 0.15 (0.21–0.78)	0.33 ± 0.11 (0.17–0.59)	0.21 ± 0.09 (0.05–0.40)	0.10 ± 0.07 (0.02–0.28)	0.60 ± 0.30 (0.18–1.34)
CFH Group 1 and *ARMS2* Group 1 ($n = 4$)	0.33 ± 0.12 (0.22–0.49)	0.25 ± 0.06 (0.18–0.32)	0.12 ± 0.05 (0.08–0.19)	0.05 ± 0.07 (0.00–0.15)	0.39 ± 0.24 (0.18–0.72)

CFH genotype: Group 1: two risk alleles; Group 2: one risk, one non-risk allele; Group 3: one risk, one protective allele; Group 4: two non-risk alleles; Group 5: one non-risk, one protective allele; Group 6: two protective alleles.
ARMS2 genotype: Group 1: two risk alleles; Group 2: one risk, one non-risk allele; Group 3: two non-risk alleles.
C2/*BF* genotype: Group 1: one or two risk alleles; Group 2: two non-risk alleles; Group 3: one or two protective alleles, no risk alleles.
C3 genotype: Group1: two non-risk alleles; Group 2: one risk, one non-risk allele; Group 3: two risk alleles.

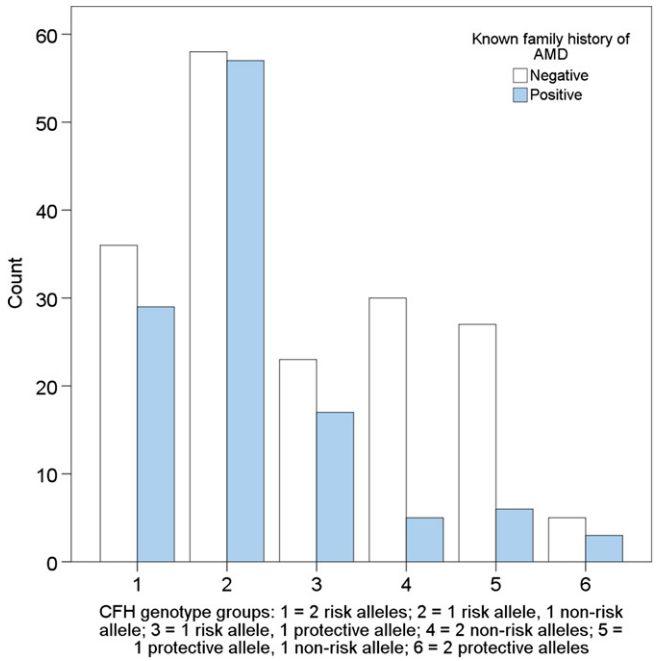

Fig. 2. Bar chart showing the proportion of subjects with and without a known family history of AMD according to *CFH* genotype.

allele; Group 3: two risk alleles. There was no statistically significant association between *C3* group and family history of AMD, or sex in this study sample (Pearson Chi square: $p > 0.05$, for both). There was no significant association between *C3* group and either age, BMI, MP optical density at any degree of retinal eccentricity or MP optical density Area, cigarette smoking, dietary intake of L or Z, or serum concentrations of L or Z (One way ANOVA: $p > 0.05$, for all).

3.5. Assessment of combined CFH and ARMS2 association

Further to the findings of Rivera et al. of a multiplicative effect on disease risk with homozygosity of risk alleles for both *CFH* and *ARMS2*, we also investigated associations in subjects with this high risk genotype (Rivera et al., 2005). Subjects homozygous for risk alleles at both the *CFH* (Group 1, above) and *ARMS2* (Group 1, above) loci ($n = 4$) had significantly lower MP optical density at 0.5° retinal eccentricity (Mean ± SD = 0.26 ± 0.06 versus 0.37 ± 0.17; Independent samples t test: $p = 0.022$) and at 1° retinal eccentricity (Mean ± SD = 0.12 ± 0.05 versus 0.24 ± 0.13; Independent samples t test: $p = 0.015$) compared with subjects who did not have this particular genotype ($n = 286$). All four subjects were unrelated to each another. This association approached statistical significance for MP optical density Area (Mean ± SD = 0.38 ± 0.24 versus 0.72 ± 0.42; Independent samples t test: $p = 0.058$). Both groups were statistically comparable in terms of dietary intake of L and Z, and serum concentrations of L and Z (Independent samples t test: $p > 0.05$, for all).

4. Discussion

This study investigated the relationship between the major AMD risk genes, MP optical density, and serum concentrations of L and Z in 302 healthy subjects, aged between 21 and 66 years. The mean MP optical density of all genotyped subjects at 0.5° retinal eccentricity was 0.37 ± 0.17 optical density units, which is comparable to previous studies that used HFP to measure MP optical density at this eccentricity (Beatty et al., 2001; Ciulla et al., 2001; Hammond and Caruso-Avery, 2000; Loane et al., 2007; Mellerio et al., 2002; Nolan et al., 2007; Snodderly et al., 2004). To our knowledge, this is the largest study to date investigating the association between AMD risk genes and MP.

MP carotenoids are derived entirely from diet, but macular levels of these carotenoids are subject to influence by genetic background, as shown in a classic twin study (Liew et al., 2005). There is a large body of evidence to suggest that MP may protect against the development and/or progression of AMD (Loane et al., 2008). This putative protective effect is based on the known properties of MP as a pre-receptoral filter of actinic short-wavelength blue light, and its antioxidant capacity, including the ability to quench singlet oxygen (Krinsky and Deneke, 1982) and inhibit the peroxidation of membranous phospholipids (Lim et al., 1992). It has been estimated that MP absorbs approximately 40% of damaging short-wavelength irradiation before its incidence on the photoreceptors and the retinal pigment epithelium (RPE) (Snodderly et al., 1984). This is deemed to be particularly important, as it has been shown by Ham et al. that exposure to short-wavelength blue light can result in photochemical retinal injury in primates (Ham, et al., 1976). Furthermore, MP is thought to preserve foveal S-cone sensitivity, protecting it from short-wavelength blue light damage in humans (Haegerstrom-Portnoy, 1988). It has also been shown that the administration of antioxidants can prevent light-induced retinal damage in rat retinas (Organisciak et al., 1999). More recently, dietary supplementation with L in mice has been reported to suppress laser-induced CNV formation, and to inhibit the associated inflammatory and angiogenic molecules related to CNV pathogenesis (Izumi-Nagai et al., 2007). This inhibitory effect of L on inflammatory and angiogenic processes was also confirmed *in vitro* by the application of L to specific cell cultures including human ARPE-19 cells (Izumi-Nagai et al., 2007). Furthermore, in early 2008, Hollyfield et al. were the first to demonstrate a molecular pathway from the initial oxidative damage to tissue molecules, leading to activation of inflammation via the complement system and the development of an antibody-mediated immune response, and histopathological changes typical of AMD (Hollyfield et al., 2008).

In summary, there is a substantial body of evidence that cumulative (photo)-oxidative stress, which results in inflammation, is involved in the pathogenesis of AMD, and that this mechanism of retinal injury can be prevented by the administration of tissue-relevant antioxidants.

Because inflammation promotes generation of reactive oxygen intermediates (ROIs), and, conversely, because oxidative stress promotes further inflammation, we hypothesized that maculae with unregulated inflammation (i.e., eyes of subjects with the risk alleles of *CFH*, *ARMS2*, *C2*/*BF*, or *C3*) and/or unregulated generation or compartmentalization of mitochondrial ROIs (i.e., subjects with the *ARMS2* risk allele(s)) would represent a high oxidative stress environment with a consequential depletion of antioxidants in this tissue, therefore exhibiting low MP optical density.

In 2005, *CFH* and *ARMS2* were identified as the major risk genes for AMD, which together have been estimated to account for over 50% of risk for developing this disease (Edwards et al., 2005; Gotoh et al., 2009; Hageman et al., 2005; Haines et al., 2005; Jakobsdottir et al., 2005; Rivera et al., 2005; Seddon et al., 2007). Subsequently, other genes associated with the complement system, namely *C2*/*BF* and *C3*, were also found to play a lesser role in the genetic predisposition to this condition (Gold et al., 2006; Maller et al., 2007; Yates et al., 2007). The association of the variant *CFH Y402H* haplotype with increased risk for AMD, and the subsequent association of certain haplotypes of *C2*/*BF* and *C3* with increased risk for AMD, represents evidence that inflammation is important

in the pathogenesis of AMD. CFH and BF are key regulators of the alternative complement pathway in humans. The complement pathway is integral to the body's innate immune system. C3 is the most abundant complement component, and is central to activation of the complement cascade (Walport, 2001). The ultimate end result of complement activation is cell lysis, and if this process is not properly regulated it results in uncontrolled inflammation and damage to normal cells and tissues (Walport, 2001). CFH is the major complement regulatory protein, without which the regulation of complement activation is lost completely, leading to continuous activation of C3 and uncontrolled inflammation (Anderson et al., 2002). The presence of complement factor proteins in drusen represents yet further evidence that inflammation plays an important role in the pathogenesis of AMD (Crabb et al., 2002; Hollyfield et al., 2008; Johnson et al., 2000). However, in this study we did not detect any significant association between CFH risk alleles and MP optical density at any degree of retinal eccentricity, MP optical density Area or serum concentrations of L or Z.

ARMS2 is located on chromosome 10q26 and significant LD exists between it and other genes, in particular *HTRA1*, which contains SNPs that have also been implicated in increased AMD risk (Hughes et al., 2007; Leveziel et al., 2007). The high level of LD has made it difficult to determine which of these genes is primarily responsible for the increased risk of AMD (attributable to this region of chromosome 10q26). However, a recent report suggests that a single SNP (rs10490924), which changes the coding sequence of *ARMS2*, may account for the association between chromosome 10q26 and increased risk for AMD (Kanda et al., 2007). Although the precise role of *ARMS2* in AMD disease aetiology has yet to be elucidated, its role in mitochondrial function is thought to be important (Rivera et al., 2005). Recently, it has been demonstrated that the normal protein coded for by *ARMS2* is associated with mitochondria, and they were able to localize this protein product to the ellipsoid region of the photoreceptors, which are known to be rich in mitochondria (Fritsche et al., 2008; Hoang et al., 2002). It has therefore been postulated that the role of *ARMS2* in AMD pathogenesis relates to mitochondrial function and/or its involvement in cell turnover and/or the removal of cellular debris from the photoreceptor-RPE complex. It is also noteworthy that there appears to be a multiplicative effect on risk for AMD with each additional *ARMS2* variant allele, in that heterozygosity confers an increased odds ratio for development of AMD of 2.83 (95% CI: 1.91–4.20), whereas homozygosity confers an increased odds ratio of 32.83 (95% CI: 12.53–86.07) (Hughes et al., 2007), although not all studies have replicated this large multiplicative effect (Conley et al., 2006; Rivera et al., 2005; Ross et al., 2007). In this study, we report no significant association between *ARMS2* risk alleles and MP optical density at any degree of retinal eccentricity, MP optical density Area, or serum concentration of L or Z.

Rivera et al. reported that combined homozygous risk alleles at both *ARMS2* and *CFH Y402H*, conferred an increased odds ratio of 57.6 (95% CI: 37.2–89.0) for the development of AMD, when compared with subjects with the respective non-risk haplotypes of these genes (Rivera et al., 2005). We report significantly lower MP optical density centrally (at 0.5° and at 1° retinal eccentricity) in association with this homozygous high risk combination of alleles. One biologically plausible rationale for this finding rests on the fact that homozygosity for the *CFH* risk alleles is associated with unregulated inflammation and, therefore, excessive production of ROIs and a consequential depletion of local antioxidants, including the macular carotenoids. Similarly, increased risk associated with *ARMS2* through mitochondrial dysfunction in the photoreceptor outer segments may result in failure to regulate or compartmentalize ROIs generated in these organelles by the respiratory chain (Beatty et al., 2000), with a consequential depletion of local antioxidants, such as L and Z. Moreover, such a failure to compartmentalize mitochondrially-generated ROIs will promote inflammatory changes with subsequent further ROI production, thus representing a self-perpetuating cascade of ROI production and inflammatory processes. However, our observation of lower MP optical density centrally in association with homozygosity for the risk haplotypes of both *CFH* and *ARMS2* should be interpreted with caution, because of the large discrepancy in sample size between the groups being compared, and further investigation of these associations in a larger cohort is certainly warranted prior to drawing any meaningful conclusions regarding this association.

In conclusion, we did not observe any significant independent association between *CFH*, *ARMS2*, *C2*/*BF*, or *C3* genotype and MP optical density (or serum concentrations of its constituent carotenoids) in this study sample. Our finding of significantly lower MP optical density levels centrally in subjects homozygous for risk alleles at both *CFH* and *ARMS2* loci should be interpreted with caution, as there was a very large discrepancy between the subject numbers in each comparison group. Independent replication of these findings in future studies may suggest that accelerated depletion of the macular carotenoids occurs in the presence of unregulated inflammation and/or unregulated generation/compartmentalization of ROIs in subjects with this particular combination of risk alleles. However, independent replication of these findings in a larger study cohort is necessary prior to drawing any firm conclusions. We trust that these preliminary findings will inform further research into the gene/environment interaction that plays a role in the pathogenesis of this blinding disease. (Hughes et al., 2007; Kim et al., 2008; Seddon et al., 2006).

Disclosure

Edward Loane: None; Gareth J McKay: None; John M Nolan and Stephen Beatty do consultancy work for nutraceutical companies, in a personal capacity, and as directors of NutraSight Consultancy Limited.

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