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Can Genetic Associations Change with Age? *CFH* and Age-Related Macular Degeneration

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Abstract
Background:

Genetic variation in the gene encoding complement factor H (*CFH*) on chromosome 1q31 has repeatedly been associated with increased risk of Age Related Macular Degeneration (AMD); however previous studies have had inadequate numbers of participants across a sufficiently wide age range to determine whether the association varies by age.

Methods:

We conducted a genetic case-control study using data from 2,294 cases and 2,294 controls selected from the Melbourne Collaborative Cohort Study, matched on age, sex and region-of-origin. Four consistently replicated *CFH* single nucleotide polymorphisms (SNPs) were genotyped: rs1061170 (Y402H); rs2274700; rs393955 and rs800292; their relationship with AMD prevalence was determined across the age range 48–86 years.

Results:

A difference in genotype frequencies was seen across age groups, where the low risk homozygote prevalence rose with each increasing age group. Associations with early AMD were strongly modified by age for three of the four SNPs (interaction P-value 0.01 - 0.00003). An inverse association between the high risk homozygote for each SNP and early AMD was observed in the younger age groups (Odds Ratios (OR) range 0.37 – 0.48 for age < 55 years), reversing to a positive association with increasing age (OR 1.87 – 2.8 for age > 75 years).

Conclusions:

The direction of associations for this gene change from inverse to risk with increasing age. These findings have important implications for predictive models for AMD and potentially other age related diseases which extrapolate risks from older cohorts, as they assume homogeneity of association by age, which might not exist.

INTRODUCTION

Age-related macular degeneration (AMD) refers to pathological changes in the central area of the retina (1) and is the most important cause of irreversible visual loss in elderly populations of the developed world (2). In early AMD, clinical signs of retinal disease can be detected on routine examination but there are usually no symptoms. In late stage AMD, vision threatening complications of choroidal neovascularization (CNV) or atrophy develop which can lead to severe irreversible central vision loss (1). AMD is considered a complex genetic disease whereby environmental factors interact with a genetic predisposition to disease (3). Certain characteristics of AMD present challenges to attempts to unravel its genetic basis. Firstly, it occurs later in life, with a peak prevalence after the eighth decade, thus, only one generation in the affected age range is typically available for study, with parents usually deceased and children too young to have developed the disease(4). Secondly, AMD displays phenotypic heterogeneity where different genes may underlie the different phenotypes so that genetic studies may fail to identify any one causative gene or region(4). Despite these obstacles, a number of genetic variants have been associated with AMD. In fact, unlike other common chronic diseases, AMD is unusual in that several genes of large 'effect' have been associated with disease(5).

One of the most widely replicated genetic associations in AMD is for variants in the Complement factor H (*CFH*) gene located at chromosome 1q31. There are biologically plausible mechanisms for the involvement of *CFH* genetic variants in AMD; CFH acts as a regulator of activation of the alternative pathway of the complement cascade, a key component of immune defense(6). Abnormalities in the structure or function of complement pathway regulatory proteins can lead to an imbalance in normal homeostasis of the complement system, resulting in 'bystander' damage to healthy tissues(7).This phenomenon is thought to account for substantial tissue damage in a variety of diseases including AMD as well as Alzheimer's disease and atherosclerosis (7).

A variant in the *CFH* gene, known as the Y402H polymorphism (SNP) (rs1061170), was independently identified in 2005 by four separate research groups who reported that it was strongly associated with the odds of developing AMD(8-11). The reported Odds Ratios (ORs) ranged from 2.45 to 3.33 for early and late AMD combined, and from 3.45 to 7.4 for late, vision threatening AMD. Since the Y402H SNP discovery, several other genetic variants of the *CFH* gene have been associated with AMD (12, 13).

Many studies have demonstrated age and smoking to be the two major non-genetic risk factors for AMD. Most genetic studies adjust for these as confounders (14-19), but have had inadequate numbers of participants, across a sufficiently wide age range, to determine whether age modifies associations between genetic factors and AMD. In recent years, studies on longevity have identified a strong genetic component in the determination of lifespan (20, 21), suggesting that genetic differences should exist between younger and older cohorts due to differential survival, with an enrichment of 'longevity genes' in the elderly. These longevity genes are thought to modify susceptibility to adverse exposures – both environmental and genetic(20). We hypothesized that genetic associations in *CFH* may therefore differ with age. In our single, large case-control study, we have examined *CFH* genotype frequencies and associations between *CFH* genotype and AMD across six age-groups, to determine if age modifies these associations The four SNPs were chosen from the literature on their strength and reliability of reported associations(17), and that previously these SNPs have been observed to have the strongest associations with AMD in the Australian population.

RESULTS

Baseline demographics

There were 4,588 individuals, comprising 2,294 cases and 2,294 controls. Of the cases, 116 were classified as late AMD. For each SNP, the number of participants successfully genotyped were as follows: rs1061170 n= 4,026 (88%); rs2274700 n= 4183 (91%); rs393955 = 4423 (96%); rs800292 n= 4349 (95%). Failure rates of genotyping did not differ by age group. The allele frequencies for each SNP did not deviate significantly from Hardy-Weinberg equilibrium (*P*-values ranged from 0.1 to 0.3). Linkage disequilibrium (LD) plots show a D' (D prime) for all 4 SNPs >0.8 in 3 LD blocks with an r2 (r squared) no greater than 0.5 for any pair of SNPs.

At baseline, individuals participating in the MCCS follow up study and therefore eligible for selection for this nested case-control sample were less likely to be current smokers, less likely to be obese, and more likely to be of northern European descent than those who did not participate in the follow up study (22).

CFH Genotypes and Age

The genotype frequencies of each SNP varied by age group (Table 1) for the 2,294 controls. The low risk homozygotes were increasingly prevalent with increasing age, and the high risk homozygote prevalence decreased in the older age groups. This pattern was observed for all SNPs, except rs1061170, and reached statistical significance for rs2274700 (P-trend=0.002) and rs393955 (P-trend<0.001).

CFH and Age Related Macular Degeneration

Table 2 provides the results for early and late AMD. For each SNP, the high risk homozygote genotype had a positive and statistically significant association with early and late AMD, with the exception of rs800292 where a statistically significant association was only observed for late AMD. The ORs were of greater magnitude for late AMD, but the confidence intervals were wide, reflecting the small number of cases with late AMD (n=116).

There was strong evidence that age modified the associations between the SNPs rs1061170, rs2274700 and rs393955 and early AMD (Table 3). A pattern of changing associations with AMD with increasing age group was found for each SNP, where an inverse relationship (OR for high risk homozygotes ranged from 0.37 – 0.48 for those aged < 55 years) was observed in younger age groups, and a strong positive association was seen in the older age groups (OR for high risk homozygotes ranged from 1.87 – 2.80 for those aged >75 years) (Table 3). There were insufficient cases of late AMD to investigate effect modification by age group; furthermore 90% of cases of late AMD were aged over 70 years.

Sex and smoking status did not significantly modify any of the *CFH* genotypes & AMD associations (*P* values ranged from 0.08 to 0.9) (data not shown).

DISCUSSION

Associations between *CFH* genotype with early and late AMD were demonstrated for each SNP. However, associations with early AMD varied across age groups where inverse associations were observed in younger age groups and positive associations were seen in older age groups. *CFH* genotype frequencies varied with age.

We are unaware of any other studies that have reported changes in *CFH* genotype frequency across age groups, or effect modification by age on *CFH* genotype associations. This is the first study of sufficient size to examine effect modification by age, where the number of cases and controls in each stratum of age was comparable to - or exceeds - many complete individual studies on AMD(23). A meta-analysis conducted in 2006(23) of 8 studies pooling 3697 cases of early and late AMD and 2380 controls reported an apparent increasingly positive genetic association with age, as we have identified in this study; however in the meta-analysis age was divided into two groups only, those

with mean age < 74 and those with mean age ≥74 years. The authors suggested the apparent heterogeneity may represent variation of association with early- and late-stage AMD, as late AMD was much more prevalent in the older age group. In our analysis of early AMD alone, this change in association with age was apparent; indicating that any difference in association for early and late AMD is not the primary explanation.

Why genetic associations should change across age groups is not entirely clear; we provide some suggestions. One possible explanation is that these results are due to chance variation. As the pattern of association is apparent for all 4 SNPs, this seems unlikely. A further possibility is that the disease seen in younger age groups may be a different disease that closely resembles AMD, that is younger cases may be phenocopies of AMD observed in older people (a phenocopy is an individual marked as affected due to an identical phenotype, the underlying genetic markers associated with the phenocopy are different from the other cases in the dataset(24)), and hence the genetic associations would differ. Finally, a survivorship effect may cause the genetic background of the comparison group of controls to differ with increasing age. Survivorship is the logical error of concentrating on those that "survived" a process and inadvertently overlooking those that did not because of their lack of visibility. The survivorship effect is a combined effect of literal ‘survival’ and factors determining the likelihood of remaining in the study such as ill-health and mobility. In the current cohort from which the cases and controls were derived, those that would be controls - who were alive, well enough to attend an eye examination and had no visible signs of age-related change such as drusen or pigment change at the macular - decreased from ~55% in the younger age groups to ~40% in those above 75 years; therefore with increasing age, controls are selected from a diminishing pool of healthy individuals. Ageing studies indicate that the determination of human lifespan is complex, with a number of loci interacting to influence longevity(21). It has been demonstrated that in those individuals who survive into extreme age the prevalence of ‘risk genes’ for cancer and

cardiovascular disease is not reduced; rather it appears that enrichment of modifier or 'longevity' genes may protect individuals from the risk genes' adverse effects(20). These modifiers, although currently unknown, are the subject of intense focus in ageing research (20, 21). Studying a disease of the elderly such as AMD must take into account the possible genetic enrichment of such modifiers by selection of healthy agers, which may distort associations and will go unnoticed if sample sizes are too small to detect effect modification (25). Risk profiles for various diseases differ between younger and older populations, with a reversal of effect for some risk factors seen in the elderly (20, 26-28), resembling the reversal of effect observed in our study. A well known example of survivorship producing skewed results is the inverse association between smoking and Alzheimer's disease (29) observed in studies that failed to consider smoking-related mortality. We have previously reported on an inverse relationship between obesity and AMD in women, where a survivorship effect may have been responsible for a seemingly 'protective' association between obesity and AMD (22). Some evidence exists to suggest that *CFH* genotype may associate with mortality, complicating the relationship of CFH with age–related diseases (30-32).

AMD is a disease of late onset, with a peak prevalence occurring in the decades where considerable attrition in terms of study participation occurs due to ill health and mortality. The survivorship effect for AMD due to loss to follow up is greater for AMD than for cancer and mortality as no data linkage exists with registries of blindness or ophthalmic diagnosis as is the case with cancer and death registries, where outcomes are observed for all participants. AMD was determined at 9-11 years from baseline, and the AMD status of those who died, or did not return to follow up, could not be determined.

Although this is the first study to demonstrate a change in genetic associations across age groups in AMD, a similar pattern has been observed in one study of liver disease (33) and also in a large melanoma study (34). The authors point out that interaction with age may be missed if samples

consist of combined age groups(34); additionally there will be under or overestimates of effect size depending on the range of ages included in analyses.

Strengths of this study include its size - it is by far the largest single case-control study of AMD – and the reliability and validity of the classification of AMD status. Careful matching on age and matching on country of birth reduced confounding. An important limitation, though, is the drop out of 33% of participants (22% loss to follow-up and 11% died) in the cohort in which this study is nested; particularly the differential loss according to smoking(22).

These results have some important implications. Firstly, as it appears that *CFH* genotype frequencies change with increasing age, it seems imperative that case-control studies of AMD –and arguably all ageing diseases – ensure cases and controls have the same age distribution. Secondly, caution should be exercised in this 'age of personalized medicine' in producing models claiming to predict genetic risk of disease where the age of the target population differs from the age of the participants in the study from which the risk estimates were derived. Thirdly, the effect of survivorship on studies of elderly cohorts should not be dismissed lightly, when either environmental or genetic exposures are considered. The change in direction of associations raises the possibility that genetic associations may be modified by factors relating to the determination of lifespan, in a similar manner observed in studies of environmental exposures. We can only speculate whether the *CFH* gene is a true 'risk' gene for AMD, a modifier or longevity gene, or merely in linkage disequilibrium with such a modifier.

MATERIALS AND METHODS

Study sample

Cases and controls were selected from the Melbourne Collaborative Cohort Study (MCCS), a prospective cohort study of 41 514 people (35). Almost all (99.3%) participants were aged 40-69 years old at baseline (1990 to 1994), with approximately equal proportions of participants across each of the three age decades.

Photography to detect AMD was conducted during a MCCS follow-up study (2003-2007), 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) 49% (20,214) were not included in the final AMD risk factor analysis, leaving 21,287 participants with macular photographs graded for AMD(22).

For each AMD case identified from this sample and selected for this nested case-control study, one control was randomly selected after individual matching on age at photography (± 2 years), region of origin (northern Europe (United Kingdom and Ireland or Australia) and southern Europe (Greece and Italy)), and sex.

Comprehensive questionnaires regarding smoking, lifestyle and health conditions were completed at baseline and follow up visits. Blood samples for the genetic analysis were collected at the follow-up visit.

The study protocol was approved by the Human Research & Ethics Committees of The Cancer Council Victoria and the Royal Victorian Eye and Ear Hospital.

AMD detection and definition

Ascertainment of AMD status through fundus photography and quality control procedures have been described previously (22, 36). Early AMD was defined as the presence of drusen ≥ 125μm, with or without the presence of pigmentary abnormalities. Late AMD was defined as evidence of choroidal neovascularisation, geographic atrophy (an area of 175 μm of hypopigmentation with a choroidal vessel in its base) or a disciform scar (37). The participants were categorized on the status of their worst affected eye. Controls had bilateral normal fundi with no evidence of drusen of any size.

DNA Extraction and Genotyping

Genomic DNA was isolated from venous blood leukocytes as previously described (38). Genotyping was performed on the MassArray platform (SEQUENOM, San Diego, CA) (Murdoch Children's Research Institute, Melbourne, Australia); see Appendix 1. Randomly selected samples were independently sequenced to confirm genotypes. The genotyping primers used are provided in Supplementary Table 1.

The *CFH* SNPs genotyped were rs1061170 (Y402H); rs2274700; rs393955 and rs800292.

Statistical analysis

The allele frequency for each gene in controls was compared with those expected for a population in Hardy-Weinberg equilibrium. Chi-squared tests were used to compare genotype frequencies across the age groups (<55; 55-59; 60-64; 65-69;70-74 and >75 years). Unconditional logistic regression, adjusting for age group, sex, region of origin and smoking status (never, previous and current) was performed to estimate odds ratios (OR) for each genotype (heterozygote and homozygote) compared with the low risk (ancestral allele) homozygote as the reference group, separately for early AMD and

late AMD compared with no AMD. For the reference group, we used the ancestral allele as reported by the US National Center for Biotechnology Information in all our loci, even though for some it was not the most frequent allele, as previous studies have consistently done this(39). As these variants have previously been reported to be associated with increased risk of AMD, the genotypes homozygous for the risk allele are referred to as the high risk homozygote, and those with no risk alleles are referred to as the low risk homozygote. We also present associations per risk allele as a continuous variable (0-2) as this is common practice in genetic studies. Note, however, that for three of the variants the association with early AMD was observed to be nonlinear (P-values for likelihood ratio test comparing models for genotype and per allele: rs1061170 P=0.03, rs2274700 P=0.0002, rs393955 P=0.003, rs800292 P=0.4).

Additionally, ORs were compared to those produced by conditional logistic regression where each case is compared only to the individually matched control. The ORs were observed to be very similar; therefore, unconditional logistic regression was used for all analyses since all the controls are included in the estimation of the associations with both early and late AMD. The variables used for matching (age, sex and country of origin) were initially included as interaction terms; as the ORs were the same without these terms we have presented the simpler model.

Differences in genotype-AMD associations across age groups (<55; 55-59; 60-64; 65-69; 70-74 and >75 years), sex and smoking status (never, previous and current) were investigated by fitting interaction terms between each variable and genotype, and tested by the likelihood ratio test.

Linearity of the association between age and the log odds of AMD was assessed using the likelihood ratio test comparing models with age as a categorical variable with those with age as a pseudo-continuous variable. As the relationship was found to be non-linear, the categorical variable of age-groups was used.

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

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Conflict of interest: none declared.

MKMA had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legend

Figure 1 Odds Ratios Associated with CFH genotypes stratified by Age Group; Melbourne Collaborative Cohort Study 2003-2007

Table 1 Genotype Frequencies (%) in Controls, by Age Group; Melbourne Collaborative Cohort Study 2003-2007

	rs1061170				rs2274700				rs393955				rs800292			
Age group (years)	Low Risk homozygote TT	Heterozygote TC	High Risk Homozygote CC	Total	Low Risk homozygote TT	Heterozygote TC	High Risk Homozygote CC	Total	Low Risk homozygote TT	Heterozygote TG	High Risk Homozygote GG	Total	Low Risk homozygote TT	Heterozygote TC	High Risk Homozygote CC	Total
<55	31 (34.8)	42 (47.2)	16 (18.0)	89	10 (10.6)	48 (51.1)	36 (38.3)	94	28 (26.2)	57 (53.3)	22 (20.6)	107	2 (2.0)	33 (32.7)	66 (65.4)	101
55-59	84 (36.1)	105 (45.1)	44 (18.9)	233	41 (15.9)	113 (43.8)	104 (40.3)	258	88 (30.7)	137 (47.7)	62 (21.6)	287	12 (4.4)	89 (33.0)	169 (62.6)	270
60-64	86 (38.7)	96 (43.2)	40 (18.0)	222	40 (17.1)	99 (42.3)	95 (40.6)	234	90 (34.8)	118 (45.6)	51 (19.7)	259	18 (7.2)	89 (35.5)	144 (57.4)	251
65-69	83 (39.0)	94 (44.1)	36 (16.9)	213	36 (16.8)	114 (53.3)	64 (29.9)	214	90 (38.8)	112 (48.3)	30 (12.9)	232	11 (5.0)	81 (36.5)	130 (58.6)	222
70-74	218 (33.1)	323 (49.0)	118 (17.9)	659	104 (15.5)	349 (51.9)	220 (32.7)	673	232 (33.1)	374 (53.4)	95 (13.6)	701	27 (3.9)	243 (34.7)	430 (61.4)	700
>75	217 (36.4)	280 (47.0)	99 (16.6)	596	114 (18.4)	322 (51.9)	184 (29.7)	620	236 (37.6)	312 (49.8)	79 (12.6)	627	49 (7.7)	207 (32.5)	381 (59.8)	637
Total	719 (35.7)	940 (46.7)	353 (17.5)	2012	345 (16.5)	1045 (49.9)	703 (33.6)	2093	764 (34.5)	1110 (50.2)	339 (15.3)	2213	119 (5.5)	742 (34.0)	1320 (60.5)	2181
P-value				0.9				0.02				0.002				0.105
P-trend				0.9				0.002				<0.001				0.24

P-value from chi-squared test with 10 degrees of freedom
P-trend – P-value from non-parametric test for trend

Table 2 Odds Ratios for Early and Late AMD Associated with CFH genotypes; Melbourne Collaborative Cohort Study 2003-2007

		Early AMD				Late AMD			
SNP	Genotype	OR	95% CI		*P*-value	OR	95% CI		*P*-value
rs1061170	TT	ref			0.0001	ref			0.003
	TC	1.07	0.93	1.23		1.27	0.77	2.10	
	CC	1.47	1.23	1.75		2.37	1.40	4.01	
	per C allele	1.18	1.08	1.29	<0.0001	1.85	1.40	2.45	<0.0001
rs2274700	TT	ref			<0.0001	ref			0.001
	TC	0.89	0.75	1.06		2.02	0.90	4.51	
	CC	1.28	1.06	1.53		3.39	1.53	7.51	
	per T allele	1.18	1.08	1.29	<0.0001	2.25	1.64	3.10	<0.0001
rs393955	TT	ref			<0.0001	ref			0.01
	TG	1.06	0.93	1.21		1.50	0.91	2.47	
	GG	1.58	1.33	1.88		2.36	1.36	4.11	
	per G allele	1.22	1.12	1.33	<0.0001	2.02	1.51	2.71	<0.0001
rs800292	TT	ref			0.1	ref			0.007
	TC	1.02	0.77	1.35		1.33	0.39	4.49	
	CC	1.16	0.88	1.53		2.52	0.79	8.10	
	per T allele	1.11	1.00	1.22	0.05	2.22	1.47	3.35	<0.0001

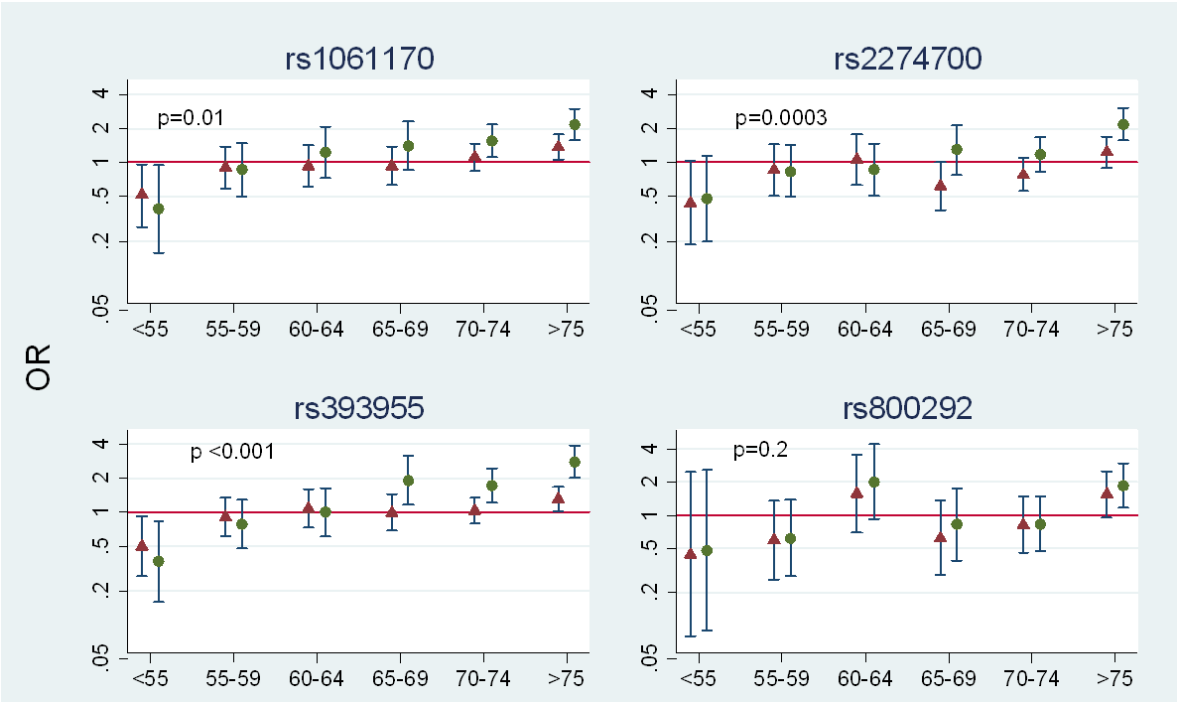
OR = odds ratio estimate from multivariable logistic regression, adjusting for age group, smoking status, grouped country of origin, sex
95% CI = 95% confidence interval
P-value from likelihood ratio test comparing multivariable logistic regression models with and without each SNP.

Table 3 Odds Ratios for Early AMD Associated with CFH genotypes stratified by Age Group; Melbourne Collaborative Cohort Study 2003-2007

		<55 years				55-59 years				60-64 years				65-69 years				70-74 years				>75 years				
SNP		OR	95% CI		*P*-value	OR	95% CI		*P*-value	OR	95% CI		*P*-value	OR	95% CI		*P*-value	OR	95% CI		*P*-value	OR	95% CI		*P*-value	Age interaction p-value*
rs1061170	TT	ref			0.04	ref			0.8	ref			0.6	ref			0.2	ref			0.03	ref			<0.0001	0.01
	TC	0.52	0.27	0.97		0.91	0.59	1.38		0.93	0.61	1.42		0.93	0.63	1.38		1.12	0.85	1.47		1.37	1.06	1.77		
	CC	0.39	0.16	0.95		0.87	0.50	1.50		1.23	0.73	2.07		1.41	0.86	2.30		1.56	1.11	2.18		2.19	1.59	3.00		
rs2274700	TT	ref			0.2	ref			0.8	ref			0.6	ref			0.0007	ref			0.007	ref			<0.0001	0.0003
	TC	0.44	0.19	1.04		0.86	0.51	1.45		1.06	0.63	1.78		0.62	0.38	1.01		0.78	0.56	1.10		1.24	0.90	1.71		
	CC	0.48	0.20	1.15		0.84	0.50	1.43		0.87	0.51	1.47		1.30	0.78	2.16		1.18	0.83	1.67		2.19	1.57	3.06		
rs393955	TT	ref			0.02	ref			0.6	ref			0.9	ref			0.02	ref			0.004	ref			<0.0001	<0.0001
	TG	0.50	0.27	0.92		0.90	0.61	1.34		1.08	0.73	1.59		0.99	0.69	1.44		1.03	0.80	1.34		1.30	1.02	1.66		
	GG	0.37	0.16	0.83		0.79	0.48	1.29		1.00	0.61	1.62		1.92	1.16	3.19		1.72	1.22	2.44		2.80	2.02	3.89		
rs800292	TT	ref			0.6	ref			0.5	ref			0.1	ref			0.2	ref			0.8	ref			0.02	0.2
	TC	0.44	0.08	2.46		0.60	0.26	1.36		1.58	0.70	3.55		0.62	0.29	1.36		0.82	0.46	1.49		1.55	0.95	2.52		
	CC	0.48	0.09	2.59		0.62	0.28	1.38		2.01	0.92	4.43		0.83	0.39	1.76		0.84	0.47	1.49		1.87	1.17	2.98		

OR = odds ratio estimate from multivariable logistic regression, adjusting for grouped country of origin, sex and smoking status
95% CI = 95% confidence interval
P-value from likelihood ratio test comparing multivariable logistic regression models with and without each SNP. Both models adjusted for age, sex, country of birth
P-value* from likelihood ratio test comparing multivariable logistic regression models with and without interaction terms between age group and CFH genotype.

Figure 1 Odds Ratios Associated with CFH genotypes stratified by Age Group; Melbourne Collaborative Cohort Study 2003-2007



Circle – High risk homozygote, 95% CI
Triangle – High risk heterozygote, 95% CI
P-value* from likelihood ratio test comparing multivariable logistic regression models with and without interaction terms between age group and CFH genotype