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***Chlamydia pneumoniae* infection, complement factor H variants and age-related macular degeneration**

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Abstract

Background/aims—Impaired inhibition of the alternative complement pathway by complement factor H (CFH) is linked to age-related macular degeneration (AMD) based on the strong association between *CFH* variant and AMD. *Chlamydia pneumoniae* (*C pneumoniae*) infection can trigger the alternative pathway, but the evidence for an association between *C pneumoniae* and AMD is contradictory. This study investigated whether *C pneumoniae* infection is associated with AMD and whether the presence of *C pneumoniae* modulates AMD risk conferred by *CFH* variants.

Methods—Genomic DNA extracted from peripheral blood of 148 advanced AMD patients and 162 controls was subjected to Taqman and PCR-RFLP for the *CFH* polymorphism and PCR for the *C pneumoniae* gene. Genomic DNA was also examined from microdissected macular cells from 59 AMD and 16 age-matched non-AMD archived slides. χ^2 testing was performed for case-control analysis.

Results—*C pneumoniae* infection was associated with increased risk of AMD (OR = 2.17, $p < 0.017$). A *CFH* variant was also linked to increased risk of AMD (OR = 1.98, $p < 0.0001$). However, no relationship was found between risk-conferring *CFH* variant and *C pneumoniae* (OR = 1.81, $p = 0.08$).

Conclusion—There is a possible association between AMD and *C pneumoniae* infection, although CFH may not be directly involved in the pathogenesis of *C pneumoniae* infection-mediated AMD.

Age-related macular degeneration (AMD) is the leading cause of blindness among older people in the world.¹ The prevalence of AMD increases dramatically with age, resulting in significant medical, social and economic costs to patients and a considerable burden on society. Its prevalence is expected to increase with the overall ageing of the population.

While the pathophysiological basis of AMD remains to be elucidated, the current paradigm supports a role for environmental triggers amidst a genetically predisposed backdrop. Indeed,

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studies at several loci have suggested a strong genetic component to AMD.^{2,3} The most commonly documented genetic association is between the *complement factor H (CFH)* Y402H polymorphism (SNP) and AMD,^{4–7} and meta-analyses have demonstrated a significant role for the *CFH* Y402H SNP in Caucasian AMD patients at the population level.^{8–10} *CFH* is an inhibitor of the alternative complement pathway, and functional studies of the Y402H *CFH* SNP suggest that it possesses impaired complement inhibitory activities. These studies are in accordance with the proposed role in AMD of inflammation in general and complement overactivity in particular, both of which can lead to tissue damage if not properly controlled.¹¹

Chronic *Chlamydia pneumoniae (C pneumoniae)* infection has been linked to AMD in a limited number of small case-control studies.^{12–14} Serum anti-*C pneumoniae* antibodies are reported to be higher in AMD patients compared with controls, and an increased titre of serum antibody against *C. pneumoniae* has been linked to rapid progression of AMD.¹⁴ Using immunohistochemistry and polymerase chain reaction (PCR) techniques, Kalayoglu *et al* have demonstrated the presence of *C pneumoniae* in AMD neovascular membranes.¹⁵ In contrast, two studies by Robman *et al* and Kessler *et al* demonstrated no significant association between *C pneumoniae* and AMD or AMD CNV, respectively.^{16,17} The conflicting data regarding the role for *C pneumoniae* in AMD pathogenesis warrant further studies to evaluate the link, if any, between chronic *C pneumoniae* infection and AMD risk.

C pneumoniae activates the alternative complement pathway or induces a chronic inflammatory state, which might contribute to the pathogenesis of AMD.^{11,18} Furthermore, it is possible that in patients with the risk-conferring *CFH* variant, *C pneumoniae* infection represents the trigger for the alternative pathway, which thereby runs uninhibited due to impaired *CFH* activity in these patients. In this study, we investigate whether *C pneumoniae* infection is associated with AMD and whether there is any relationship among chronic *C pneumoniae* infection, the *CFH* SNP, and risk of AMD using our previously reported cohort of individuals with and without AMD.^{19–23}

MATERIALS AND METHODS

Subjects

The description of the eligibility criteria and recruitment of participants into this study has been previously described.^{21,23} In brief, all participants were recruited from a National Eye Institute (NEI) cohort, which was approved by NEI Institutional Review Boards, and each participant signed the informed consent at enrolment. All participants were Caucasians residing in the greater Washington, DC area. Only patients with advanced AMD and controls with normal retina were recruited. Clinical diagnosis of advanced AMD was based upon fundus photographs graded as geographic atrophy involving the centre of the fovea and/or choroid neovascularisation associated with large drusen in at least one eye. The controls have no or few small drusen. A total of 148 patients with advanced AMD and 162 non-AMD controls were enrolled. Their peripheral blood cells were collected for genomic DNA extraction using a QIAamp DNA Blood Maxi kit (Qiagen, Valencia, California).

Archived paraffin-embedded ocular slides from 59 pathologically diagnosed advanced geographic or neovascular AMD and 16 age-matched cases with normal retina were also examined. The macular cells on these slides were manually microdissected, and their genomic DNA was extracted as described previously.^{21,24,25}

Analysis of CFH SNP genotyping

The SNP genotyping was performed by Taqman SNP Genotyping Assay (Applied Biosystems, Foster City, California), assay ID# C_2530286_20 for *CFH* intron (rs380390).

Detection of *C pneumoniae* DNA

DNA isolated from peripheral blood cells was subjected to PCR amplification of the *C pneumoniae* 16S rRNA gene using with a primer pair of CPN90/CPN91, for which the sequences are 5'-GGT CTC AAC CCC ATC CGT GTC GG-3' and 5'-TGC GGA AAG CTG TAT TTC TAC AGT T-3'.²⁶ DNA isolated from microdissected cells was first incubated with 20 μ l of TE buffer containing 0.5 mg/ml proteinase K at 37°C for overnight. After incubation at 90°C, 5 μ l of DNA solution was subjected to PCR with ³²P-labelled primer pair of CPN90/CPN91. A total volume of 25 μ l containing 10 ng of blood DNA or 5 μ l of DNA from microdissection, 1X PCR buffer, 0.4 mM dNTP, 2.0 mM MgCl₂ and 2 units of AmpliTag Gold (Applied Biosystems) was amplified for 7 min at 95°C, followed by 60 cycles of denaturation at 94°C for 45 s, annealing beginning at 65°C and ending at 52°C for 45 s, and extension at 72°C for 1 min. The annealing temperature was lowered by 1°C every four cycles until it reached 52°C, and this annealing temperature was maintained until the end of the cycling process. The 197 bp products of the PCR amplification were subjected for polyacrylamide gel electrophoresis and visualised by ethidium bromide staining or autoradiography.

Statistical analysis

Data for *C pneumoniae* status, *CFH* alleles, and AMD versus control status were entered into contingency tables using the GraphPad Prism software (GraphPad Software, San Diego, California). χ^2 analyses were performed to test for differences in these categorical variables between two or more groups. Specifically, χ^2 tables were used to assess for associations between *CFH* variants and AMD in all patients and in patients with the *C pneumoniae* risk factor, *C pneumoniae* infection and AMD in all patients and in patients with the risk-conferring *CFH* allele. Statistical significance was established when $p < 0.05$ for all analyses.

Odds ratios and relative risk for AMD were also calculated for the presence of one risk factor (risk-conferring *CFH* allele or *C pneumoniae* positivity) and for the presence of both risk factors.

RESULTS

The demographic information from the two data samples (peripheral blood and archived slides) is summarised in table 1.

CFH intron SNP was closely associated with AMD risk

The *CFH* intron G/C SNP (rs380390) and *CFH*-Y402H are in complete linkage disequilibrium (LD), and both the C allele at rs380390 and the Y402H SNPs have been shown to be significantly associated with AMD.⁴²¹ Our study similarly found an association between the C allele at rs380390 and risk of AMD. Among the 148 AMD patients, the numbers of G/G, G/C and C/C allele were 51, 53 and 44, respectively. Among the 162 control subjects, the numbers of G/G, G/C and C/C allele were 76, 70 and 16, respectively. χ^2 analysis demonstrated a statistically significant association between presence of the C allele at this position and risk of AMD ($p < 0.0001$).

C pneumoniae infection associated with increased risk of AMD

C pneumoniae DNA was identified in the peripheral blood and macular cells of both advanced AMD patients and controls using PCR and PAGE (fig 1). As delineated in table 2, 20.3% of

AMD patients exhibited chronic *C pneumoniae* infection, while only 10.5% of controls did, suggesting an association between *C pneumoniae* infection and AMD risk ($p < 0.017$).

However, no difference was found in the archived samples, which most likely resulted from the small sample size and sample material, as well as a relatively low incidence of *C pneumoniae* infection in this population (table 2).

CFH variant was not involved in the pathogenesis of *C pneumoniae*-associated AMD

χ^2 analysis indicated that the presence of *C pneumoniae* infection did not affect the risk of AMD in patients with the risk-conferring *CFH* variant. Similarly, the *CFH* SNP did not affect the risk of AMD in patients with chronic *C pneumoniae* infection (table 3). Interestingly, a higher frequency of *C pneumoniae* infection (positive *C pneumoniae* DNA) seemed to relate to the risk-conferring *CFH* variant (C allele) in the dry AMD patients. Since this study only included 35 dry AMD patients, the data would have even less impact.

The presence of the C allele of *CFH* (risk-variant) led to a relative risk (RR) = 1.411 (95% CI 1.202 to 1.658) and odds ratio (OR) = 1.980 (95% CI 1.427 to 2.746) of AMD, which represented a statistically significant association ($p < 0.0001$). The presence of chronic *C pneumoniae* infection led to an RR = 1.423 (95% CI 1.189 to 1.702) and OR = 2.168 (95% CI 1.376 to 3.416), which was also significant ($p = 0.0008$). In contrast, the presence of both factors (*C pneumoniae* infection and C allele of *CFH*) did not increase the RR or OR over that seen with one factor alone, with RR = 1.314 (95% CI 1.023 to 1.688) and OR = 1.813 (0.967 to 3.399) ($p = 0.0840$).

DISCUSSION

AMD development and progression have been associated with increasing antibody titre to *C pneumoniae*.¹⁴ Moreover, CNV specimens from AMD patients have been reported to be significantly more likely to show evidence of *C pneumoniae* by immunohistochemistry, when compared with non-AMD CNV and non-AMD eyes.¹⁵ Macrophages are shown to produce the proangiogenic vascular endothelial growth factor (VEGF) after infection with *C pneumoniae*, providing one mechanism by which *C pneumoniae* infection may predispose to CNV development. In contrast to these reports of positive associations between AMD and *C pneumoniae* infection, there are also negative findings in the literature.^{16 17}

This study confirms the potential association between AMD and *C pneumoniae* infection, with a significantly higher incidence of the *C pneumoniae*'s genetic signature in blood from 148 AMD patients than in blood from 162 control patients. Our finding of *C pneumoniae* DNA in macular cells from the pathological slides also suggests a possible role for *C pneumoniae* in AMD.

C pneumoniae is an obligate intracellular pathogen responsible for 6–20% of cases of community-acquired pneumonia.²⁷ Most infections are mild or asymptomatic. The micro-organism is disseminated through blood circulation and has a particular attraction for vascular tissues, where it may result in chronic infection.²⁸ Circulating *C pneumoniae* elementary bodies and/or *C pneumoniae* reticulate bodies in macrophages and other cells in the AMD lesions serve as infective triggers for AMD.²⁹ Recently *C pneumoniae* has been posited as a potentially important factor for several systemic diseases such as asthma,³⁰ multiple sclerosis³¹ and cardiovascular diseases.³²

As a microbe, *Chlamydia* can also activate the alternative pathway of complement, which is strongly implicated in AMD pathogenesis.^{45 1833 34} Indeed, chronic inflammation from *C pneumoniae* might be one of the triggers that activates the alternative complement pathway

and ultimately leads to complement overactivity in patients with the risk variant of *CFH*, which possesses impaired complement inhibitory function. If so, we would expect that the presence of both the risk-conferring variant of *CFH* and *C pneumoniae* positivity would result in an increased risk of AMD, as compared with the presence of either the risk-conferring *CFH* variant or *C pneumoniae* positivity alone. Our findings demonstrate that individually each factor—*CFH* risk allele and *C pneumoniae* gene—is associated with AMD risk, but that the presence of the second factor does not significantly increase this risk. However, it is possible that such gene–gene interaction may not be detected in a study of relatively small sample size in our study population.

The link between *C pneumoniae* and AMD is still controversial, which may be due to difficulties in verifying laboratory techniques for the detection of the micro-organism in the chronic infection stage.^{35,36} Our data resulting from a relatively small study certainly support a positive association between chronic *C pneumoniae* infection and AMD. Our study fails to find any additive or a combined effect of *C pneumoniae* and the risk-conferring *CFH* variants on AMD. While the association between *C pneumoniae* and AMD may not be directly linked to an impaired complement alternative pathway, it may be mediated by other inflammatory mechanisms such as macrophages, inflammatory cytokines and/or chemokines. For example, it is possible that macrophages infected with *C pneumoniae* may upregulate adhesion molecules, promote recruitment of inflammatory cellular migration through the blood–brain/retina barrier and/or facilitate homing of inflammatory cells into the macula.³⁷ Further investigations are warranted to parse out the underlying mechanisms by which *C pneumoniae* might promote AMD development and progression.

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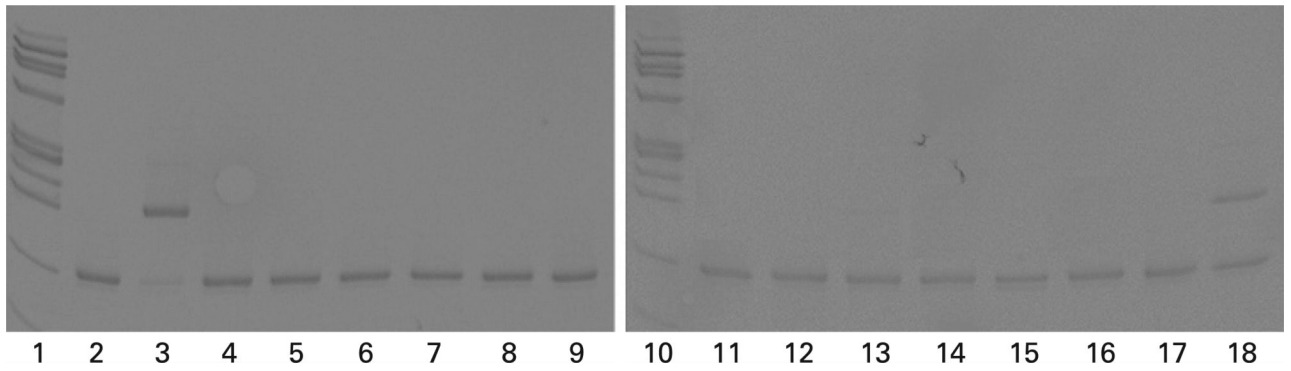


Figure 1. Amplification of *Chlamydia pneumoniae* in peripheral blood DNA. Lanes 1 and 10, ϕ x 174 DNA/*Hae III* fragments. Lanes 2–9, DNA from AMD patients. Lanes 11–18, DNA from non-AMD controls.

Table 1

Demographic data of the participants

Group	NEI cohort		Archived slide	
	AMD	Control	AMD	Control
No	148	162	59	16
Age, mean (SD)	79 (8)	66 (11)	83 (9)	72 (8)
Female	78	92	25	5
Male	70	70	17	7
Unknown gender	0	0	17	4

AMD, age-related macular degeneration; NEI, National Eye Institute.

Table 2Detection of *Chlamydia pneumoniae* DNA signature in the participants

Specimen	Age-related macular degeneration		Control	
	Total cases	<i>C pneu</i> ⁺ (%)	Total cases	<i>C pneu</i> ⁺ (%)
Archived eye DNA	59	2 (3.3%)	16	1 (6.2%)
Blood DNA	148	30 (20.3%)	162	17 (10.5%)

C pneu⁺, positive *C pneumoniae* DNA.

Table 3
Distribution of complement factor H SNP and *C pneumoniae* DNA among the participants

Complement factor H intron	AMD						Control			
	Wet	<i>C pneum</i> ⁺ in wet	Dry	<i>C pneum</i> ⁺ in dry	W or D unknown	<i>C pneum</i> ⁺ in unknown	Total	<i>C pneum</i> ⁺ in total	Total	<i>C pneum</i> ⁺
G/G	38	8 (21.0%)	10	3 (30.0%)	3	1	51	12 (23.5%)	76	3 (4.0%)
G/C	32	4 (12.5%)	14	5 (35.7%)	7	0	53	9 (17.0%)	70	11 (15.7%)
C/C	28	5 (17.9%)	11	4 (36.4%)	5	0	44	9 (20.5%)	16	3 (18.8%)
Total	98	17 (17.3%)	35	12 (34.2%)	15	1	148	30 (20.3%)	162	17 (10.5%)

AMD, age-related macular degeneration; *C pneum*⁺, positive *C pneumoniae* DNA; Dry, dry AMD; Unknown, unknown AMD subtype; Wet, wet AMD.