Genomic Copy Number Variations of the Complement Component *C4B* Gene Are Associated With Chronic Central Serous Chorioretinopathy

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Submitted: May 26, 2015
Accepted: July 23, 2015

Citation: Breukink MB, Schellevis RL, Boon CJF, et al. Genomic copy number variations of the complement component *C4B* gene are associated with chronic central serous chorioretinopathy. *Invest Ophthalmol Vis Sci*. 2015;56:5608–5613. DOI:10.1167/iovs.15-17343

Purpose. Chronic central serous chorioretinopathy (cCSC) has recently been associated to variants in the complement factor H gene. To further investigate the role of the complement system in cCSC, the genomic copy number variations in the complement component 4 gene (*C4*) were studied.

Methods. *C4A* and *C4B* copy numbers were analyzed in 197 cCSC patients and 303 healthy controls by using a Taqman copy number determination assay. Copy numbers of *C4A*, *C4B*, and the total *C4* load were compared between cases and controls, by using a Fisher exact test. For this analysis Bonferroni correction was performed for three tests, and P values < 0.017 were considered to be significant. A logistic regression model was constructed to calculate the odds ratios (ORs) of each of the *C4B* copy numbers, using two copies as a reference. For this model P values < 0.05 were considered to be significant.

Results. *C4B* genomic copy numbers differed significantly between cCSC patients and healthy controls ($P = 0.0018$). Absence of *C4B* significantly conferred risk of cCSC ($P = 0.039$, OR = 2.61 [95% confidence interval (CI) = 1.05–6.52]), whereas three copies of *C4B* significantly decreased the risk of cCSC ($P = 0.014$, OR = 0.45 [95% CI = 0.23–0.85]). The *C4A* genomic copy numbers and total *C4* load did not significantly differ between cases and controls.

Conclusions. This study showed that copy numbers of *C4B* are significantly associated with cCSC. Carrying no copies of *C4B* significantly increases the risk of cCSC, whereas carrying three *C4B* copies is protective. These findings reinforce the hypothesis of a possible involvement of the complement system in the pathogenesis of cCSC.

Keywords: chronic central serous chorioretinopathy, cCSC, complement component 4, *C4*, *C4A*, *C4B*

Chronic central serous chorioretinopathy (cCSC) is characterized by fluid accumulation under the neuroretina. It has been postulated that this serous fluid derives from the choroid and that it leaks through a dysfunctional retinal pigment epithelium, causing a detachment of the neuroretina.[1–4] Classically, cCSC patients are relatively young (middle-aged) men who are still professionally active.[5] Besides male sex, also the use of corticosteroids, type A personality, and stress have been associated with cCSC.[4–6] Although the exact pathophysiological mechanism of the disease remains unknown, we and others have previously suggested involvement of the complement system, and in particular the complement factor H gene (*CFH*) in cCSC.[7,8] Interestingly, *CFH* variants that confer increased risk in cCSC have previously been described to be protective in age-related macular degeneration, and vice versa.[8–10]

The complement system consists of three major pathways: the classical, the lectin, and the alternative pathway.[11] CFH is involved in the alternative pathway and can influence C3b production by blocking one of the two C3-convertases (C3bBb). The classical and lectin pathways also play an important role in C3b production mediated by the other C3-convertase (C4b2a), of which the complement component 4 (C4) protein is a key factor.[12]

Copy number variations of the *C4* gene have been associated with several eye diseases and autoimmune disorders (e.g., Vogt-Koyanagi-Harada and Behçet's disease).[13–15] In addition, Banlaki et al.[16] have found that the genomic copy number of *C4B* is associated with cortisol release after adrenocorticotropic hormone (ACTH) stimulation. This is of particular interest considering that stress, which appears to be associated with cCSC,[4] has a strong influence on the hypothalamic–pituitary–adrenal (HPA) axis and increases ACTH release.[17]

Because of its role in the complement system, and its association with the HPA axis, we hypothesized that copy number variation in the *C4* gene may be associated with cCSC. In this study we assessed the copy number variations of the *C4* gene in a cCSC cohort.

Table 1. Demographics of the Study Population

	cCSC Patients	Controls	*P* Value
No. of subjects	197	303	NA
Sex, male/female	154/43	226/77	0.392
Age, mean ± SD, y	53 ± 10	53 ± 11	0.755
Age range, y	29–74	29–77	NA

NA, not annotated; SD, standard deviation.

Materials and Methods

Subjects

In this study, 197 patients diagnosed with cCSC who visited the outpatient clinic of the Department of Ophthalmology at the Radboud University Medical Center, Nijmegen, the Netherlands, were included (Table 1). The diagnosis cCSC was based on an extensive ophthalmologic examination including fundoscopy, spectral-domain optical coherence tomography, fluorescein angiography, and indocyanine green angiography. The definition of typical cCSC used in this study was based on the previously published subgroups by de Jong et al.,[8] and patients in this study underwent phenotyping by an experienced retina specialist (CJFB) (Fig. 1A–F). Additionally, a total of 303 control subjects were recruited from the blood bank of the Radboud University Medical Center ($n = 154$) and the European Genetic Database (EUGENDA, www.eugenda.org; provided in the public domain by the University Hospital of Cologne, Cologne, Germany and the Radboud University Medical Center) ($n = 149$) (Table 1). For this last group, fundus photographs were graded to rule out any ophthalmologic abnormalities at the moment of inclusion. Informed consent for the use of DNA for genetic studies was obtained from all subjects. This study followed the guidelines of the Declaration of Helsinki and was approved by the local ethics committee.

Copy Number Determination

DNA was isolated from peripheral blood by using standard procedures. *C4A* and *C4B* copy numbers were determined by real-time PCR using Taqman genotyping assays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The FAM-labeled *C4A* (Hs07226349_cn) or *C4B* (Hs07226350_cn) Taqman copy number assay was combined with the VIC-labeled Ribonuclease P (*RNaseP*) reference assay (catalog No. 4403326), and Taqman genotyping mastermix (catalog No. 4381656). All samples were tested in duplicate for *C4A* and *C4B* on 384-wells plates by using 10 ng DNA in a total reaction volume of 10 μL.

Samples with known copy numbers for either *C4A* (0–4) or *C4B* (0–3) were kindly provided by C. Yung Yu.[18] These samples were included as a reference on each plate to facilitate accurate copy number determination, using the method described previously.[18] In each run the amplification efficiencies of the *C4A*/*C4B* and *RNaseP* probes were calculated by using a serial dilution (50–1.56 ng) of a sample with two *C4A* and *C4B* copies. The primer efficiencies of the probes were compared and deemed similar if they differed <2%; this was the case in all runs. Therefore, the efficiencies were not incorporated into the calculations of the copy numbers. Polymerase chain reaction was performed with a 7900HT thermocycler (Applied Biosystems, Thermo Fisher Scientific) using the following program: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Data were analyzed with the Copycaller software (V2.0; Applied Biosystems, Thermo Fisher Scientific). Copy numbers determined by the Copycaller software were corrected by using the trend line based on the reference samples, as described before.[18] If the results were inconsistent between the Copycaller output and the values corrected with the trend line, samples were retested on a new plate in triplicate.

Statistics

The comparison of *C4A*, *C4B*, and total *C4* copy number distribution between cCSC patients and controls was performed by means of a Fisher exact test using SPSS Statistics (V20; IBM Corp., Armonk, NY, USA). Bonferroni correction for multiple testing was performed for three tests and P values < 0.017 were considered to be statistically significant. A logistic regression model was constructed to determine the odds ratios (ORs) for the various copy numbers of *C4B*. According to previously published studies, two genomic copy numbers of *C4B* are considered to be most common in the healthy population.[15,19] We were able to confirm this in our cohort, and therefore this copy number was set as reference. In this model, P values < 0.05 were considered to be significant. Graphs were generated by using Graphpad Prism (V5; Graphpad Software, San Diego, CA, USA).

Results

The copy numbers of *C4A* and *C4B* were successfully determined in 197 cCSC cases and 303 controls. No significant difference was observed between cases and controls for the *C4A* genomic copy number (range: 0–6, $P = 0.649$; Fig. 2A).

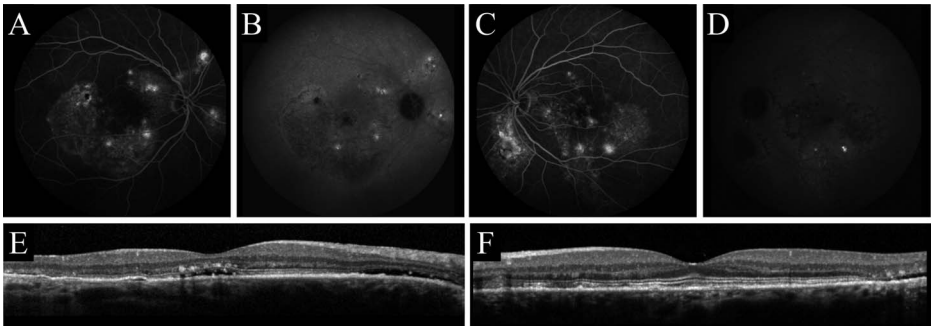

Figure 1. Example of FA of an RE (**A**) and an LE (**C**), ICG angiography of an RE (**B**) and an LE (**D**), and spectral-domain optical coherence tomography of an RE (**E**) and an LE (**F**) imaging of the phenotypic characteristics of the cCSC cohort used for this study. (**A–F**) The RE and LE of a patient demonstrate diffuse hyperfluorescent areas of leakage on FA and ICG angiography (**A–D**) and subretinal fluid beneath the fovea (**E**), illustrative for typical cCSC. FA, fluorescein angiography; ICG, indocyanine green; LE, left eye; RE, right eye.

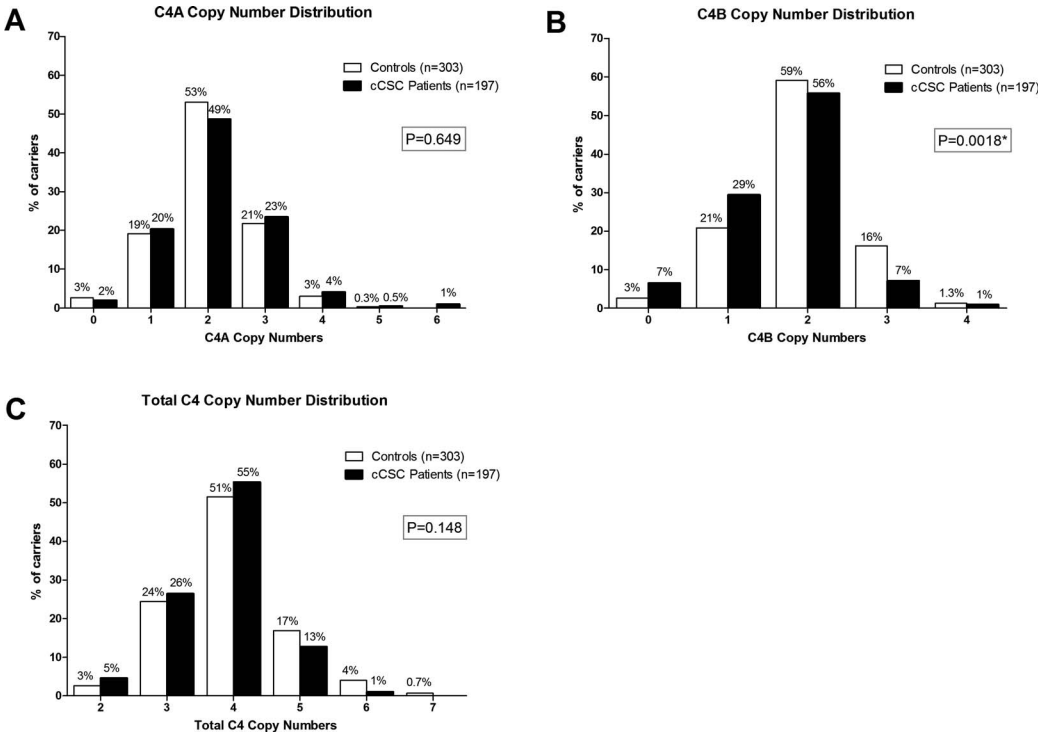

Figure 2. Distribution of the genomic copy numbers of *C4A* (**A**), *C4B* (**B**), and total *C4* (**C**) in patients with cCSC and controls. Displayed *P* values were generated with a Fisher exact test. To correct for multiple testing, *P* values < 0.017 were considered to be significant.

The *C4B* distribution was significantly different between cCSC patients and controls (range: 0–4, $P = 0.0018$; Fig. 2B). Overall, cases carried lower copy numbers of *C4B* than the control population. The total *C4* genomic copy number was not different in cases compared to controls ($P = 0.148$; Fig. 2C). Age and sex were not associated with *C4A*, *C4B*, or total *C4* genomic copy number (Table 2; data for *C4A* and total *C4* not shown).

To assess the effect size of the different copy numbers of *C4B* on development of cCSC, a logistic regression was performed (Table 2). The logistic regression model based on the distribution of *C4B* between cases and controls was significant ($P = 0.0035$; Table 2). Carrying no copies of *C4B* conferred increased risk of cCSC ($P = 0.039$, OR = 2.61, 95% confidence interval [CI] = 1.05–6.52). A similar trend was observed for carriers of one copy of *C4B*, but the results were not significant ($P = 0.080$, OR = 1.47, 95% CI = 0.96–2.26). Carrying three *C4B* copies was associated with a significantly decreased risk of cCSC ($P = 0.014$, OR = 0.45, 95% CI = 0.24–0.85), whereas no significant association with cCSC was observed in individuals carrying four copies of *C4B* ($P = 0.81$).

Discussion

Our study results demonstrated that cCSC patients have a significantly different *C4B* load as compared to healthy controls ($P = 0.0018$). Carrying no copies of *C4B* was associated with an increased risk of cCSC (OR = 2.61, 95% CI = 1.05–6.52), whereas carrying three *C4B* copies was associated with a decreased risk of cCSC (OR = 0.45, 95% CI = 0.23–0.85). No association with cCSC was observed in individuals carrying four *C4B* copies, which is likely due to the limited sample size of this group (cases, $n = 4$; controls, $n = 2$). No significant differences were observed between cases and controls for *C4A* and total *C4* load.

The *C4* gene lies within the *RP-C4-CYP21-TNX* (*RCCX*) locus located in the major histocompatability complex (MHC) region III on chromosome 6 of the human genome.[19] The MHC region contains an elevated level of genomic copy number variations that are presumably present to increase immunologic diversity.[20] Duplications and deletions in the region have led to the formation of haplotypes containing variable copies of the *RCCX* locus in the human population (Fig. 3B).[20–22] Haplotypes containing two or more duplications of the *RCCX* locus show extensive variability in their gene content, generally with complete duplications of the *C4* gene (Fig. 3B).[21,23] The *C4* gene encodes the C4 protein, of which two variants have been described (C4A/C4B), differing in only four amino acids encoded by exon 26 (Fig. 3A).[24]

Copy number variations of either *C4A* or *C4B* have been associated with several systemic diseases with ocular involvement, such as Vogt-Koyanagi-Harada disease, Behçet's disease, and systemic lupus erythematosus (SLE).[13–15] Hou et al.[13] have shown that a lower copy number of *C4A* and *C4B* increases the risk of Vogt-Koyanagi-Harada disease, an autoimmune disorder characterized by bilateral granulomatous panuveitis. The same group[14] has also demonstrated that higher copy numbers of *C4A* confer risk of Behçet's disease, an autoinflammatory disease, which presents with acute anterior uveitis. Several studies[15,25,26] have shown an association between low copy numbers of *C4* and an increased risk for SLE, an autoimmune disease that is typically mediated by immune complexes. In the past, CSC has been described in SLE patients,[27,28] but it remains unclear whether this is a primary manifestation of SLE, or whether it is a consequence of corticosteroid treatment for SLE.[29,30] Several studies have reported a positive linear correlation between serum C4 and *C4* genomic copy number,[31–33] suggesting that the lower number of *C4B* copies in cCSC patients leads to lower systemic C4B levels. This may indicate that an overall lower activity of the complement system might be present in cCSC patients.

Table 2. Logistic Regression Model for *C4B* Load

Overall Significance Model, $P = 0.0035$					
***C4B* Copy Number**	**cCSC Patients, *n***	**Controls, *n***	***P* Value**	**OR**	**95% CI**
Age	197	303	0.605	NA	NA
Sex	197	303	0.346	NA	NA
0	13	8	0.039	2.613	1.048–6.518
1	58	63	0.080	1.469	0.956–2.259
2	110	179	Ref	1	NA
3	14	49	0.014	0.445	0.234–0.849
4	2	4	0.808	0.809	0.145–4.503

Ref, reference.

Recently, low copy numbers of *C4B* have been associated with hyperreactivity of the HPA axis.[16] Banlaki et al.[16] have shown that in patients with adrenal incidentaloma and low (<2 copies) *C4B* genomic copy number, baseline ACTH is significantly reduced as compared to high (≥2 copies) genomic copy number of *C4B*. Moreover, a significantly higher cortisol response is observed after ACTH stimulation in the patients with low *C4B* genomic copy number.[16]

These results are of interest in the context of cCSC because of the described clinical associations with stress and the use of corticosteroids that both exert physiological effects at the level of the HPA axis.[17] Various relatively small studies have studied cortisol levels in cCSC patients. Although 24-hour urine samples show elevated cortisol levels in cCSC patients in certain studies,[34,35] these results are not observed in single serum measurements during set times in other studies.[36,37] These discrepancies could be explained by variable cortisol fluctuations between individuals during the day, and therefore changes in endogenous cortisol levels cannot be ruled out as a hallmark of cCSC. It is possible that patients with cCSC generally have normal cortisol levels but respond differently to stimulation of the HPA axis. Stress, which also appears to be associated with cCSC,[2,38] stimulates the HPA axis and could lead to temporarily elevated cortisol levels in patients as compared to healthy individuals. How high levels of cortisol can lead to subretinal fluid accumulation is currently unknown. A study in rats suggests that the disease mechanism could be mediated by binding of corticosteroids to the mineralocorticoid receptor.[39] In this study, activation of the mineralocorticoid receptor causes vascular effects similar to those observed in cCSC,[39] but the underlying pathways still remain to be elucidated.

The mechanism through which low copy numbers of *C4B* may lead to hyperresponsiveness of the HPA axis is unclear. It has been hypothesized that it is not the *C4B* gene, but rather the neighboring *CYP21A2* gene, that mediates this effect.[16] The *CYP21A2* gene encodes the enzyme 21-hydroxylase, which plays an important role in the steroid metabolism pathway by converting progesterone and 17-α-hydroxyprogesterone to 11-deoxycorticosterone and 11-deoxycortisol, respectively. Because of the genomic structure of the *RCCX* locus, variation in the *CYP21A2* gene is in high linkage disequilibrium with variation in the neighboring *C4* gene (*C4A* or *C4B*).[16] Therefore, further exploration of the precise structure and specific variations present in the *RCCX* locus may reveal new insights into the pathogenesis of cCSC.

The current study and previous studies identified an association between complement genes and cCSC,[7,8] suggesting that the complement system may be dysregulated in cCSC. Taken together, these findings may indicate that the immune system, influenced by environmental factors such as stress, could play a pivotal role in the pathophysiology of cCSC. Further studies are necessary to determine the physiological effects of genetic variation at the *C4* gene and the *RCCX* locus in cCSC.

Acknowledgments

We thank C. Yung Yu, DPhil, for providing the positive control samples (Center for Molecular and Human Genetics, The Research Institute, City, Ohio, USA). We thank Joannes M. M. Groenewoud

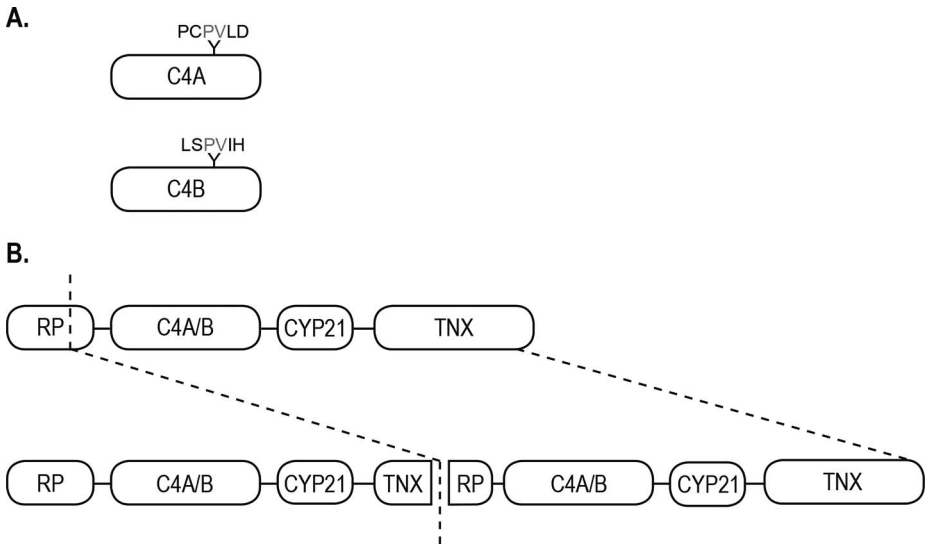

Figure 3. A schematic illustration of the *RCCX* locus. (**A**) A display of the two variants of the *C4* gene, differing in four amino acids in exon 26 (p.1101–1106). (**B**) An example of the structure of the *RCCX* locus with a single copy (*upper panel*) and two copies (*lower panel*) of the *C4* gene. In case of duplication, a complete *C4A* or *C4B* gene and the *CYP21* gene are duplicated, while the *RP* and *TNX* genes are only partially duplicated. The *dotted lines* indicate the approximate site where during recombination the duplication has occurred in the past (Figure based on Banlaki et al. 2013).[22]

(MSc) for his statistical support (Department of Health Evidence, Radboudumc, Nijmegen, the Netherlands).

Supported by the Macula Vision Research Foundation, MD Fonds, Landelijke Stichting voor Blinden en Slechtzienden, Gelderse Blindenstichting, Stichting Nederlands Oogheelkundig Onderzoek, Stichting Blindenhulp, Stichting A.F. Deutman Oogheelkunde Researchfonds, Nijmeegse Oogonderzoek Stichting, Janivo Stichting, Retina Netherlands, Stichting Blinden Penning, Oogfonds, Gisela Thier Fellowship of Leiden University, and the Radboud Institute for Molecular Life Sciences. The authors alone are responsible for the content and writing of the paper.

Disclosure: **M.B. Breukink**, None; **R.L. Schellevis**, None; **C.J.F. Boon**, None; **S. Fauser**, None; **C.B. Hoyng**, None; **A.I. den Hollander**, None; **E.K. de Jong**, None

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