RESEARCH ARTICLE

Human Mutation

OFFICIAL JOURNAL
HGVS
HUMAN GENOME VARIATION SOCIETY
www.hgvs.org

Cadherin 5 is Regulated by Corticosteroids and Associated with Central Serous Chorioretinopathy

Carl Schubert,[1†] Anders Pryds,[2†] Shemin Zeng,[3†] Yajing Xie,[1] K. Bailey Freund,[4] Richard F. Spaide,[4] John C. Merriam,[1] Irene Barbazetto,[4] Jason S. Slakter,[4] Stanley Chang,[1] Inger C. Munch,[2] Arlene V. Drack,[3] Jasmine Hernandez,[3] Suzanne Yzer,[1] Joanna E. Merriam,[1] Allan Linneberg,[5] Michael Larsen,[2] Lawrence A. Yannuzzi,[4] Robert F. Mullins,[3*] and Rando Allikmets[1,6*]

[1]Department of Ophthalmology, Columbia University, New York City, New York; [2]Department of Ophthalmology, Glostrup Hospital, University of Copenhagen, Copenhagen, Denmark; [3]Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa City, Iowa; [4]Vitreous-Retina-Macula Consultants of New York, New York City, New York; [5]Research Centre for Prevention and Health, the Capital Region of Denmark, Copenhagen, Denmark; [6]Department of Pathology & Cell Biology, Columbia University, New York City, New York

Communicated by Andreas Gal.

Received 5 November 2013; accepted revised manuscript 10 March 2014.
Published online 24 March 2014 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22551

ABSTRACT: Central serous chorioretinopathy (CSC) is characterized by leakage of fluid from the choroid into the subretinal space and, consequently, loss of central vision. The disease is triggered by endogenous and exogenous corticosteroid imbalance and psychosocial stress and is much more prevalent in men. We studied the association of genetic variation in 44 genes from stress response and corticosteroid metabolism pathways with the CSC phenotype in two independent cohorts of 400 CSC cases and 1,400 matched controls. The expression of *cadherin 5* (*CDH5*), the major cell–cell adhesion molecule in vascular endothelium, was downregulated by corticosteroids which may increase permeability of choroidal vasculature, leading to fluid leakage under the retina. We found a significant association of four common *CDH5* SNPs with CSC in male patients in both cohorts. Two common intronic variants, rs7499886:A>G and rs1073584:C>T, exhibit strongly significant associations with CSC; $P = 0.00012$; odds ratio (OR) = 1.5; 95%CI [1.2;1.8], and $P = 0.0014$; OR = 0.70; 95%CI [0.57;0.87], respectively. A common haplotype was present in 25.4% male CSC cases and in 35.8% controls ($P = 0.0002$; OR = 0.61, 95% CI [0.47–0.79]). We propose that genetically predetermined variation in *CDH5*, when combined with triggering events such as corticosteroid treatment or severe hormonal imbalance, underlie a substantial proportion of CSC in the male population.

Hum Mutat 35:859–867, 2014. Published 2014 Wiley Periodicals, Inc.*

KEY WORDS: *CDH5*; Cadherin 5; central serous chorioretinopathy; genetic association; retinal disease

Introduction

Central serous chorioretinopathy (CSC, MedGen 147591) is a disorder of the choroid and the retina characterized by serous detachment of the neurosensory retina and focal angiographic leakage at the level of the retinal pigment epithelium (RPE) secondary to choroidal vascular exudation [Spaide et al., 1996; Wang et al., 2008]. The most common symptoms are a central relative scotoma, metamorphopsia, reduced contrast sensitivity, and reduced color saturation. The majority of patients with CSC have a favorable prognosis but some patients may experience significant vision loss because of degenerative RPE changes and photoreceptor atrophy after recurrent or persistent serous retinal detachment [Wang et al., 2002; Piccolino et al., 2005]. The disorder presents in acute and chronic forms. Acute CSC cases often resolve spontaneously in 3–4 months [Yannuzzi, 2010]. Cases that progress to chronic CSC have recurrent or persistent detachments for 6 months or longer [Gass, 1977; Yannuzzi et al., 1992].

It is currently believed that CSC is primarily an exudative choroidal vasculopathy since indocyanine-green (ICG) angiography has revealed choroidal vascular hyperpermeability and staining of choroidal vessels [Spaide et al., 1996; Yannuzzi et al., 2003; Pryds et al., 2010]. This is supported by enhanced depth-imaging optical coherence tomography (EDI-OCT) having demonstrated increased thickness of the choroid in patients with CSC and reduction of choroidal thickness after photodynamic therapy [Imamura et al., 2009; Pryds and Larsen, 2012]. Although CSC is considered a vascular disorder, the primary exudative disturbance resulting in a macular detachment is thought to be nonvasogenic, that is, not associated with proliferation of choroidal vessels, which distinguishes CSC from other forms of macular detachment associated with neovascularization of the choroid and eventual disciform

Additional Supporting Information may be found in the online version of this article.

†These authors contributed equally to this study and should be considered as co-first authors.

*Correspondence to: Rober F. Mullins, Department of Ophthalmology and Visual Sciences, Carver College of Medicine, University of Iowa, Iowa City, IA. E-mail: robert-mullins@uiowa.edu; Rando Allikmets, Department of Ophthalmology, Columbia University, Eye Institute Research Annex, Rm. 202, 630 West 168th Street, New York City, NY 10032. E-mail: rla22@columbia.edu

Contract grant sponsors: Macula Vision Research Foundation; NIH/NEI EY017451; NIH/NEI EY013435; Foundation Fighting Blindness; Øjenforeningen; Danish Eye Research Foundation; Danish Eye Health Society; the Velux Foundation; the Macula Foundation.

© 2014 The Authors. *Human Mutation published by Wiley Periodicals, Inc.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

scarring, such as in CNV in AMD [Yannuzzi, 1986; Spaide et al., 2008]. The mechanism whereby choroidal hyperpermeability becomes symptomatic involves RPE detachment and detachment of the neurosensory retina, which is presumably caused by increased hydrostatic pressure in the choroid.

The main risk factors for CSC are either endogenous or exogenous corticosteroid imbalance, [Garg et al., 1997; Carvalho-Recchia et al., 2002; Yannuzzi, 2010] type A personality, episodes of psychosocial stress, and pregnancy, especially with complications such as (pre)eclampsia [Tittl et al., 1999; Carvalho-Recchia et al., 2002]. Other, more minor risk factors include hypertension, elevated plasma testosterone levels and infections (e.g., *H. pylori*) [Mauget-Faysse et al., 2002; Ahnoux-Zabsonre et al., 2004].

The prevalence of CSC (suggested at 1:10,000) is highest in men and has a peak incidence at 47.5 years of age [Tittl et al., 1999; Haimovici et al., 2004; Wang et al., 2005]. The female to male gender ratio is reported to be from 1:3 to 1:4. Familial cases of CSC are very rare, but the existence of familial cases may very likely be obscured by misdiagnosis of age-related macular degeneration (AMD) in many instances, especially in chronic CSC. The few reports suggesting genetic predisposition to CSC include a description of fundus lesions compatible with CSC in 35 out of 80 relatives of chronic CSC patients [Weenink et al., 2001]. Another study reported that two to four siblings were affected with CSC in each of the families of 10 CSC patients, including a parent and offspring pair. The chronic course of the disease was found in 80% of all eyes presenting with CSC [Oosterhuis, 1996]. CSC has been also reported in monozygotic twins [Wyman, 1963]. The mode of inheritance remains obscure.

Although the disease seems to be caused largely by external, environmental factors, this study tested the hypothesis that the existence of familial cases suggests a genetic component directing the response to the external stimuli, such as stress and hormonal imbalance by influencing choroidal endothelial permeability.

Materials and Methods

Ethics Statement

Informed consent was obtained from all patients and controls prior to enrollment and the study followed the guidelines of the Declaration of Helsinki. The study was approved by the institutional review boards at all centers (protocol numbers IRB-AAAA4242, H-D-2008-046, and M05.07.034).

All mouse experiments were performed in accordance with the ARVO resolution for use of laboratory animals and with approval of the University of Iowa Animal Care Committee.

Study Cohorts

Patients with acute and chronic CSC were enrolled for the genetic study in the period from September 2008 to June 2011. Study patients and ethnically matched controls were recruited prospectively at three large referral centers, at the Vitreous, Macula and Retina Consultants and at Columbia University in New York City, New York, United States and at the Glostrup Hospital in Copenhagen, Denmark. The 145 US patients and matched controls served as the primary study cohort, whereas the 254 Danish patients and matched controls served as the replication cohort. The mean age in the Danish cohort was 54.1 years (range 17–84 years) and the mean age in the US cohort was 48.6 years (range 18–79 years). Of the 254 Danish patients, 180 patients (71%) were men, whereas 110 (76%) of the 145 US patients were men. The ethnicity of the US cohort was predominantly European American with mostly Eastern European descent, whereas the Danish cohort included predominantly (94%) Danes and Northern Europeans.

Study procedures included determination of best-corrected visual acuity (BCVA, Snellen), slit-lamp biomicroscopy examination, indirect funduscopy, fundus autofluorescence imaging, OCT, and fluorescein angiography (FA). EDI-OCT was used from 2009. ICG angiography was performed when FA was inconclusive. The diagnosis of CSC was made according to guidelines described in a recent review. [Wang et al., 2008] The study enrolled both patients with acute CSC and patients with chronic CSC of any duration, during active and inactive phases of the disease without consideration of prior treatment or treatment response. Patients with choroidal neovascularization secondary to CSC were included if there was no sign of other eye disease, such as drusen in AMD. Exclusion criteria included the presence of retinal disease other than CSC.

Control groups consisted of ethnically matched individuals who had eye exams to document normal macular findings upon funduscopy or color fundus photography and who had no history of any retinal disease. The US control cohort included 368 subjects (mean age 68.8, range 52–75 years, 168 (46%) males), who had also been involved in our previous studies of AMD [Gold et al., 2006]. The Danish cohort consisted of 171 individuals (mean age 55.7 years, range 23–92 years, 84 (49%) males). In addition, 1,010 individuals of Danish descent from the Inter99 study [Jorgensen et al., 2003; Munch et al., 2010], who received eye exams were included in the study after an eye exam.

Human Donor Eyes

Human donor eyes were obtained from the Iowa Lions Eye Bank (Iowa City, IA) following consent of the donors' families. All experiments conformed to the Declaration of Helsinki. For some experiments, immunofluorescence was performed as described previously [Mullins et al., 2007] using an anti-vascular endothelial (VE) cadherin antibody (Chemicion, Temecula, CA).

Sequencing and Genotyping

DNA was isolated from whole blood by column purification (Qiagen DNA Blood Maxi 51194). SNP genotyping was performed by TaqMan assay (Applied Biosystems) in the two cohorts of CSC patients and unaffected controls to determine allele frequencies in these cohorts. Population frequencies were also determined, for reference, from dbSNP, 1000 Genomes, and the Inter99 study. Genotyping of SNPs in the Inter99 population was performed by KBiosciences using the PCR KASPar genotyping system. (KBiosciences, Hoddesdon, UK, www.kbioscience.co.uk) Call-rates for all SNPs were above 98%. Replicates were run for 350 individuals and the replication rate was 100%.

The coding and flanking intronic sequences of the *CDH5* gene (MIM #601120) were analyzed by direct Sanger sequencing in 96 patients from the New York cohort. Primer sequences and PCR conditions are available upon request. Sequencing was carried out by Genewiz (Genewiz, www.genewiz.com). Allele and genotype frequencies were compared between cases and controls with standard statistical tests, such as 2×2 table and Fisher's exact test.

Cell Culture

The C166 endothelial cell line (ATCC, Rockville, MD) was cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and 1% penicillin—streptomycin (Sigma–Aldrich, St. Louis, MO).

Cells were grown to confluency and then treated with medium only or with prednisolone (Sigma–Aldrich; 100–300 ng/ml). Following 6 hr of exposure, total RNA was isolated using a commercial kit (Qiagen RNeasy kit, Valencia, CA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA as described previously [Skeie et al., 2010]. Quantification of specific mRNA was performed by SYBR Green real-time PCR using an ABI Prim 7700 Sequence Detection System (Applied Biosystems) [Mullins et al., 2006]. Triplicate reactions were performed for each sample. For some experiments, testosterone (Sigma–Aldrich; 100 and 10 ng/ml) was included to assess the role of a corticosteroid without known impact on CSC in humans.

Mouse Injections

All animal experiments were performed in accordance with the ARVO resolution for use of laboratory animals and with approval of the University of Iowa Animal Care Committee. Mice (129SVEV, Jackson Laboratories) were anesthetized using ketamine/xylazine mix (0.1 ml/20 g of weight at a concentration of 17.5 mg/ml of ketamine and 2.5 mg/ml of xylazine) and were injected into the subretinal space under an operating microscope with either 3 μl balanced saline solution or 3 μl triamcinolone (40 mg/ml, Kenalog-40) a corticosteroid reported to exacerbate CSC in humans [Kocabora et al., 2008]. The injection technique involved proptosing the eye, then making a conjunctival peritomy, followed by a scleral puncture using a 30 g needle posterior to the limbus. A blunt tip 33 g needle on a Hamilton syringe was then threaded into the subretinal space and the injection was delivered. As injection itself may affect gene expression levels in the choroid, steroid injected eyes were compared with the saline injected eyes from different animals. The visual presence of a retinal bleb was used to verify the subretinal location of the injection. A total of five mice were injected with saline and five were injected with triamcinolone. Ages ranged from 5 months to 5.5 months. Male (n = 5) and female (n = 5) mice were divided between steroid and control injection groups.

Twenty-four hours after the injections, mice were sacrificed by CO_2 asphyxiation followed by cervical dislocation, eyes were removed, and the neural retina was removed from the posterior pole. The remaining posterior pole containing RPE, choroid, and sclera was used for RNA isolation and cDNA synthesis as described above.

Organ Cultures

Human organ cultures of RPE/choroid were utilized as described previously [Skeie et al., 2010]. Briefly, extramacular 4 mm punches of RPE and choroid were collected from eyes from two donors and were placed in modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. Punches were collected in a paired fashion with pairs at an equivalent distance from the macula. Half of the cultures were also exposed to 100 ng/ml prednisolone. Cultures were incubated for 20 hr at 37°C in 5% CO_2, and were then either snap frozen in liquid nitrogen or fixed in one half strength Karnovsky fixative. Frozen samples were used for RNA isolation and cDNA synthesis as described above for C166 cells.

Ultrastructural Analysis

Organ cultures were postfixed in osmium tetraoxide, dehydrated, and embedded in Spurr's resin as described previously [Mullins et al., 2007]. Ultrathin sections were collected on an ultramicrotome onto formvar coated grids and viewed on a transmission electron microscope. Qualitative assessment was performed on intercellular junctions of the RPE and the choroid. A total of 188 endothelial cell junctions from six pairs of cultures were evaluated. For quantitative experiments, the length of overlap of junctional complexes was measured in a masked fashion in images of EC junctions in the choroid using the broken line tool in ImageJ [Abramoff et al., 2004]. Pixels were converted to nanometers using an internal 500 nm scale bar in the images. Statistical significance was determined using a two-tailed t-test.

Analysis of *CDH5* Expression

The levels of mRNA for the VE-cadherin genes were assessed using quantitative PCR [Mullins et al., 2006]. Primer sequences and PCR conditions are available upon request. Levels were normalized using the CRALBP gene (*RLBP1*) as a reference gene for human RPE-choroid organ cultures, *Icam2* as a reference gene for mouse eyes, or *Rpl19* for mouse cultured endothelial cells. Fold change was determined using the ddCT method as described previously [Mullins et al., 2006] and using a commercial software package (DataAssist^{TM} v2.0.software; Applied Biosystems). Significance was determined with a cutoff P value of 0.05 using the Student's t-test.

Results

Defining and Screening of Candidate Genes for CSC

Candidate genes for CSC were selected based on their function from three main pathways implicated in CSC: stress response, steroid metabolism, and choroidal/endothelial permeability (Supp. Table S1). Some genes implicated in hypertension, pre-eclampsia, and infection response/inflammation were also analyzed as these pathways are also sometimes associated with CSC. Common variants (SNPs) in these genes were selected based on haplotype tagging (htSNPs) and in some cases previous results from association studies were also taken into account in SNP selection for genotyping. In total, 82 SNPs from 44 candidate genes were screened (Supp. Table S1). Initial screening was performed on the entire New York (US) cohort of cases and controls; SNPs with suggestive association were followed up in the replication cohort from Copenhagen (Denmark). SNPs from four genes, *HSP90*, *CFH*, *MAPK1*, and *CDH5* were significant in the joint analysis in the two cohorts. Of these, association of one SNP each from *HSP90* and *MAPK1*, and two SNPs from *CFH* genes were marginally significant ($P\sim0.005$–0.02) in one of the two cohorts and in the joint analysis, which could indicate a clinically insignificant finding (Supp. Table S2). However, four out of 10 SNPs from the *CDH5* gene were independently statistically significantly associated with the CSC phenotype in both cohorts (Table 1) and strongly associated in the male subgroup in the joint analysis including data from both cohorts (Table 2).

Variants in *CDH5* are Associated with CSC

Cadherin 5 (CDH5, also known as VE-cadherin) is located in a six-cadherin cluster on the long arm of chromosome 16. It is composed of 12 exons, of which 11 are coding, over a 37 kb genomic locus.

To determine whether common variants of the *CDH5* gene are associated with CSC, a selection of 10 SNPs were screened in a cohort of 148 CSC patients and 368 disease-free, ethnically matched, controls from New York. Nine of ten SNPs were located in

Table 1. Association of SNPs in the *CDH5* Gene with CSC in all Cohorts

	Minor allele frequency number of cases							Comparisons		
	Columbia		Danish		Combined			Columbia	Danish	Combined
	CSC	Control	CSC	Control	CSC	Control		CSC and controls	CSC and controls	CSC and controls
rs10852432:C>T	47.9	53.2	51.0	53.5	49.9	53.4	OR	0.81	0.90	0.87
N	144	348	246	1055	390	1403	*P* value	0.13	0.32	0.08
rs2344564:C>T	29.7	27.0	28.2	20.2	28.8	24.8	OR	1.14	1.55	1.22
N	145	358	248	168	393	526	*P* value	0.39	0.009	0.06
rs7499886:C>G	50.7	43.8	45.9	40.4	47.7	41.2	OR	1.32	1.25	1.30
N	144	360	245	1050	389	1410	*P* value	0.046	0.025	0.001
rs1130844:C>T	42.4	49.4	42.4	48.3	42.4	48.6	OR	0.75	0.79	0.78
N	144	362	243	1049	387	1411	*P* value	0.04	0.018	0.002
rs1073584:T>C	25.9	33.0	25.8	30.5	25.8	31.1	OR	0.71	0.79	0.77
N	145	361	246	1060	391	1421	*P* value	0.027	0.04	0.004

Table 2. Association of *CDH5* SNPs with CSC in Male Cohorts

	Minor allele frequency number of cases							Comparisons		
	Columbia		Danish		Combined			Columbia	Danish	Combined
	CSC	Control	CSC	Control	CSC	Control		CSC and controls	CSC and controls	CSC and controls
rs10852432:C>T	48.2	56.4	50.0	53.7	49.3	53.8	OR	0.72	0.83	0.83
N	109	157	172	538	281	695	*P* value	0.062	0.13	0.037
rs2344564:C>T	29.1	24.1	28.7	18.3	28.9	22.1	OR	1.30	1.80	1.43
N	110	158	174	82	284	240	*P* value	0.19	0.011	0.012
rs7499886:C>G	50.9	37.4	46.8	40.1	48.4	39.5	OR	1.73	1.35	1.47
N	109	163	171	537	280	700	*P* value	0.0018	0.014	0.00012
rs1130844:C>T	44.5	51.8	40.6	49.1	42.1	49.7	OR	0.74	0.74	0.78
N	109	163	171	536	280	699	*P* value	0.09	0.0066	0.0024
rs1073584:T>C	27.3	37.7	24.9	31.2	25.8	32.7	OR	0.62	0.71	0.70
N	110	162	173	539	283	701	*P* value	0.012	0.013	0.0014

non-coding regions (in promoter, and introns 1, 5, and 7) and one was a c.384 C>T, synonymous variant (rs1130844:C>T; Fig. 1, Tables 1 and 2, and Supp. Table S3).

Statistically significant associations were found with four individual, mostly haplotype-tagging (ht), SNPs including rs7499886:A>G (χ^2 = 5.3, P = 0.046) and rs1073584:C>T (χ^2 = 5.6, P = 0.027) (Table 1). Interestingly, by dividing the cohort by gender it appeared that the association was driven almost exclusively by the genotype frequencies in males (Table 2 and Supp. Table S4). For all other variants, after dividing the data by gender, the association in the larger (110 out of 148) male group became statistically stronger; the strongest association with CSC in the male cohort was observed with a variant in intron 1, rs7499886:A>G (χ^2 = 9.7, P = 0.0018), resulting in an odds ratio (OR) of 1.73 (95% confidence interval (CI) [1.23;2.46]).

These results were confirmed in an independent cohort of CSC patients (n = 254) and documented CSC-free matched controls (n = 171) obtained at the University of Copenhagen, Denmark (Tables 1 and 2). To increase the power of statistical analyses, data from more Danish population-based controls, derived from the Inter99 study [Jorgensen et al., 2003; Munch et al., 2010] were added to the study for variants where genotype frequencies were available. The Inter99 study included 6108 Danes, of whom 1,010 (513 men, 497 women) were clinically confirmed to be free of CSC, diabetic retinopathy and macular dystrophy. The allele frequencies did not differ significantly between disease-free and general population controls; however, we included in this study only those controls who were confirmed disease-free after ophthalmic examination, that is, 513 men and 497 women of Danish descent.

The same variants were also associated with CSC in the Danish male cohort; rs7499886:A>G (χ^2 = 5.3, P = 0.014) and rs1073584:C>T (χ^2 = 5.6, P = 0.013) (Table 2). The two sets of data corroborated each other in that most of the genotyped SNPs were not only statistically significantly associated with CSC but the allele/genotype frequencies and the extent and direction of association were very similar in the two cohorts (Tables 1 and 2, and Supp. Tables S3 and S4). Consequently, the joint analysis of the two cohorts resulted in highly significant association for most analyzed variants with CSC in males, even after correction for multiple testing (0.05/10 = 0.005). Variants from intron 1 (rs7499886:A>G) and intron 7 (rs1073584:C>T) were associated with CSC; P = 0.00012; OR = 1.47; 95% CI [1.2;1.8], and P = 0.0014; OR = 0.70; 95% CI [0.57;0.87], respectively (Table 2).

These associations were highly significant when the entire CSC cohorts were compared with the controls. No statistically significant differences were observed ($P > 0.05$ for all comparisons) between the acute and chronic forms of CSC; however, this observation needs to be confirmed in larger cohorts since separation by the form of the disease rendered some subgroups very small for enough statistical power and thereby prone to chance findings. In addition, defining acute and chronic forms is not always unequivocal; acute patients, especially of younger age, can develop chronic form of the disease with time.

Sequencing of the *CDH5* Gene

To determine if rare, highly penetrant, variants in *CDH5* could be associated with CSC, we sequenced the entire ORF including 11 exons and flanking intronic sequences in 96 CSC patients from the NY cohort. Sequencing revealed some known and some new common variants but identified rare heterozygous amino acid-changing variants in only three out of 96 patients. These included missense variants c.343G>A, p.E115K, c.896G>A, p.R299Q, and c.2018C>T, p.P673L, the latter two of which have been seen very rarely in the Exome Variant Sequencing project. Altogether, direct sequencing of

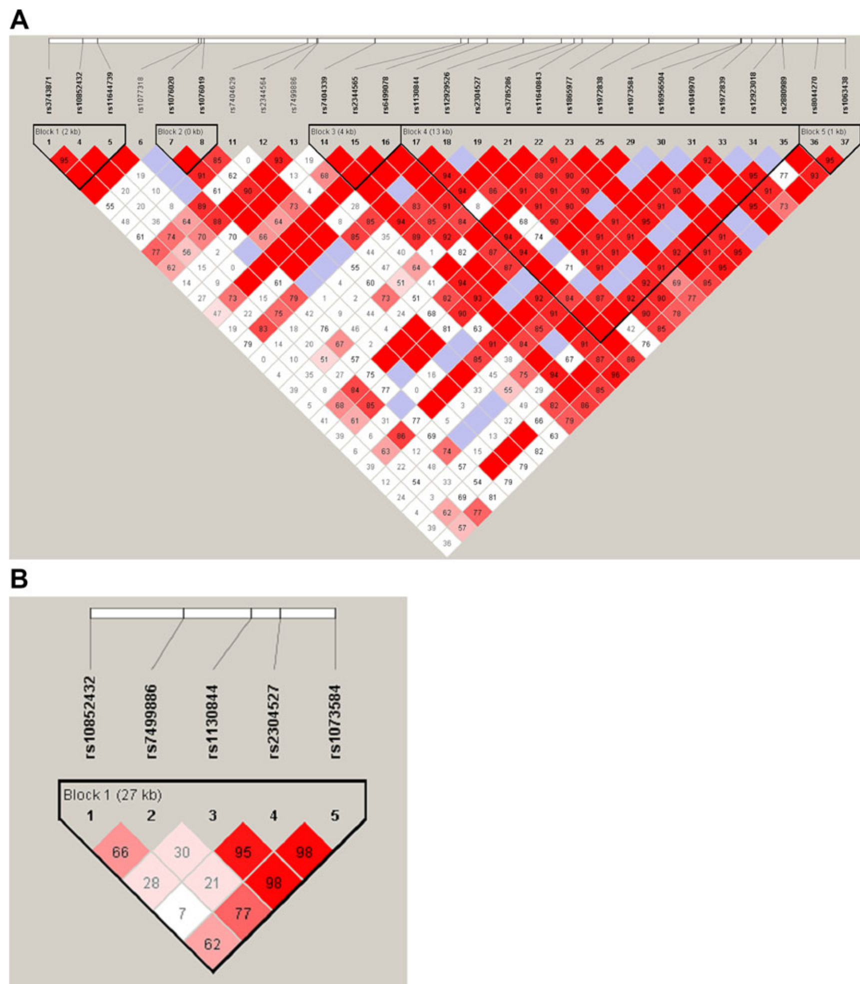

Figure 1. Haplotype structure of the *CDH5* locus. **A**: Linkage disequilibrium and haplotype structure of the entire *CDH5* locus. **B**: structure of the locus as defined by the five out of 10 htSNPs typed in this study.

Table 3. Haplotype Frequencies in the *CDH5* Gene as Defined by Five htSNPs

Haplotypes	rs10852432: C>T 5′ UTR	rs7499886: C>G 5′ UTR	rs1130844: C>T Exon 2	rs2304527: G>T Intron 5	rs1073584: T>C Intron 7
H1			C	T	T
H2			T	T	C
H3	C	A			C
H4	T	G			T
H5	C	G			T

Haplotypes	OR [CI]	*P*	CAS	CON
H1	0.61 [0.47–0.79]	0.0002	0.25	0.36
H2	1.45 [1.06–1.97]	0.0196	0.22	0.16
H3	1.57 [1.21–2.04]	0.0006	0.39	0.29
H4	0.66 [0.50–0.88]	0.0041	0.21	0.28
H5	0.44 [0.22–0.88]	0.017	0.022	0.05

OR, odds ratio; CI, 95% confidence interval; CAS, cases; CON, controls.

CDH5 in 96 CSC subjects suggests no significant role for rare *CDH5* alleles in the disorder.

Haplotype Analyses

Linkage disequilibrium (LD) analysis showed extensive LD across the entire *CDH5* gene (Fig. 1). Three SNPs in the exons 3–8 region were in virtually complete LD as were the variants in promoter (5′UTR) and exon 2 (Fig. 1A and B). Haplotype estimation in cases and controls identified the most frequent protective haplotype (H1) in 25% of cases versus 36% of controls (P = 0.0002; OR = 0.61, 95% CI [0.47–0.79]) (Table 3). Homozygotes for this haplotype were present in 5.9% of the CSC cases and in 15.3% of the controls (OR = 0.35, 95% CI [0.19;0.63]). A common at-risk haplotype (H3)

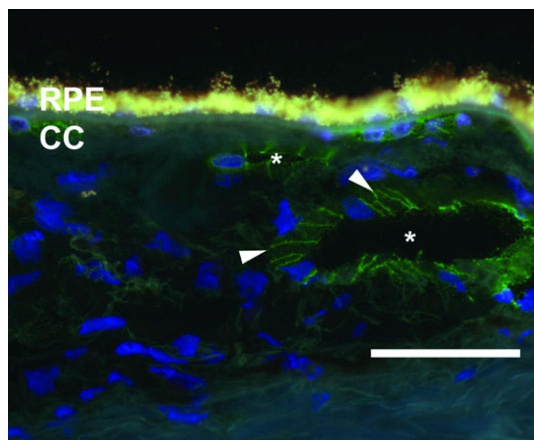

Figure 2. Immunohistochemical labeling of CDH5 (green fluorescence) in the choroid of an 86-year-old female donor. Note labeling in the choriocapillaris (CC) and at the margins of endothelial cells in larger choroidal vessels. Arrowheads indicate intercellular junctions and asterisks indicate vessel lumens. The yellow autofluorescence in the RPE is due to lipofuscin and the blue fluorescence is due to nuclear counterstaining with DAPI. Scale bar = 50 μm.

was found in 39% of cases and in 29% of controls (P = 0.0006; OR = 1.6, 95% CI [1.21–2.04]). The SNPs that distinguish the risk and protective haplotypes are mainly contained in a region between promoter and exon 8; SNPs outside this area, for example, these in the promoter (rs6499077:C>G, rs9940044:A>G, rs11860610:A>G) or after exon 8, add little effect.

Localization and Expression of the *CDH5* Protein

The protein encoded by the *CDH5* gene is a calcium-binding cell–cell adhesion glycoprotein composed of five extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail. CDH5 is localized to endothelial cell junctions, including those between endothelial cells in the human choroid. As described for other vascular beds, CDH5 protein was localized to choriocapillaris and larger vessels in Sattler's and Haller's layers of the human choroid. Labeling was notable in larger vessels at the interfaces between individual endothelial cells (Fig. 2). Therefore, CDH5 is localized to intercellular junctions also in human eyes where it functions as a classic cadherin allowing choroidal endothelial cells to adhere in a homophilic manner. The protein plays an important role in endothelial cell biology through control of the cohesion and organization of the intercellular junctions. Phosphorylation or decreased mRNA expression are associated with disassembly of CDH5 molecules and increased vascular permeability [Dejana et al., 2008; Harris and Nelson, 2010]. We investigated the possibility that *CDH5* is a plausible candidate gene for CSC by determining whether its expression is affected by corticosteroids in cultured endothelial cells, in human RPE-choroid organ cultures, and in murine eyes in vivo.

Prednisolone Suppresses *Cdh5* Expression in Endothelial Cells

The C166 endothelial cell line was exposed to prednisolone for 6 hr. Treatment resulted in significantly reduced *Cdh5* expression (fold change >-2.5, P < 0.05) at all tested concentrations (Fig. 3A) when normalized to expression of *Rpl19*. In contrast, treatment of C166 EC with testosterone, a steroid that has not been linked to changes in CSC incidence or severity, shows no significant change in *CDH5* expression.

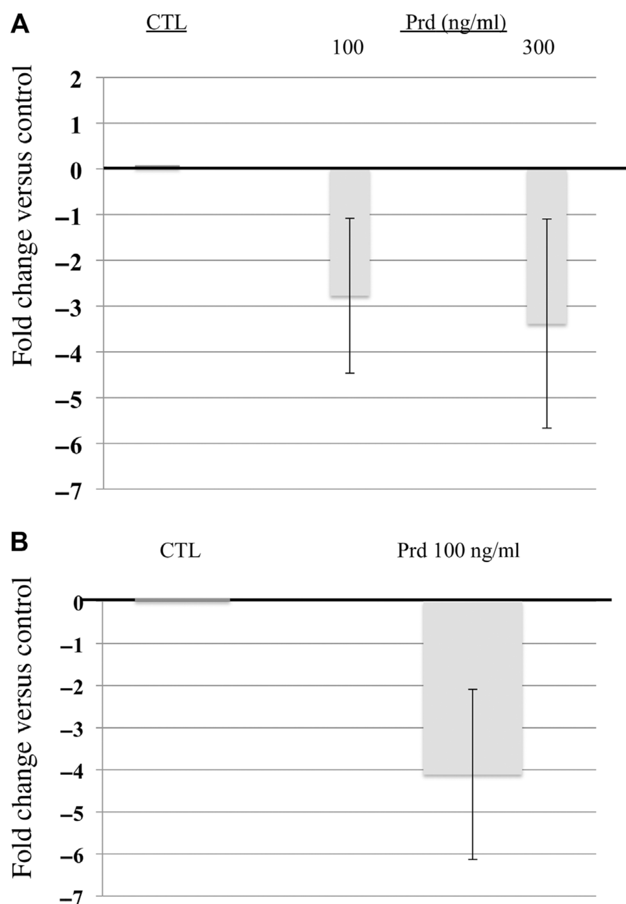

Figure 3. Suppression of the *CDH5* gene by steroids in vitro. **A**: Normalized level of *Cdh5* in mouse endothelial cells shows striking down regulation of mRNA following treatment with prednisolone (Prd) at two concentrations. Expression of *Cdh5* was significantly reduced in all steroid treated cultures (P < 0.05). **B:** Organ cultures of human RPE/choroid downregulate expression of the *CDH5* gene when exposed to prednisolone. When compared with the expression levels of *RLPB1*, *CDH5* expression in prednisolone treated cultures was suppressed by greater than fourfold compared with the control cultures (CTL; P < 0.05). In all cases, no fold change is set to 0 and error bars indicate standard deviation.

Prednisolone Reduces *CDH5* Expression and Affects Junctional Ultrastructure in Human Organ Cultures

When human RPE-choroid organ cultures were exposed to prednisolone, the expression levels of *CDH5* were significantly reduced (P = 0.011, fold change >-4×) compared with the control cultures (Fig. 3B). In order to assess whether corticosteroid treatment is capable of altering the microanatomy of endothelial cell junctional contacts, transmission electron microscopy was performed on 12 organ cultures (n = 6 prednisolone and n = 6 control) from two donors with no known history of ophthalmic disease. Qualitatively, no obvious changes were noted in RPE cell junctional complexes.

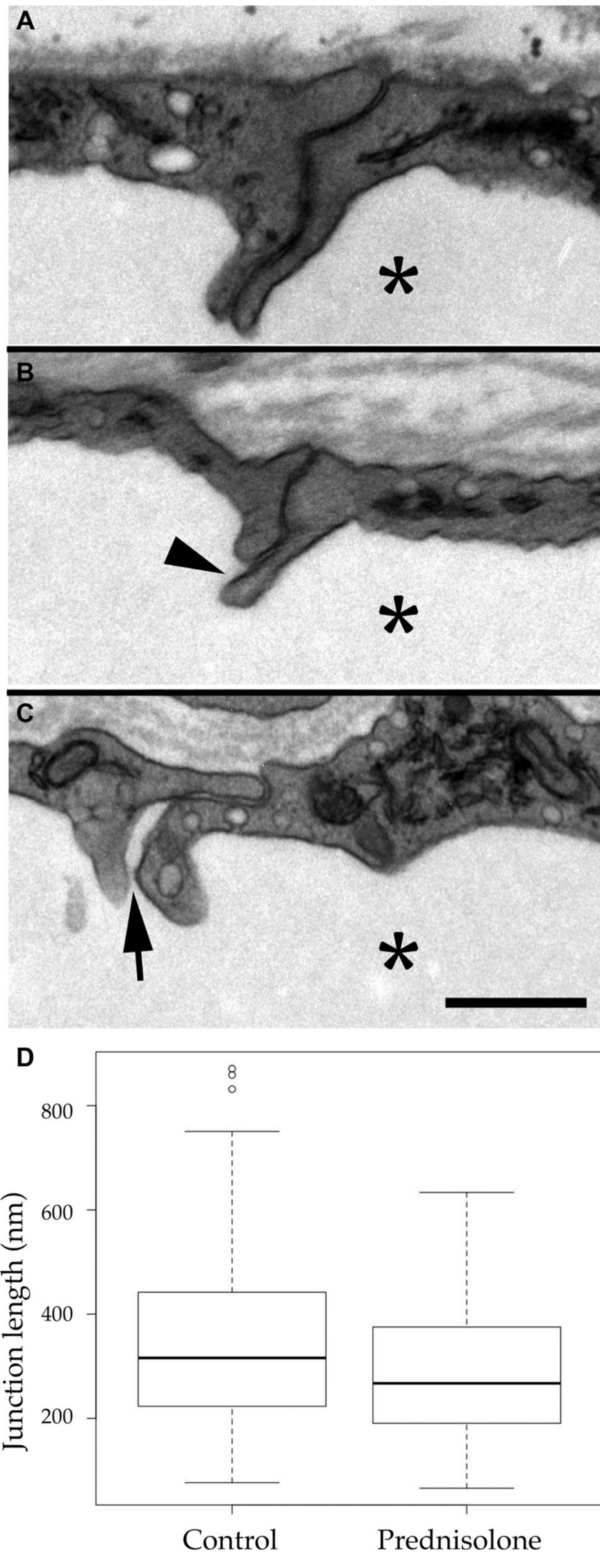

Figure 4. Anatomy of intercellular endothelial junctions in organ cultures of human RPE-choroid. **A–C**: Choriocapillaris endothelial cell junctions. In each case the vascular lumen (asterisk) is oriented toward the bottom of the micrograph. Compared with the control cultures (**A**), cultures treated with prednisolone displayed more EC junctions with misaligned or overhanging adjacent leaflets (**B**, arrow). In rare cases, there was a gap between the EC leaflets in steroid treated cultures (**C**, arrow). Scale bar = 500 nm. **D**: Distribution of junctions in control and prednisolone treated cultures in the outer choroid. Prednisolone treated cultures exhibited decreased junction size ($P < 0.05$).

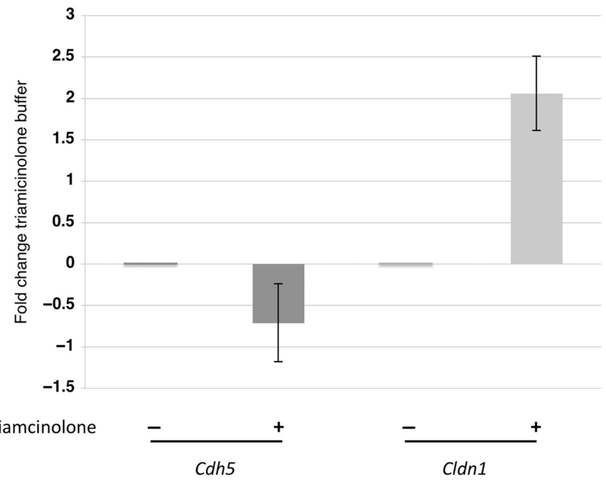

Figure 5. Reduced *Cdh5* expression in mouse RPE-choroid-sclera in eyes injected with triamcinolone. When delivered by subretinal injection, triamcinolone significantly decreased expression of *Cdh5* compared with the saline injected eyes, $P = 0.02$. In contrast, expression of the tight junction component *Cldn1* increased in steroid treated eyes. No fold change is set to 0.

The majority of choroidal intercellular junctions appeared grossly similar in steroid-treated and control cultures. However, qualitative alterations in junctional structure were observed in some junctions of the choriocapillaris in prednisolone treated cultures more notably than in control cultures. This included increased overhang of one of the leaflets and/or reduced contact between EC (Fig. 4B). In some cases, prednisolone treated cultures showed a substantial gap in the junctional contacts of up to 50 nm (Fig. 4C); although relatively rare in steroid-treated cultures, this phenomenon was not observed at all in any of the control cultures.

Quantification of intercellular junction size was also performed on transmission electron micrographs from the choroid of control and prednisolone treated organ cultures, with the length of membrane overlap between adjacent endothelial cell leaflets determined masked to treatment. In the choriocapillaris, junctions showed a trend toward decreased overlap in prednisolone treated cultures, but this did not reach statistical significance (32% decrease, $P = 0.07$). The overlap of EC leaflets was decreased in the outer choroid, with a mean junction size of 360nm in control cultures and 287 nm in prednisolone treated cultures (Fig. 4D). This 25% difference was statistically significant ($P < 0.05$).

Corticosteroids Reduce *Cdh5* Expression in Choroid In Vivo

In order to determine whether corticosteroids reduce *Cdh5* expression in vivo, mice were given subretinal injections of triamcinolone. Corticosteroid-injected eyes showed a significant decrease in *Cdh5* mRNA levels in the posterior pole when compared with the saline injected eyes ($P < 0.05$, >70% reduction) (Fig. 5). Interestingly, expression of claudin-1, a gene which product is a major component of tight junctions and is likely to increase resistance across the RPE, was increased by triamcinolone (Fig. 5; $P = .022$, fold change = 2×).

Discussion

CSC is rarely thought of as a disease with a (significant) genetic component, as most cases are sporadic and the triggers, such as

type A personality, corticosteroid treatment, and strong psychosocial stress suggest mostly "environmental" causality. In addition, acute cases often resolve in a few months and chronic cases are rarer. However, one could suggest that the reaction to strong exogenous and also endogenous hormonal stimuli is genetically modulated, similar to variable warfarin response in patients with different *CYP2C9* alleles [Higashi et al., 2002]. In addition, familial cases, albeit rare, have been described. These lines of reasoning prompted us to investigate the genetic basis of CSC and, together with expression/functional studies, led to CDH5 as the primary target.

CDH5 is an essential gene for vascular homeostasis with complex regulation at the mRNA, protein, and post-translational levels [Deleuze et al., 2007; Gavard, 2009]. Based on its role in cell–cell adhesion, and hence its potential involvement in the choroidal vascular changes associated with CSC clinically, we hypothesized that stimuli that contribute to CSC may do so through an effect on CDH5. We found that prednisolone in cultured EC and human organ cultures, and triamcinolone in mice in vivo, all significantly reduced expression of *CDH5* mRNA, which likely results in increased vascular permeability in vivo. We propose that this is a possible mechanism for the vascular changes in the choroid seen in patients with CSC.

Organ culture of human donor RPE-choroid offers excellent opportunities to examine the impact of molecular changes in the context of a relatively authentic physiological environment, which is important for studying conditions like CSC in which the entire RPE/Bruch's membrane/choroid complex is affected. We present ultrastructural evidence that reduced CDH5 expression is related to disassembly of EC–EC junctions (Fig. 4). However, although we observed EC gaps and nonoverlap more often in corticosteroid-treated than control cultures, in our system of overnight incubation, the majority of junctions appeared similar between groups.

There are some limitations to the functional part of our study. First, cells and eyes used in these experiments were not selected on the basis of known susceptibility to CSC, or *CDH5* genotypes, and a comparison of samples from patients with and without CSC would be a powerful, although less tractable, approach to understanding CSC. Recent developments in the use of patient specific inducible pluripotent stem cells from affected and unaffected patients, which show considerable promise in understanding pathogenesis of other diseases [Tucker et al., 2011], may offer novel insights into the pathogenesis of CSC. One necessary assumption of our experiments is that a generalizable response of the endothelium to corticosteroids can model aspects of CSC. The attenuation of choroidal EC contacts is consistent with clinical findings in CSC. Moreover, it was not our goal to determine a dose response curve, but to determine whether at any concentration corticosteroids impact *CDH5* expression and anatomy of intercellular junctions. It is interesting, however, that 6 hr of exposure of EC to prednisolone was sufficient to suppress expression of *CDH5*, suggesting that corticosteroids may directly affect gene expression of *CDH5* rather than this suppression relying on de novo expression of upstream regulator genes.

The endothelial cells of the choriocapillaris are fenestrated, and are thus much more permeable than the continuous capillaries of the retina, which has the additional structural components that form the blood–retina barrier to compensate for weakness of CDH5. This might suggest that in normal physiology, the RPE is constantly exposed to plasma filtrate. However, both the behavior of ICG and tracer studies have shown that the diaphragmed fenestrae of the choriocapillary endothelium are selectively permeable and show charge- and size-based exclusion of larger macromolecules [Pino and Essner, 1981]. The microenvironment of the RPE is therefore susceptible to substantial changes in composition depending on the condition of the underlying choriocapillaris. We propose that molecular and structural changes in the choroid may alter the microenvironment of the RPE in a way that affects its transport and barrier capabilities. Interestingly, this effect appears to be downstream of steroid exposure as organ cultures exposed to prednisolone appeared to possess normal RPE intercellular junctions and functional studies suggest that RPE junctions become less permeable to horseradish peroxidase in corticosteroid-exposed cells (Zeng and Mullins), which is also consistent with elevated claudin-1 expression in vivo. In summary, the choroidal endothelium and, specifically, CDH5 appear to be targets of corticosteroids in human and animal eyes.

The functional studies were supported by genetic analysis of the *CDH5* gene. Screening of htSNPs from the entire *CDH5* locus revealed strong association of independent variants, and haplotypes tagged by these, with the CSC phenotype. Interestingly, the association was largely driven by the male part of the both cohorts. CSC is known to be much more prevalent in males; the male:female ratio being, on average, 80:20. Therefore, the association with males is not overly surprising; however, we have no straightforward explanation for this observation.

Similar to the functional part of the study, the genetics part has also some limitations, the main of which is the relatively small samples sizes of both discovery and replication cohorts, which could result in chance findings. However, we suggest that the results are valid for at least the following four reasons:

(1) The association is observed in both discovery and replication cohorts and is remarkably similar in associated variation, and in the strength and direction of association (Tables 1 and 2).
(2) The association is observed in males, who account for 80% of the disease.
(3) Functional/expression studies support the genetic findings and vice versa. The reduction of CDH5 expression following corticosteroid exposure, which results in the attenuation of cell–cell contacts in the choroidal vasculature, provides a plausible mechanism by which the fluid leakage under the retina, the main feature of CSC, occurs.
(4) Involvement of a glucocorticoid response pathway in CSC, specifically through the mineralocorticoid receptor, has been demonstrated in a recent study [Zhao et al., 2012].

If confirmed, the accumulated data could serve as a basis for pretreatment genetic screening of male patients to identify those who are at subtantially elevated risk for developing CSC as a consequence of corticosteroid treatment. If applicable, alternative treatments should be considered for cases of high CSC risk.

In conclusion, this study describes the first gene/locus associated with CSC and confirms the influence of the corticosteroid imbalance to vascular permeability in the pathophysiology of CSC. It suggests that steroid-induced suppression of *CDH5* expression may be associated with genetic variation in the gene and results in the major feature of this, relatively common ocular disease, the leakage of fluid into the subretinal space, therefore further strengthening the proposed model of CSC that increased choroidal vascular permeability plays a major role in this disorder.

Acknowledgment

The authors thank Mr. Miles Flamme-Wiese for assistance with morphological experiments.

Disclosure statement: The authors have no disclosures regarding this manuscript.

References

Abramoff MD, Magelhaes PJ, Ram SJ. 2004. Image processing with ImageJ. Biophoton Int 11:36–42.

Ahnoux-Zabsonre A, Quaranta M, Mauget-Faysse M. 2004. Prevalence of Helicobacter pylori in central serous chorioretinopathy and diffuse retinal epitheliopathy: a complementary study. J Fr Ophtalmol 27:1129–1133.

Carvalho-Recchia CA, Yannuzzi LA, Negrao S, Spaide RF, Freund KB, Rodriguez-Coleman H, Lenharo M, Iida T. 2002. Corticosteroids and central serous chorioretinopathy. Ophthalmology 109:1834–1837.

Dejana E, Orsenigo F, Lampugnani MG. 2008. The role of adherens junctions and VE-cadherin in the control of vascular permeability. J Cell Sci 121 (Pt 13):2115–2122.

Deleuze V, Chalhoub E, El-Hajj R, Dohet C, Le Clech M, Couraud PO, Huber P, Mathieu D. 2007. TAL-1/SCL and its partners E47 and LMO2 up-regulate VE-cadherin expression in endothelial cells. Mol Cell Biol 27:2687–2697.

Garg SP, Dada T, Talwar D, Biswas NR. 1997. Endogenous cortisol profile in patients with central serous chorioretinopathy. Br J Ophthalmol 81:962–964.

Gass JD. 1977. Photocoagulation treatment of idiopathic central serous choroidopathy. Trans Am Acad Ophthalmol Otolaryngol 83 (3 Pt 1):456–467.

Gavard J. 2009. Breaking the VE-cadherin bonds. FEBS Lett 583:1–6.

Gold B, Merriam JE, Zernant J, Hancox LS, Taiber AJ, Gehrs K, Cramer K, Neel J, Bergeron J, Barile GR, Smith RT, AMD Genetics Clinical Study Group, et al. 2006. Variation in factor B (BF) and complement component 2 (C2) genes is associated with age-related macular degeneration. Nat Genet 38:458–462.

Haimovici R, Koh S, Gagnon DR, Lehrfeld T, Wellik S. 2004. Risk factors for central serous chorioretinopathy: a case-control study. Ophthalmology 111:244–249.

Harris ES, Nelson WJ. 2010. VE-cadherin: at the front, center, and sides of endothelial cell organization and function. Curr Opin Cell Biol 22:651–658.

Higashi MK, Veenstra DL, Kondo LM, Wittkowsky AK, Srinouanprachanh SL, Farin FM, Rettie AE. 2002. Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. JAMA 287:1690–1698.

Imamura Y, Fujiwara T, Margolis R, Spaide RF. 2009. Enhanced depth imaging optical coherence tomography of the choroid in central serous chorioretinopathy. Retina 29:1469–1473.

Jorgensen T, Borch-Johnsen K, Thomsen TF, Ibsen H, Glumer C, Pisinger C. 2003. A randomized non-pharmacological intervention study for prevention of ischaemic heart disease: baseline results Inter99. Eur J Cardiovasc Prev Rehabil 10:377–386.

Kocabora MS, Durmaz S, Kandemir N. 2008. Exacerbation of central serous chorioretinopathy following intravitreal triamcinolone injection. Graefes Arch Clin Exp Ophthalmol 246:1783–1786.

Mauget-Faysse M, Kodjikian L, Quaranta M, Ben Ezra D, Trepsat C, Mion F, Megraud F. 2002. [Helicobacter pylori in central serous chorioretinopathy and diffuse retinal epitheliopathy. Results of the first prospective pilot study]. J Fr Ophtalmol 25:1021–1025.

Mullins R, Kuehn M, Faidley E, Syed N, Stone E. 2007. Differential macular and peripheral expression of bestrophin in human eyes and its implication for Best disease. Invest Ophthalmol Vis Sci 48:3372–3380.

Mullins R, Skeie J, Malone E, Kuehn M. 2006. Macular and peripheral distribution of ICAM-1 in the human choriocapillaris and retina. Mol Vision 12:224–235.

Munch IC, Ek J, Kessel L, Sander B, Almind GJ, Brondum-Nielsen K, Linneberg A, Larsen M. 2010. Small, hard macular drusen and peripheral drusen: associations with AMD genotypes in the Inter99 Eye Study. Invest Ophthalmol Vis Sci 51:2317–2321.

Oosterhuis JA. 1996. Familial central serous retinopathy. Graefes Arch Clin Exp Ophthalmol 234:337–341.

Piccolino FC, de la Longrais RR, Ravera G, Eandi CM, Ventre L, Abdollahi A, Manea M. 2005. The foveal photoreceptor layer and visual acuity loss in central serous chorioretinopathy. Am J Ophthalmol 139:87–99.

Pino R, Essner E. 1981. Permeability of rat choriocapillaris to hemeproteins. Restriction of tracers by a fenestrated endothelium. J Histochem Cytochem 29: 281–290.

Pryds A, Larsen M. 2012. Choroidal thickness following extrafoveal photodynamic treatment with verteporfin in patients with central serous chorioretinopathy. Acta Ophthalmol 90:738–743.

Pryds A, Sander B, Larsen M. 2010. Characterization of subretinal fluid leakage in central serous chorioretinopathy. Invest Ophthalmol Vis Sci 51:5853–5857.

Skeie JM, Fingert JH, Russell SR, Stone EM, Mullins RF. 2010. Complement component C5a activates ICAM-1 expression on human choroidal endothelial cells. Invest Ophthalmol Vis Sci 51:5336–5342.

Spaide RF, Campeas L, Haas A, Yannuzzi LA, Fisher YL, Guyer DR, Slakter JS, Sorenson JA, Orlock DA. 1996. Central serous chorioretinopathy in younger and older adults. Ophthalmology 103:2070–2079; discussion 2079–80.

Spaide RF, Koizumi H, Pozonni MC. 2008. Enhanced depth imaging spectral-domain optical coherence tomography. Am J Ophthalmol 146:496–500.

Tittl MK, Spaide RF, Wong D, Pilotto E, Yannuzzi LA, Fisher YL, Freund B, Guyer DR, Slakter JS, Sorenson JA. 1999. Systemic findings associated with central serous chorioretinopathy. Am J Ophthalmol 128:63–68.

Tucker BA, Scheetz TE, Mullins RF, DeLuca AP, Hoffmann JM, Johnston RM, Jacobson SG, Sheffield VC, Stone EM. 2011. Exome sequencing and analysis of induced pluripotent stem cells identify the cilia-related gene male germ cell-associated kinase (MAK) as a cause of retinitis pigmentosa. Proc Natl Acad Sci USA 108:E569–E576.

Wang M, Munch IC, Hasler PW, Prunte C, Larsen M. 2008. Central serous chorioretinopathy. Acta Ophthalmol 86:126–145.

Wang M, Sander B, la CM, Larsen M. 2005. Clinical characteristics of subretinal deposits in central serous chorioretinopathy. Acta Ophthalmol Scand 83:691–696.

Wang MS, Sander B, Larsen M. 2002. Retinal atrophy in idiopathic central serous chorioretinopathy. Am J Ophthalmol 133:787–793.

Weenink AC, Borsje RA, Oosterhuis JA. 2001. Familial chronic central serous chorioretinopathy. Ophthalmologica 215:183–187.

Wyman GJ. 1963. Central serous retinopathy in twins. Am J Ophthalmol 55:1265.

Yannuzzi LA. 1986. Type A behavior and central serous chorioretinopathy. Trans Am Ophthalmol Soc 84:799–845.

Yannuzzi LA. 2010. Central serous chorioretinopathy: a personal perspective. Am J Ophthalmol 149:361–363.e1.

Yannuzzi LA, Slakter JS, Gross NE, Spaide RF, Costa DL, Huang SJ, Klancnik JM, Jr., Aizman A. 2003. Indocyanine green angiography-guided photodynamic therapy for treatment of chronic central serous chorioretinopathy: a pilot study. Retina 23:288–298.

Yannuzzi LA, Slakter JS, Kaufman SR, Gupta K. 1992. Laser treatment of diffuse retinal pigment epitheliopathy. Eur J Ophthalmol 2:103–114.

Zhao M, Celerier I, Bousquet E, Jeanny JC, Jonet L, Savoldelli M, Offret O, Curan A, Farman N, Jaisser F, Behar-Cohen F. 2012. Mineralocorticoid receptor is involved in rat and human ocular chorioretinopathy. J Clin Invest 122:2672–2679.