Clinical Research Article

The Cortisol Response of Male and Female Choroidal Endothelial Cells: Implications for Central Serous Chorioretinopathy

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Abstract

Context: Central serous chorioretinopathy (CSC) is a severe ocular disease characterized by fluid accumulation under the retina and abnormalities in the underlying vascular layer, the choroid. CSC has a striking prevalence in males of 80% to 90% of total patients. Corticosteroids are the most pronounced extrinsic risk factor for CSC. Choroidal endothelial cells (CECs) are important for the vascular integrity of the choroid, but the effects of corticosteroid effects in these cells are unknown.

Objective: We aimed to reveal the potential steroidal contribution to CSC.

Method: We characterized the expression of the glucocorticoid, mineralocorticoid, and androgen receptor in the human choroid using immunohistochemistry. Using RNA-sequencing, we describe the cortisol response in human CECs derived from 5 male and 5 female postmortem donors.
**Results:** The glucocorticoid receptor was highly expressed in the human choroid, whereas no to minimal expression of the mineralocorticoid and androgen receptors was observed. The extensive transcriptional response to cortisol in human primary cultured CECs showed interindividual differences but very few sex differences. Several highly regulated genes such as ZBTB16 (log2 fold change males 7.9; females 6.2) provide strong links to choroidal vascular regulation.

**Conclusions:** The glucocorticoid receptor predominantly mediates the response to cortisol in human CECs. Interindividual differences are an important determinant regarding the cortisol response in human cultured CECs, whereas intrinsic sex differences appear less pronounced. The marked response of particular target genes in endothelial cells to cortisol, such as ZBTB16, warrants further investigation into their potential role in the pathophysiology of CSC and other vascular conditions.

**Key Words:** cortisol, sex differences, choroidal endothelial cells, central serous chorioretinopathy

Central serous chorioretinopathy (CSC) is an eye disease with a poorly understood pathogenesis that frequently occurs in males and is highly associated with corticosteroid exposure (1-4). Hallmarks of CSC include the accumulation of subretinal fluid, which manifests between the neuroretina and the retinal pigment epithelium (RPE), and abnormalities in the underlying vascular layer of the eye (the choroid) that nourishes the outer retina (1,2,5). Typical choroidal abnormalities in CSC are hyperpermeability and increased choroidal thickness, which is mainly attributed to dilatation of choroidal veins (1,3,6-9). These choroidal changes are presumed to damage the outer blood-retinal barrier, which is formed by the tight junctions of the RPE, leading to the fluid accumulation under the retina (8,9). This fluid often preferentially occurs in the cone photoreceptor-rich central area of the retina due to its specific anatomical and functional characteristics (10). As such, this subretinal fluid can lead to irreversible damage to photoreceptors, visual loss, and loss of quality of life (11). As available treatment options are not effective for a substantial proportion of CSC patients, there is an imminent need for the development of novel therapeutic strategies (2,12).

Steroid hormones play an important role in the pathogenesis of CSC and may even (sub)acutely trigger the disease (1,4). Exposure to corticosteroids has been found to be the most pronounced risk factor for CSC, with a described odd ratio of up to 37 for systemic steroid use (1,13-16). Interestingly, supporting evidence for nonsystemic routes of administration has been reported as well, including oral, intranasal, intra-articular, and topical administration of corticosteroids (15,17). Furthermore, high levels of endogenous corticosteroids as found in Cushing’s syndrome are associated with CSC (18-21). In line with these findings, both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), the 2 corticosteroid-binding receptors, have been postulated to play a role in CSC pathogenesis (1,4,22,23). Another peculiarity of CSC is that males are predominantly affected, representing approximately 85% of the cases, which may point to a role for androgens (17). Taken together, steroid hormones are likely to play an important role in the pathogenesis of CSC. Nevertheless, disease mechanisms by which steroids may induce CSC are currently unidentified.

The phenotype of endothelium differs per tissue, which may be related to specific functions such as barrier function, transendothelial transport, and regulation of vascular tone (5,24). The choroidal endothelial cell (CEC) plays an important role in the regulation of vascular permeability and vascular tone (25,26). At the inner part of the choroid, the choriocapillaris endothelium is fenestrated (27), meaning that small molecules such as glucose can diffuse freely to support the highly metabolically active photoreceptors (26). The transcriptional response to cortisol in different types of endothelium has remained unexplored. Given the particularly specialized phenotype of the CEC (5) and because the response to corticosteroids is known to be heterogenic, there may be particular value to study CSC disease mechanisms in a species- and tissue-specific manner (28).

The current study describes immunostaining of the human choroid for the steroid receptors that have been implicated in CSC [ie, the GR, MR, and androgen receptor (AR)]. We also performed RNA sequencing on human primary CECs of both males and females that were treated with cortisol to identify genes potentially involved in CEC dysfunction.

**Materials and Methods**

**Human Donor Eyes**

Donor eyes from 10 human cadavers (both eyes; 5 male, 5 female; mean age 80.3 ± 9.8 years; range 58-94 year)
were obtained at either the Department of Anatomy and Embryology at Leiden University Medical Center (LUMC; Leiden, The Netherlands; n = 8) or the Dutch Brain Bank (Amsterdam, the Netherlands; n = 2) within 24 h postmortem. Written informed consent for organ and tissue donation was obtained from all donors, and the current study followed the tenets of the Declaration of Helsinki. Medical histories were not available.

Immunofluorescent Staining

Biopsies of the central RPE/choroid complex and neuroretina were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and cut into 7 µm sections. Citrate buffer (MKCH9616; Sigma-Aldrich, Steinheim, Germany) was used for antigen retrieval, and blocking was performed with 5% bovine serum albumin (A7906; Sigma-Aldrich) in phosphate buffered saline (B. Braun Medical, Oss, the Netherlands) for 60 min at room temperature. Sections were mounted with Gold Antifade mounting medium (ThermoFisher Scientific). The central region of the RPE/choroid complex was dissected from the donor eyes and incubated in a 2% collagenase type II (C2674; Sigma-Aldrich) for 60 min in a shaking water bath at 37°C. The cell suspension was filtered on a 30 µm strainer (MACS SmartStrainer; Miltenyi Biotec, Leiden, the Netherlands) and seeded on fibronectin-coated (F1141; Sigma-Aldrich) plates in EGM2-MV (CC-3202; Lonza, Walkersville, MD, USA) culture medium without hydrocortisone to avoid additional exposure to steroids. Cells were grown to confluence and passaged twice with a magnetic-activated cell sorting procedure (CD31 Microbead Kit; Miltenyi Biotec) according to manufacturer’s instructions. Per eye 1 confluent 12-well plate was used for treatment, consisting of 6 technical replicates for both the control (0.01% ethanol) and cortisol (1 µM; H088; Sigma-Aldrich) condition. The cortisol concentration was based on previous experiments, in which 1 µM was found to be a saturating concentration for primary CECs under these culture conditions (22). As such, the results of this study are expected to indicate maximum pharmacological efficacy, rather than potency. To mimic circadian exposure, treatment was performed according to an intermittent paradigm, which consisted of cortisol treatment from t = 0 to t = 4, vehicle from t = 4 to t = 20, and cortisol treatment from t = 20 to t = 24. Pilot data showed that this regimen led to a stronger induction of messenger RNAs than either 24-h continuous treatment or a single 4-h treatment.

RNA Isolation and Whole Transcriptome Analysis

RNA isolation was performed according to standard procedures using TriPure (Roche, Basel, Switzerland). Equal amounts from the RNA of each technical replicate from both eyes were pooled into a vehicle and a cortisol-treated sample for each donor. RNA sequencing was performed at the Beijing Genome Institute (Beijing, China) using a NovaSeq platform (illumina, Hong Kong, China). Library preparation was performed with Illumina Truseq Prep Kit (Illumina). Subsequently, complimentary DNA fragments were amplified and purified for 150 bp paired-end sequencing. Approximately 90 million clean reads per sample were obtained. All data were processed using the BIOPET Gentrap pipeline version 0.9, developed at the LUMC (https://github.com/biopet/biopet). This pipeline includes quality control (with FastQC version 0.11.2), quality trimming (with Sickle version 1.33), adapter clipping (with Cutadapt version 1.10), RNA sequence alignment (with STAR version 2.6.0a and GRCh38 as reference genome), gene read quantification (with HTSeq-count version 0.6.1p1 and Ensembl gene annotation version 87). Afterward, a differential gene expression analysis was performed using the voom/limma method (29).
Culture of Primary Human Umbilical Vein Endothelial Cells

Primary human umbilical vein endothelial cells (HUVECs) obtained from the Department of Vascular Surgery at LUMC were pooled from 5 different donors and cultured on fibronectin (Sigma-Aldrich). The HUVECs were used for experiments at passage number 4 or 5. EGM-Plus culture medium (CC-5035; Lonza) without hydrocortisone was used. For gene expression analysis, HUVECs were cultured in technical triplicates per experimental condition.

Gene Expression Analysis by Quantitative Polymerase Chain Reaction

Synthesis of complimentary DNA and quantitative polymerase chain reaction was performed according to standard protocols using M-MLV reverse transcriptase and SYBR Green master mix (all Promega Benelux, Leiden, the Netherlands). For analysis of relative gene expression, the 2-ΔΔ Ct method was used with LRP10 as a housekeeping gene (30). For statistical analysis, a 1-way analysis of variance with Dunnett’s test for multiple comparisons was used. Dose-response curves were analyzed with a Friedman analysis of variance and Dunn’s test for multiple comparisons. Differences were considered significant when the P-value was <0.05. Primer sequences are available in Supplementary Table 1 (31).

Protein Quantification

Western blot procedures were performed according to a standardized protocol. Briefly, 0.8 mg/mL protein in radioimmunoprecipitation assay buffer was separated on an electrophoresis gel and transferred to a nitrocellulose membrane. After 1-h blocking in 5% milk powder at room temperature, membranes were incubated with the primary antibodies anti-zinc finger and BTB domain-containing protein 16 (anti-ZBTB16; 1:2500, MA5-15667, ThermoFisher Scientific) or anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000, NB300-325, Novus Biologicals, CO, USA) overnight at 4°C. Secondary antibodies were applied for 90 min at room temperature [antimouse immunoglobulin G (H+L), 1:2000, W4021; antirabbit immunoglobulin (H+L), 1:2000, W401B; Promega, the Netherlands]. Protein quantification for glyceraldehyde 3-phosphate (1:50, NB300-325, Novus Biologicals, USA) was also performed using Wes automated western blotting (ProteinSimple, San Jose, CA, USA) at a protein concentration of 0.8 mg/mL.

Results

The Cortisol Response in the Human Choroid Is Mediated by the Glucocorticoid Receptor

Both the GR and the MR have been suggested to play a role in the pathophysiology of CSC (3,4,22). Another steroid receptor of interest may be the AR, due to the high incidence of CSC in males compared to females (17). Therefore, we characterized the expression of these nuclear receptors in the human RPE/choroid complex and retina by immunostaining (Fig. 1). The RPE/choroid complex stained positive for the GR including the CECs (Fig. 1A), whereas MR staining was minimal (Fig. 1D). The retina and kidney showed a diffuse staining pattern for the GR (Fig. 1B and 1C), whereas cell-specific staining of the MR was observed (Fig. 1E). MR staining was apparent in the collecting ducts of the human kidney, used as positive control (Fig. 1F). AR immunoreactivity was absent in both the retina and the RPE/choroid complex (Fig. 1G and 1H), while the positive control, human prostate carcinoma tissue, showed clear staining (Fig. 1I).

Male and Female Cortisol Responses in Cultured Human Choroidal Endothelial Cells

The predominance of GR immunoreactivity in CECs is in line with our earlier data pointing to the GR as a mediator of cortisol effects in cultured CECs (22). To study the cortisol response of human CECs in detail in both sexes, we performed RNA sequencing on human primary CECs cultures derived from both eyes of 5 males and 5 females that were treated with 1 µM cortisol. Multidimensional scaling was used to visualize the overall similarity between the included samples (Fig. 2). The control and cortisol-treated samples derived from the same individual were overall similar, whereas individuals could be distinguished from each other on the multidimensional scaling, establishing individual variation as the main driver for transcriptome diversity. Conversely, the samples did not stratify based on male or female sex, suggesting that the transcriptomes of male and female CECs in general are not fundamentally different from each other in these cultured primary CECs (Fig. 2). While all males clearly showed Y-chromosomal gene expression under basal conditions, these did not drive the overall variation in the individual transcriptomes, and none of the cortisol-regulated genes were found to be on this chromosome.

An overview of the differentially expressed genes (DEGs) by cortisol is shown in an MA plot and volcano plot, with labels on the top 10 of up- and downregulated genes in both males and females (Fig. 3). For the male group, 153 genes (103 upregulated, 50 downregulated) were found to
be differentially expressed ($P < 0.05$). For the female group, there were 130 DEGs (80 upregulated, 50 downregulated). We performed a separate VOOM analyses for the male ($n = 5$) and female ($n = 5$) group to identify DEGs with potential sex-specific regulation. Although some genes may suggest sex-specific transcriptional regulation of the cortisol response in CECs (Fig. 4), it should be taken into account that interindividual differences showed a substantial impact on the average gene expression (Fig. 5). In general, the upregulated genes showed a higher similarity between the sexes compared to the downregulated genes (Fig. 5). For some genes, the fold change induced by cortisol differed consistently in all individuals between the male and female group, indicating that these genes may

Figure 1. Steroid receptor expression in the human eye. Expression of the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and androgen receptor (AR) in the human choroid (A, D, G) and neuroretina (B, E, H). As positive controls, human kidney tissue (E, F) and human prostate carcinoma tissue (I) were used. Nuclear receptors are shown in green (Alexa Fluor 488), and choroid sections show VE-cadherin in red (Alex Fluor 647) as a biomarker for endothelial cells. Nuclei are stained blue with 4',6-diamidino-2-phenylindole. The GR is diffusely expressed in both the choroid and neuroretina, whereas MR immunoreactivity is sparse in the human choroid and restricted to specific cell types within the neuroretina. Expression of the AR has not been observed within either the choroid or the neuroretina. The retinal pigment epithelium shows autofluorescence due to accumulation of lipofuscin. Abbreviations: CC, choriocapillaris; CECs, choroidal endothelial cells; RPE, retinal pigment epithelium.
be candidates for sex-specific regulation by cortisol. These included \textit{RASD1} (log2 fold change: males 1.8, females 0.8) and \textit{FAM26D} (log2 fold change: males −3.1, females −0.9) for the male group. For the female group, these genes were \textit{CXCL5} (log2 fold change: males 0.7, females 1.5), \textit{SULT1B1} (log2 fold change: males 1.5, females 0.7), and \textit{NR1D1} (log2 fold change: males −0.7, females −1.6) (Figs. 4 and 5). However, there were no genes identified with formal sex-specific regulation by cortisol.

### Cortisol Interferes With Signaling of Key Endothelial Cell Functions

Interestingly, several of the top 10 DEGs could be related in literature to EC barrier function and/or neovascularization (Fig. 3). The strongest upregulated gene in both males and females was \textit{ZBTB16} (log2 fold change: males 7.9, females 6.2), a gene that has been linked to hypertension and myocardial fibrosis in a rat model for arterial hypertension (32) and to vascular endothelial growth factor (VEGF)-induced phosphorylation of endothelial nitric oxide synthase (eNOS) in human umbilical cord vascular endothelium cells (HUVECs) (33). Other upregulated genes in the top 10 of both sexes include \textit{ANGPTL4} (log2 fold change males 2.5; females 2.5), \textit{HIF3A} (log2 fold change males 1.9; females 1.7), and \textit{SPARCL1} (log2 fold change males 1.7; females 1.8) (Fig. 3). Interestingly, \textit{ANGPTL4} has been shown to affect EC junctions and induce vascular hyperpermeability (34–36). \textit{HIF3A} is a gene typically upregulated in response to hypoxia, with a myriad of stress response functions including the induction of angiogenic factors (37). \textit{SPARCL1} is an extracellular matrix protein that has been linked to cellular adhesion, migration, and proliferation (38,39). A consistent downregulation in both sexes was only observed for \textit{PLAU} (log2 fold change males −1.1; females −1.1), which is a protease involved in blood coagulation, cell adhesion, and choroidal neovascularization (40).

### Cortisol Induces a Rapid and Sustained Induction of the Transcription Factor ZBTB16

The transcription factor \textit{ZBTB16} was found to be the strongest induced DEG in both sexes. In order to validate these findings, primary HUVECs of 5 pooled donors and primary CECs from an independent female donor were treated with cortisol and evaluated for \textit{ZBTB16} expression at both the messenger RNA and protein level. Primary HUVECs demonstrated a dose-dependent induction of \textit{ZBTB16} to cortisol treatment, which was blocked by the addition of the GR antagonist mifepristone, showing that \textit{ZBTB16} induction by cortisol is not restricted to primary CECs (Fig. 6A). Moreover, in line with earlier findings in primary CECs (22), the canonical GR target genes \textit{FKBP5}, \textit{GILZ}, and \textit{PER1}, showed a dose-dependent induction in HUVECs (Fig. 6A).

Protein expression of \textit{ZBTB16} upon cortisol stimulation was validated in both primary CECs and HUVECs. Cortisol treatment of primary CECs demonstrated sustained protein expression of \textit{ZBTB16} after 6, 12, and 24 h (Fig. 6B). Intriguingly, \textit{ZBTB16} expression was not observed at any time in the control samples, indicating that a binary induction occurs rather than an increase of pre-existing expression. In order to confirm these findings, the primary CECs of the left and right eye of this independent donor were cultured in technical duplicates in a separate experiment for 6 h, yielding similar results (Fig. 6C).

### Discussion

In the present study, we established by immunohistochemistry that the GR is the predominant cortisol receptor in the human choroid, and we evaluated the genome-wide transcriptional response to cortisol in human primary CECs, derived from postmortem donor eyes of both males and females with unknown medical history. Although a spectrum of genes was differentially expressed upon cortisol exposure, male and female responses were found to be largely similar. Many well-known corticosteroid-responsive genes were induced, including \textit{FKBP5}, \textit{GILZ}, and \textit{PER1} (41,42).

The highest DEG by cortisol was \textit{ZBTB16}, a transcription factor also known as promyelocytic leukemia zinc finger protein, which also showed a sustained induction at the protein level in both CECs and HUVECs (Fig. 6). Other cortisol-regulated genes were earlier shown to be related to key EC functions, including \textit{ANGPTL4}, \textit{HIF3A}, \textit{SPARCL1},
and PLAU. As many of these genes are not known as highly specific to the phenotype of the CEC (26), they may not only be relevant for the pathogenesis of CSC, but also for other aspects of vascular physiology and disease.

Even though CSC can be caused by synthetic glucocorticoids with high selectivity for GR, MR antagonists such as eplerenone and spironolactone have been widely studied as a treatment option for chronic CSC, mainly based on animal studies and retrospective studies in humans on small cohorts (1,4,43,44). A meta-analysis on 145 eyes of CSC patients across 5 studies evaluated the efficacy of MR antagonists compared to either placebo treatment or observation. A modest positive effect of MR antagonists on visual acuity was reported after 1 and 2 months of treatment, but this effect did not remain significant over time (44). Of note, the effect of MR antagonists on complete subretinal fluid resolution—which has been found to be the desired effect of treatment rather than changes in subretinal fluid height or visual acuity—was not assessed in these studies (2,44). In addition, retrospective studies on potential treatments are especially problematic in CSC, because even chronic CSC often shows a variable clinical course with episodes of spontaneous improvement, and up to 30% of placebo-treated patients showed a complete resolution of subretinal fluid after one year of follow-up in the VICI trial (45, 46). The only two prospective randomized controlled trials (the SPECTRA trial and VICI trial) have not found evidence of a supporting role for treatment.

Figure 3. Messenger RNA regulation in male and female primary choroidal endothelial cells after cortisol treatment. A MA plot (top panel) and volcano plot (bottom panel) showing differentially expressed genes (DEGs) for the male and female group. The top 10 of up- and downregulated DEGs are tagged with the gene symbol. Red indicates DEGs that have been found to be significant only in females, whereas blue indicated DEGs that have been found to be significant only in males.
Figure 4. Overlay volcano plots visualize similarities and differences in differentially expressed genes between the sexes. Differentially expressed genes are categorized into statistically significant regulation in both sexes (top plot), females only (middle plot), and males only (bottom plot). Blue indicates the male group; red indicates the female group.
MR antagonists in CSC treatment (12,45). In one of these studies, the VICI trial, eplerenone treatment (n = 57) was compared to placebo (n = 57) in chronic CSC. After 12 months, additional therapy was required for 4 patients in the eplerenone group and 7 patients in the placebo group, however a positive effect on visual outcome for eplerenone was not observed (45). Our immunohistochemical findings argue against a pivotal role for the MR in human choroidal endothelium. However, MR staining was present in a subset of retinal cells, which could be a lead for MR-mediated disease mechanisms. In line with these histological findings, our previous pharmacological data in primary cultured human CECs showed GR-mediated but not MR-mediated cortisol responses (22). Still, it should be noted that expression of the MR in ECs can be dependent on specific contexts (i.e. pro-inflammatory conditions or neovascularization) (47,48), which may explain why we observed a lack of MR immunoreactivity in the human choroid by immunohistochemistry. Of note, the rs2070951 variant of the NR3C2 gene has previously been associated with hypertension and chronic stress (49,50), but also with CSC (23). Specifically, the GA haplotype of rs2070951, a loss-of-function variant, has been associated with increased activation of the renin-angiotensin-aldosterone axis and hypertension in males, and is also associated with an increased risk for CSC (23,49). The association of this MR variant with CSC was not significant for females, however this may also be due to the limited number of female CSC patients included in this study (23). In contrast, the CA haplotype of rs2070951, a gain-of-function variant, is associated with stress resilience and lower blood pressure, and is also protective against CSC (23,50,51). Thus, while CECs robustly respond to cortisol, direct MR-mediated effects appear unlikely, although systemic MR-mediated effects may play a role in the pathogenesis of CSC (23,49).

CSC is characterized by choroidal thickening, dilatation of the large choroidal veins with congestion of blood flow, hyperpermeability of the capillary layer of the choroid (choriocapillaris), and subretinal fluid accumulation (1,2,6,52). The biomolecular mechanism of the choroidal dysfunction is unclear. Our findings indicate that cortisol may affect the barrier function and/or the vascular tone of the choroid.
choroid. For example, the most strongly induced gene found in this study, ZBTB16, has been shown to be a mediator of VEGF-induced phosphorylation of eNOS in HUVECs (33). This eNOS phosphorylation leads to an increased production of nitric oxide, of which 1 of the main effects in vivo is vasodilation (52,53). Spaide et al recently described an analogy between CSC and varicose veins as both being diseases that result from (chronic) venous insufficiency (52). Interestingly, in surgically removed varicose veins, eNOS shows increased expression, which further merits investigation into the potential role of eNOS in CSC (52). Therefore, based on the results of the current study, it may be speculated that the enlarged vascular lumens frequently observed in CSC patients may be, at least in part, attributed to vasodilation induced by increased nitric oxide production. Cortisol may also contribute to the development of CSC by the strong induction of ANGPTL4 that we observed in this study, which has been shown to affect the integrity of EC junctions leading to

Figure 6. Cortisol induces a rapid and sustained induction of ZBTB16. (A) In primary human umbilical vein endothelial cells, the canonical corticosteroid target genes FKBP5, GILZ, and PER1 show dose-dependent induction by cortisol on the messenger RNA level, which was also observed for ZBTB16. The vertical dotted lines represent the half maximal effective concentration (EC50). Simultaneous incubation of cortisol (1 μM) with the glucocorticoid receptor antagonist mifepristone (1 μM) blocked the upregulation of ZBTB16. (B) Human primary choroidal endothelial cells treated with cortisol for either 6, 12, or 24 h demonstrate a rapid and sustained induction on the protein level. (C) For validation purposes, human primary choroidal endothelial cells of an independent donor have been cultured in biological duplicates of both the left and the right eye and were treated with cortisol for 6 h.
hyperpermeability (35), 1 of the main clinical signs of CSC (2). These considerations warrant further investigation on the functional level, such as vasodilatation measurements or assessment of barrier function.

It should be noted that the DEGs in our data set do not necessarily represent an exclusive phenotype of the CEC but rather contain a multitude of genes that are expressed throughout a variety of vascular ECs. This raises the question why an individual may specifically develop CSC upon corticosteroid exposure, while glucocorticoid effects on ECs in other organs appear to remain without clinical consequence. Although speculative, preexistent ocular abnormalities and the highly specialized functional and anatomical ocular microenvironment may determine a “first hit” vulnerability to develop CSC upon exposure to corticosteroids (5). For example, the recently proposed hypothesis of chronic venous overload of choroidal blood flow in CSC may take effect as a “first hit” (7,52). Subsequently, pathological effects of corticosteroids act as a “second hit” on this stressed vasculature. This would then lead to CEC dysfunction, damage to the outer blood-retina barrier formed by the tight junctions of the RPE, and ultimately subretinal fluid accumulation. This hypothesis is also in line with the fact that subretinal fluid accumulation occurs most frequently in the highly specialized macular region in CSC, where the choriocapillaris density and shear stress is highest and the neurosensory retina is most dependent on a healthy functioning choroid (2,5,25,54).

The striking prevalence of CSC in males may be another approach to reveal some of the biomolecular mechanisms underlying the pathophysiology of CSC. Interestingly, elevated steroid hormone levels (androstosterone, estrone, etiocholanolone, androstenedione), as well as imbalances between steroid hormones, have been reported in male CSC patients (20). Importantly, the current study is based on ex vivo cultured CECs in which culture conditions were standardized, thus not taking into account the in vivo hormonal context. Of note, we did not observe significant expression of the androgen or estrogen receptors in our RNA sequencing data set, nor did another single cell RNA sequencing study on freshly isolated human CECs (26). However, intrinsic cellular sexual dimorphism in the cortisol response could independently play a role in the high prevalence of CSC in males. Yet, in the current study we observed largely similar cortisol responses of CECs between males and females, and therefore we did not find any evidence for potential intrinsic cellular sexual dimorphism. This may also be explained by the limited number of donors (5 males, 5 females) and the relatively high interindividual variation in the cortisol response. An example of a gene that may be of interest for further investigation into intrinsic sexual dimorphism of the cortisol response is RASD1, which was consistently more highly induced in the male group (average log2 fold change 1.75) compared to the female group (average log2 fold change 0.84). Although there is no evidence that RASD1 may be linked to CSC, genetic variations of RASD1 have been found to be associated with an increased risk for ischemic stroke (55). On the other hand, scleral thickness has been described to be greater in males compared to females, which may be another explanation for the sex differences in CSC, since an increased scleral thickness may impede venous outflow (52).

There are several limitations of our study. Although we implemented a species- and site-specific approach to study the pathophysiology of CSC, our study relies on primary cultured human cells under standardized conditions, which are passaged twice before they are of sufficient quantity for the experiments and may therefore be subject to dedifferentiation (22,56). Second, the donor eyes are obtained from postmortem donors with unknown medical histories. Conversely, an advantage of our approach is the use of human tissue and isolation of CECs that are derived from the choroid, the primarily affected tissue in CSC. This advantage is reinforced by the fact that animal models recapitulating the choroidal abnormalities and subretinal fluid accumulations as observed in humans with CSC do not exist. Nonetheless, in this study we only focused on CECs, not taking into account other cell types that may be affected in CSC, such as the RPE or in the sclera. Although we did not observe AR expression in CECs, the high male preponderance in CSC may still be explained by crosstalk between corticosteroids and androgens in other cell types outside the choroid, a mechanism that has been linked to a sexual dimorphic response to corticosteroids (57,58).

In this study, we described the genome-wide cortisol response of human CECs, identified the GR as their predominant steroid receptor, and found that there were striking interindividual differences in cellular transcriptomes but no intrinsic sex differences. The corticosteroid-responsive genes that we identified offer ample opportunities to further understand physiological as well as pathophysiological consequences of corticosteroid effects on the vasculature. Moreover, it would be valuable to compare the differential cortisol response between stem cell-derived ECs derived from healthy individuals and CSC patients and/or to study CEC responses in 3D culture models that recapitulate the niche of the RPE/choroid complex (59). In addition, patient-specific studies in CSC may include analysis on GR signaling and its interaction with glucocorticoid response elements in or nearby the promoter regions of genes such as ZBTB16 (eg, by luciferase reporter assays or chromatin immunoprecipitation). Finally, stem cell research can pave the way for drug and toxicity screening on a large scale, enabling both patient-specific CSC research and the development of therapeutic strategies within the framework of personalized medicine.
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Disclosures: The authors have nothing to disclose.

Data Availability: Some or all data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Kaye R, Chandra S, Sheth J, Boon CJF, Sivaprasad S, Lotery A. Central serous chorioretinopathy: an update on risk factors, pathophysiology and imaging modalities. Prog Retin Eye Res. 2020;79:100865.
2. van Rijssen TJ, van Dijk EHC, Yzer S, et al. Central serous chorioretinopathy: towards an evidence-based treatment guideline. Prog Retin Eye Res. 2019;73:100770.
3. Nicholson B, Noble J, Forooghian F, Meyerle C. Central serous chorioretinopathy: update on pathophysiology and treatment. Surv Ophthal. 2013;58(2):103-126.
4. Daruich A, Matet A, Dirani A, et al. Central serous chorioretinopathy: recent findings and new physiopathology hypothesis. Prog Retin Eye Res. 2015;48:82-118.
5. Brinks J, van Dijk E, Klaassen I, et al. Exploring the choroidal vascular system and its molecular and structural roles in health and disease. Prog Retin Eye Res. Published online July 2021. doi:10.1016/j.preteyeres.2021.100994
6. Dansingani KK, Balaratatamasingam C, Naysan J, Freund KB. En face imaging of pachychoroid spectrum disorders with swept-source optical coherence tomography. Retina. 2016;36(3):499-516.
7. Spaida RF. Choroidal blood flow: review and potential expla- nation for the choroidal venous anatomy including the vortex vein system. Retina. 2020;40(10):1851-1864.
8. Prünte C, Flammer J. Choroidal capillary and venous congestion in central serous chorioretinopathy. Am J Ophthalmol. 1996;121(1):26-34.
9. Piccolino PC, Borgia L. Central serous chorioretinopathy and indocyanine green angiography. Retina. 1994;14(3):231-242.
10. van Dijk EHC, Boon CJF. Serous business: Delineating the broad spectrum of diseases with subretinal fluid in the macula. Prog Retin Eye Res. 2021;84:100955.
11. Breukink MB, Dingemans AJ, den Hollander AI, et al. Chronic central serous chorioretinopathy: long-term follow-up and vision-related quality of life. Clin Ophthalmol. 2017;11:39-46.
12. van Rijssen TJ, van Dijk EH, Tsonaka R, et al. Half-dose photodynamic therapy versus eplerenone in chronic central serous chorioretinopathy (SPECTRA): a randomized controlled trial. Am J of Ophthalmol. Published online June 2021. doi:10.1016/j.ajo.2021.06.020
13. Haimovici R, Koh S, Gagnon DR, Lehrfeld T, Wellik S. Central Serous Chorioretinopathy Case-Control Study Group. Risk factors for central serous chorioretinopathy: a case-control study. Ophthalmology. 2004;111(2):244-249.
14. Rim TH, Kim HS, Kwak J, Lee JS, Kim DW, Kim SS. Association of corticosteroid use with incidence of central serous chorioretinopathy in South Korea. JAMA Ophthalmol. 2018;136(10):1164-1169.
15. Nicholson BP, Atchison E, Idris AA, Bakri SJ. Central serous chorioretinopathy and glucocorticoids: an update on evidence for association. Surv Ophthalmol. 2018;63(1):1-8.
16. Araki T, Ishikawa H, Iwahashi C, et al. Central serous chorioretinopathy with and without steroids: A multicenter survey. PLoS One. 2019;14(2):e0213110.
17. Kitzmann AS, Pulido JS, Diehl NN, Hodge DO, Burke JP. The incidence of central serous chorioretinopathy in Olmsted County, Minnesota, 1980-2002. Ophthalmology. 2008;115(1):169-173.
18. Haimovici R, Rumelt S, Melby J. Endocrine abnormalities in patients with central serous chorioretinopathy. Ophthalmology. 2003;110(4):698-703.
19. van Dijk EH, Dijkman G, Biersnas NR, van Haalen FM, Pereira AM, Boon CJ. Chronic central serous chorioretinopathy as a presenting symptom of Cushing syndrome. Eur J Ophthalmol. 2016;26(5):442-448.
20. Schellevis RL, Altay L, Kalsinshag A, et al. Elevated steroid hormone levels in active chronic central serous chorioretinopathy. Invest Ophthalmol Vis Sci. 2019;60(10):3407-3413.
21. Brinks J, van Haalen FM, van Rijssen TJ, et al. Central serous chorioretinopathy in active endogenous Cushing’s syndrome. Sci Rep. 2021;11(1):2748.
22. Brinks J, van Dijk EHC, Habeeb M, et al. The effect of corticosteroids on human choroidal endothelial cells: a model to study central serous chorioretinopathy. Invest Ophthalmol Vis Sci. 2018;59(13):5682-5692.
23. van Dijk EHC, Schellevis RL, van Bergen MGJM, et al. Association of a haplotype in the NR3C2 gene, encoding the mineralocorticoid receptor, with chronic central serous chorioretinopathy. JAMA Ophthalmol. 2017;135(5):446-451.
24. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res. 2007;100(2):158-173.
25. Nickla DL, Wallman J. The multifunctional choroid. Prog Retin Eye Res. 2010;29(2):144-168.
26. Voigt AP, Mulfaulk J, Mullin NK, et al. Single-cell transcriptionomics of the human retinal pigment epithelium and choroid in health and macular degeneration. Proc Natl Acad Sci U S A. 2019;116(48):24100-24107.
27. Blauwaggers HG, Holtkamp GM, Rutten H, et al. Polarized vascular endothelial growth factor secretion by human retinal pigment epithelium and localization of vascular endothelial growth factor receptors on the inner choriocapillaris: evidence for a trophic paracrine relation. Am J Pathol. 1999;155(2):421-428.
28. van Batenburg MF, Li H, Polman JA, et al. Paired hormone response elements predict caveolin-1 as a glucocorticoid target gene. PLoS One. 2010;5(1):e8839.

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29. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15(2):R29.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-408.
31. Brinks J, van Dijk EHC, Kielbasa SM, et al. Supplementary data for: the cortisol response of male and female choroidal endothelial cells: implications for central serous choriorretinopathy. FigShare. Posted August 19, 2021. https://figshare.com/articles/figure/RNAssec_CECs_Supplementary_1/15090129
32. Liška F, Mancini M, Krupková M, et al. Plzf as a candidate gene predisposing the spontaneously hypertensive rat to hypertension, left ventricular hypertrophy, and interstitial fibrosis. Am J Hypertens. 2014;27(1):99-106.
33. Hsu YH, Chen YC, Chen TH, et al. Far-infrared therapy induces the nuclear translocation of PLZF which inhibits VEGF-induced proliferation in human umbilical vein endothelial cells. PLoS One. 2012;7(1):e30674.
34. Zhou H, Yang YH, Basile JR. The Septamorphin 4D-Plexin-B1-RhoA signaling axis recruits pericytes and regulates vasculature permeability through endothelial production of PDGF-B and ANGPTL4. Angiogenesis. 2014;17(1):261-274.
35. Xin X, Rodrigues M, Umaphathi M, et al. Hypoxic retinal Muller cells promote vascular permeability by HIF-1-dependent up-regulation of angiopoietin-like 4. Proc Natl Acad Sci U S A. 2013;110(36):E3423-E3434.
36. Padua D, Zhang XH, Wang Q, et al. TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell. 2008;133(1):66-77.
37. Mammadzada P, Corredoira PM, André H, et al. The role of hypoxia-inducible factors in neovascular age-related macular degeneration: a gene therapy perspective. Cell Mol Life Sci. 2020;77(5):819-833.
38. Sullivan MM, Sage EH. Hevin/SC1, a matricellular glycoprotein and potential tumor-suppressor of the SPARC/ BM-40/Osteonectin family, Int J Biochem Cell Biol. 2004;36(6):991-996.
39. Girard JP, Springer TA. Modulation of endothelial cell adhesion by hevin, an acidic protein associated with high endothelial venules. J Biol Chem. 1996;271(8):4511-4517.
40. Das A, Boyd N, Jones TR, Talarico N, McGuire PG. Inhibition of choroidal neovascularization by a peptide inhibitor of the urokinase plasminogen activator and receptor system in a mouse model. Arch Ophthalmol. 2004;122(12):1844-1849.
41. Zalachorhas I, Verhoeve SL, Toonen LJ, et al. Isoform switching of steroid receptor co-activator-1 attenuates glucocorticoid-induced angiogenic amygdala CRH expression. Mol Psychiatry. 2016;21(12):1733-1739.
42. Drebert Z, Bracke M, Beck IM. Glucocorticoids and the non-steroidal selective glucocorticoid receptor modulator, compound A, differentially affect colon cancer-derived myofibroblasts. J Steroid Biochem Mol Biol. 2015;149:92-105.
43. Zhao M, Célerié I, Bousquet E, et al. Mineralocorticoid receptor is involved in rat and human ocular chorioretinopathy. J Clin Invest. 2012;122(7):2672-2679.
44. Wang SK, Sun P, Tandias RM, Seto BK, Arroyo JG. mineralocorticoid receptor antagonists in central serous chorioretinopathy: a meta-analysis of randomized controlled trials. Ophthalmol Retina. 2019;3(2):154-160.
45. Lotery A, Sivapradas S, O’Connell A, et al; VICI Trial Investigators. Eplerenone for chronic central serous chorioretinopathy in patients with active, previously untreated disease for more than 4 months (VICI): a randomised, double-blind, placebo-controlled trial. Lancet. 2020;395(10220):294-303.
46. van Rijssen Tj, van Dijk EHC, Scholte P, et al. Reply to comment on: Focal and diffuse chronic central serous chorioretinopathy treated with half-dose photodynamic therapy or subthreshold micropulse laser: PLACE Trial report no. 3. Am J Ophthalmol. 2020;212:187-188.
47. Zhao M, Mantel I, Gelize E, et al. Mineralocorticoid receptor antagonist limits experimental choroidal neovascularization and structural changes associated with neovascular age-related macular degeneration. Nat Commun. 2019;10(1):369.
48. Caprio M, Newfell BG, la Sala A, et al. Functional mineralocorticoid receptors in human vascular endothelial cells regulate intercellular adhesion molecule-1 expression and promote leukocyte adhesion. Circ Res. 2008;102(11):1359-1367.
49. van Leeuwen N, Caprio M, Blaya C, et al. The functional c.-2G > C variant of the mineralocorticoid receptor modulates blood pressure, renin, and aldosterone levels. Hypertension. 2010;56(5):995-1002.
50. van Leeuwen N, Bellingrath S, de Kloet ER, et al. Human mineralocorticoid receptor (MR) gene haplotypes modulate MR expression and transactivation: implication for the stress response. Psychoneuroendocrinology. 2011;36(5):699-709.
51. Klok MD, Giltay EJ, Van der Does AJ, et al. A common and functional mineralocorticoid receptor haplotype enhances optimism and protects against depression in females. Transl Psychiatry. 2011;1:e62.
52. Spaide RF, Cheung CMG, Matsumoto H, et al. Venous over-load choroidopathy: a hypothetical framework for central serous chorioretinopathy and allied disorders. Prog Retin Eye Res. Published online May 2021. doi:10.1016/j.preteyeres.2021.100973
53. Koss MC. Functional role of nitric oxide in regulation of ocular blood flow. Eur J Pharmacol. 1999;374(2):161-174.
54. Nork TM, Kim CB, Shanmuganayagam D, Van Lysel MS, Ver Hoeve JN, Folts JD. Measurement of regional choroidal blood flow in rabbits and monkeys using fluorescent microspheres. Arch Ophthalmol. 2006;124(6):860-868.
55. Dighans M, Malik R, König IR, et al; METASTROKE Consortium; CARDioGRAM Consortium; C4D Consortium; International Stroke Genetics Consortium. Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. Stroke. 2014;45(1):24-36.
56. Peavey J, Malek G. Cell line authentication in vision research and protects against depression in females. Transl Psychiatry. 2011;1:e62.
57. Spaide RF, Cheung CMG, Matsumoto H, et al. Venous over-load choroidopathy: a hypothetical framework for central serous chorioretinopathy and allied disorders. Prog Retin Eye Res. Published online May 2021. doi:10.1016/j.preteyeres.2021.100973
58. Koss MC. Functional role of nitric oxide in regulation of ocular blood flow. Eur J Pharmacol. 1999;374(2):161-174.
59. Nork TM, Kim CB, Shanmuganayagam D, Van Lysel MS, Ver Hoeve JN, Folts JD. Measurement of regional choroidal blood flow in rabbits and monkeys using fluorescent microspheres. Arch Ophthalmol. 2006;124(6):860-868.
60. Dighans M, Malik R, König IR, et al; METASTROKE Consortium; CARDioGRAM Consortium; C4D Consortium; International Stroke Genetics Consortium. Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. Stroke. 2014;45(1):24-36.
61. Peavey J, Malek G. Cell line authentication in vision research and protects against depression in females. Transl Psychiatry. 2011;1:e62.
62. Spaide RF, Cheung CMG, Matsumoto H, et al. Venous over-load choroidopathy: a hypothetical framework for central serous chorioretinopathy and allied disorders. Prog Retin Eye Res. Published online May 2021. doi:10.1016/j.preteyeres.2021.100973
63. Koss MC. Functional role of nitric oxide in regulation of ocular blood flow. Eur J Pharmacol. 1999;374(2):161-174.
64. Nork TM, Kim CB, Shanmuganayagam D, Van Lysel MS, Ver Hoeve JN, Folts JD. Measurement of regional choroidal blood flow in rabbits and monkeys using fluorescent microspheres. Arch Ophthalmol. 2006;124(6):860-868.
65. Dighans M, Malik R, König IR, et al; METASTROKE Consortium; CARDioGRAM Consortium; C4D Consortium; International Stroke Genetics Consortium. Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. Stroke. 2014;45(1):24-36.
66. Peavey J, Malek G. Cell line authentication in vision research and protects against depression in females. Transl Psychiatry. 2011;1:e62.