Complement Activation Products and Cytokines in Pachychoroid Neovasculopathy and Neovascular Age-Related Macular Degeneration

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PURPOSE. To investigate the characteristics of complement activation products and angiogenic cytokines in the aqueous humor in eyes with pachychoroid neovasculopathy (PNV) and neovascular age-related macular degeneration (nAMD).

METHODS. This was a prospective, comparative, observational study. All patients with choroidal neovascularization were classified as PNV without polyps, PNV with polyps (polypoidal choroidal vasculopathy [PCV]), or drusen-associated nAMD according to the presence or absence of pachychoroid features and soft drusen. This study included a total of 105 eyes. Aqueous humor samples were collected from 25 eyes with PNV without polyps, 23 eyes with PCV, and 24 eyes with drusen-associated nAMD before intravitreal anti-vascular endothelial growth factor (VEGF) injection and cataract surgery in 33 control eyes. Clinical samples were measured for complement component 3a (C3a), C4a, C5a, VEGF, and macrophage chemoattractant protein 1 (MCP-1) using a bead-based immunoassay.

RESULTS. C3a and MCP-1 levels were significantly higher in PCV (P = 0.032 and P = 0.039, respectively) and drusen-associated nAMD (P = 0.01 for both comparisons) than in controls, and no difference was seen in C3a and MCP-1 levels between PNV and controls (P = 0.747 and P = 0.294, respectively). VEGF levels were significantly higher in PNV (P = 0.016), PCV (P = 0.009), and drusen-associated nAMD than in controls. In PNV, the VEGF levels elevated without elevated C3a and MCP-1.

CONCLUSIONS. PNV, PCV, and drusen-associated nAMD had significantly distinct profiles of complement activation products and cytokines in the aqueous humor.

Keywords: pachychoroid neovasculopathy, neovascular age-related macular degeneration, aqueous humor, complement, cytokine

Age-related macular degeneration (AMD) is a leading cause of irreversible visual impairment in older adults.1 AMD consists of two forms, dry and wet. Dry-form AMD shows atrophy of the retinal pigment epithelium (RPE). Choroidal neovascularization (CNV) is a common clinical feature in a type of wet-form AMD referred to as neovascular AMD (nAMD). CNV in nAMD is categorized as type 1 or type 2 based on the CNV location; CNV is located under the RPE in type 1 CNV and above the RPE in type 2 CNV.2 Typically, nAMD shows adjunctive clinical features, including multiple soft drusen and relatively thinner choroid, which can be observed clinically through optical coherence tomography (OCT).3

A new type 1 CNV with peculiar clinical features in older adults is referred to as polypoidal choroidal vasculopathy (PCV) or pachychoroid neovasculopathy (PNV). PCV was proposed by Yannuzzi et al.4 in 1990, and PNV was reported in 2015 by the same research group.5 Both PCV and PNV show common clinical features, except for polypoidal lesions. The common characteristics of PCV and PNV are thick choroid (so-called pachychoroid), few or no soft drusen, choroidal vascular hyperpermeability (CVH), dilated large choroidal vessel (pachyvessel), and predisposition of Asian populations.5,6 Differences in the pathophysiology of CNV among nAMD, PNV, and PCV have been noted in many published reports, but such differences are still controversial.

The cytokines play a pivotal role in CNV development. Previous studies have reported that the cytokine levels of IL-1β, IL-6, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), and vascular endothelial growth factor (VEGF) may be involved in CNV formation. In addition, cytokine levels in the aqueous humor can be useful for predicting treatment effects.7,8 Among the
cytokines mentioned above, VEGF7–16 and MCP-17,8,10,15,16 have been considered key cytokines in the development of CNV.17–19

The complement proteins are also important in CNV development. Deposition of complement proteins has been confirmed in drusen, and previous genetic studies have suggested involvement of the complement system with nAMD pathogenesis.20–24 It has been reported that complement proteins, such as Ba, complement component 3a (C3a), and C4a, which are complement-activation products of factor B, C3, and C4, respectively, were elevated in the aqueous humor of nAMD patients.25–27 Experimental research has suggested that local complement activation could upregulate angiogenic cytokines, resulting in CNV development.

Although it is assumed that complement proteins and cytokines play an important role in CNV formation, to our knowledge no reports have compared cytokines in the aqueous humor among PNV without polyps, PCV, and nAMD. Measurement of complement proteins and cytokines in the aqueous humor may reveal the pathophysiology of each CNV subtype. In the current study, we measured C3a, C4a, C5a, VEGF, and MCP-1 in the aqueous humor of eyes with PNV without polyps, PCV, or nAMD using cytometric bead arrays.

**METHODS**

**Patients**

This prospective, comparative, observational study adhered to the tenets of the Declaration of Helsinki; the research ethics board approved the study before patient enrollment began at the Department of Ophthalmology of Fukushima Medical University Hospital, Fukushima City, Japan. All patients provided informed consent before enrollment.

All patients were over 50 years old. Seventy-two eyes of patients with treatment-naive PNV, PCV, and drusen-associated nAMD were included. Thirty-three eyes of 33 patients who had received cataract surgery were recruited as controls. All patients underwent comprehensive ophthalmic examinations, including slit-lamp biomicroscopy with a non-contact fundus lens. Color fundus photographs and fluorescein angiography (FA) images were obtained using the TRC-DX fundus camera (Topcon, Tokyo, Japan). Fundus autofluorescence and indocyanine green angiography (ICGA) images were obtained using the HRA2 (Heidelberg Engineering, Heidelberg, Germany). OCT (Spectralis; Heidelberg Engineering) and OCT angiography (PLEX Elite 9000; Carl Zeiss Meditec, Jena, Germany) were also performed. All controls underwent FA and ICGA.

**Classification**

All eyes were classified into one of three subtypes: PNV, PCV, or drusen-associated nAMD (Table 1); Figure 1 shows the representative cases. Because the definition of PNV is still evolving, PNV was classified based on the latest reports.5,8,28 In our study, PNV characteristics included pachychoroid features, no soft drusen, and no polypoidal lesions (Figs. 1A–D). We defined pachychoroid features as satisfying all of the following criteria: (1) type-1 CNV detected in one or both eyes; (2) no or only non-extensive drusen (total area ≤ 125–μm circle) or hard drusen (≤63 μm) in both eyes; (3) dilated choroidal vessels below type 1 CNV detected by ICGA and OCT; (4) CVH detected on the late-phase ICGA images; and (5) the presence of central serous chorioretinopathy (CSC)-or pachychoroid pigment epitheliopathy-related RPE abnormalities independent of CNV lesions detected by FA or a history of CSC. PCV characteristics included pachychoroid features, polypoidal lesions, and no soft drusen (Figs. 1E–H). The eyes satisfying PNV criteria and having polypoidal lesions were defined as PCV. Polypoidal lesions were diagnosed in the presence of terminal arteriole dilatations with a branching vascular network in ICGA corresponding to steep RPE elevations on OCT images. When the eyes with definitive pachychoroid features showed pachydrusen (drusen-like deposits), the eyes were classified into PCV or PNV. We defined pachydrusen based on the following criteria reported in a recent review by Yanagi28: (1) size > 125 μm (large drusen), (2) irregular outer contours with better defined outer borders, (3) scattered distribution over the posterior pole, and (4) present in isolation or in groups of only a few yellow–white deposits. Subjects with soft drusen and pachydrusen or borderline cases were excluded. Drusen-associated nAMD had soft drusen in either eye and no pachychoroid features (Figs. 1I–L). Eyes with retinal angiomatous proliferation were excluded. This category would be the closest to Caucasian nAMD.

The exclusion criteria were eyes with a history of any other retinal diseases; uveitis; glaucoma, including ocular hypertension, with the use of antiglaucoma eye drops; any intraocular surgery, including cataract surgery; high myopia (spherical equivalent < –6 diopters or axial length > 26.5 mm); or eyes with a treatment history of intravitreal anti-VEGF injections. Patients were excluded who had any systemic diseases potentially involving complement system activation, such as diabetes, autoimmune diseases, cancer, cardiovascular disease, cerebrovascular disease, or systemic corticosteroid medications. In controls, eyes with soft drusen or pachychoroid features were excluded.

**Aqueous Humor Collection**

Aqueous humor samples of eyes with PNV, PCV, or drusen-associated nAMD were aspirated before intravitreal injection of aflibercept (Eylea; Regeneron Pharmaceuticals, Tarrytown, NY, USA) or ranibizumab (Lucentis; Genentech, South San Francisco, CA, USA) under topical anesthesia using a syringe with a 30-gauge needle (Nipro, Osaka, Japan). The procedure and timing of sample collection were standardized to minimize variance due to the sampling procedure. The aqueous humor of controls was aspirated before cataract surgery in the same manner. The aqueous humor samples were immediately mixed with 2 μL of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) to prevent complement and anaphylatoxin activation. Aliquots
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FIGURE 1. Right eye of a 53-year-old male with PNV: (A) Color fundus photography revealed no drusen. (B) FA in the early phase revealed granular leakage. (C) ICGA in the late phase revealed CVH. (D) OCT along the white lines demonstrated irregular RPE detachment, serous retinal detachment (SRD), and thick choroid with pachychoroidal lesions (asterisks). Left eye of a 62-year-old female with PCV: (E) Color fundus photography revealed soft drusen and RPE abnormalities. (F) FA in the early phase revealed granular leakage. (G) ICGA in the early phase revealed polypoidal lesions and branching vascular network. (H) OCT along the white lines demonstrated SRD, thick choroid with pachychoroidal lesions (asterisks), and acute RPE elevation (polyps). Right eye of an 80-year-old male with drusen-associated nAMD: (I) Color fundus photography revealed no drusen. (J) FA in the early phase revealed granular leakage and window defects. (K) ICGA in the early phase revealed CNV. (L) OCT along the white lines demonstrated SRD, macular edema, and relatively thin choroid.

Measurement of Complement Activation Products and Cytokine Levels

Levels of anaphylatoxins (C3a, C4a, and C5a) in the aqueous humor were measured using a BD CBA Human Anaphylatoxin Kit (BD Biosciences, San Jose, CA, USA). The levels of cytokines (VEGF and MCP-1) in the aqueous humor were measured using a BD Human Soluble Protein Master Buffer Kit (BD Biosciences). The bead-based immunoassays were performed according to the manufacturer’s instructions.

Image Analysis

Soft drusen and pigmentary abnormalities were evaluated using color fundus photographs. Soft drusen were graded based on the simplified severity scale for AMD from the Age-Related Eye Disease Study. The central retinal thickness (CRT), presence of subretinal and intraretinal fluid, and subfoveal choroidal thickness (SFCT) were evaluated on OCT images. CRT and SFCT were measured using the caliper function of the Spectralis instrument. CRT was defined as the distance between the RPE surface and the internal limiting membrane surface. SFCT was defined as the distance between the hyporeflective line corresponding to Bruch’s membrane beneath the RPE and the choriocapillaris border.

Complete posterior vitreous detachment was defined as no vitreous attachment to the retina and disc surface on both vertical and horizontal spectral-domain OCT scans. The diagnosis of polypoidal lesions was confirmed by the presence of dilated polyps at the end of the branching vascular network on ICGA images. The presence of CVH was defined as multifocal hyperfluorescence seen on middle- and late-phase ICGA images, as judged by two masked retinal specialists (YK and YO). The kappa value of CVH judgment by the two graders was 0.72. When the retina specialists did not agree, a third grader (TS) made the final judgment regarding CVH. The greatest linear dimension was measured manually, using a tool in IMAGEnet software (Topcon), as the lesion dimension covering the area of CNV, including the areas of dye leakage, pigment epithelial detachment, and subretinal hemorrhage on fluorescein angiography. The CNV lesion size was calculated manually in each early ICGA image using the HRA2 Image Explorer “draw lesion” tool.

Statistical Analysis

The Mann–Whitney U test was used to compare the levels of anaphylatoxins and cytokines in the aqueous humor among each group. The Kruskal–Wallis test was used to compare the levels of anaphylatoxins and cytokines in the aqueous humor among the CNV subtypes. Post hoc analysis was performed using the Steel–Dwass procedure. Multidimensional scaling (MDS) multivariate analysis provides a means of visualizing the level of similarity of individual cases of a...
### Table 2.
Levels of Complement Activation Products and Cytokines in the Aqueous Humor of Patients with CNV and in Controls

| Analyte      | PNV (n = 25) Median (IQR) | PCV (n = 23) Median (IQR) | nAMD (n = 24) Median (IQR) | Total (N = 72) Median (IQR) | Controls (n = 33) Median (IQR) | Power² |
|--------------|---------------------------|---------------------------|----------------------------|-----------------------------|-------------------------------|--------|
| C3a (ng/mL)  | 2.34 (1.97–2.94)          | 3.12 (2.21–3.48)          | 2.95 (2.36–3.57)           | 2.7 (2.14–3.35)             | 2.2 (1.52–2.82)               | 0.004  |
| C4a (ng/mL)  | 1.47 (1.21–1.80)          | 1.77 (1.48–2.08)          | 1.97 (1.41–2.43)           | 1.69 (1.35–2.09)            | 1.48 (1.03–1.92)              | 0.11   |
| C5a (ng/mL)  | 0.018                     | 0.017                     | 0.027                      | 0.018                       | 0.032 (0.015–0.056)           | 0.049  |
| VEGF (pg/mL)| 42.0 (26.3–53.2)          | 40.4 (27.3–67.8)          | 38.6 (28.5–49.1)           | 40.1 (27.3–52.6)            | 25.3 (17.1–36.0)              | 0.001  |
| MCP-1 (pg/mL)| 273.9                    | 306.7                     | 311.9                      | 305.8                       | 201.3 (122.4–305.5)           | 0.001  |

† Mann–Whitney U test among CNV subtypes.
† Statistical power among the three categories.
‡ Mann–Whitney U test between the eyes with CNV and controls.
§ Statistical power between total eyes with CNV and controls.

### Table 3.
Demographics of Patients with CNV and Controls

| Analyte      | PNV (n = 25) | PCV (n = 23) | nAMD (n = 24) | Total (N = 72) | Controls (n = 33) |
|--------------|--------------|--------------|---------------|----------------|-------------------|
| Age (y), mean ± SD | 70.8 ± 9.4   | 72.6 ± 7.7   | 76.7 ± 8.6    | 73.5 ± 8.9    | 72.3 ± 6.3        |
| Female, n (%) | 4 (16.0)     | 6 (26.1)     | 11 (45.8)     | 21 (29.1)     | 17 (51.5)         |
| Complete PVD, n (%) | 12 (48.0) | 13 (56.5)   | 11 (45.8)     | 39 (54.2)     | 20 (60.6)         |
| Spherical equivalent (D), median (IQR) | 0.38 (–1.31 to 1.44) | 0 (–1.38 to 0.75) | –0.25 (–1.66 to 1.50) | 0.79 (–1.37 to 1.47) | 0.06 (–1.87 to 0.25) |
| CRT (μm), median (IQR) | 302 (221–441) | 414 (285–482) | 392 (348–572) | 379 (284–480) | —                 |
| SFCT (μm), median (IQR) | 340 (256–401) | 285 (226–353) | 210 (146–258) | 274 (204–351) | —                 |
| GLD (μm), median (IQR) | 3.5 (2.7–4.7) | 3.9 (3.5–5.0) | 4.9 (3.2–6.4) | 4.0 (2.9–5.2) | —                 |
| CNV size (mm²), median (IQR) | 1.4 (0.6–4.2) | 1.1 (0.7–2.5) | 2.2 (1.2–4.7) | 1.4 (0.8–3.5) | —                 |
| SRF, n (%) | 20 (80.0)    | 21 (91.3)    | 17 (70.8)     | 58 (80.5)     | —                 |
| IRF, n (%) | 1 (4.0)      | 2 (8.7)      | 8 (33.3)      | 11 (15.3)     | —                 |
| Soft drusen, n (%) | 0 (0)       | 0 (0)        | 11 (45.8)     | <0.001        | 11 (10.6)         |
| Pigmentary abnormalities, n (%) | 21 (84.0) | 20 (86.9) | 21 (87.5) | 62 (59.1) | —                 |

GLD, greatest linear dimension; SRF, subretinal fluid; IRF, intraretinal fluid.
† Kruskal–Wallis test among CNV subtypes.
† Mann–Whitney U test between the eyes with CNV and controls.

### RESULTS

#### Clinical Features

Table 3 shows the demographic and clinical characteristics of patients with PNV, PCV, or drusen-associated nAMD and of the controls. A total of 105 patients (72 patients with CNV, 33 controls) were enrolled. The mean age was 73.5 ± 8.9 years (mean ± SD) for eyes with CNV and 72.3 ± 6.3 years in the control group (P = 0.17). The mean age of the patients with drusen-associated nAMD was higher than that of the controls (P = 0.01) and the PNV group (P = 0.02) by pairwise analysis. The percentage of female patients with CNV (n = 21, 29.1%) was lower than that in the control group (n = 17, 51.5%; P = 0.28). In the 72 eyes with CNV, the number of eyes with PNV, PCV, or drusen-associated nAMD was 25 (35%), 23 (32%), and 24 (33%), respectively. There were no significant differences in age, sex, equivalent spherical power, incidence of posterior vitreous detachment, subretinal fluid and pigmentary abnormalities, greatest linear dimension, or CNV lesion size among the three subtypes. CRT for the PNV, PCV, and drusen-associated nAMD groups (median, 302, 414, and 392 μm, respectively; interquartile range [IQR], 221–441, 285–482, and 348–572, respectively; P = 0.029), incidence rates of intraretinal fluid
(numbers of eyes, 1, 2, and 8, respectively; *P = 0.011), soft drusen (numbers of eyes, 0, 0, and 11, respectively; *P < 0.001), and SFCT (median, 340, 285, and 210 μm, respectively; IQR, 256–401, 226–353, and 146–258, respectively; *P = 0.005) differed among the three subtypes. SFCT was greater in the PNV group (median, 340 μm; IQR, 257–401) than in the drusen-associated nAMD group (median, 210 μm; IQR, 146–258; *P = 0.001), whereas there were no differences in SFCT between the PNV and PCV groups (median, 285 μm; IQR, 226–353; *P = 0.301) (Fig. 2).

Concentrations of Complement Activation Products and Cytokines

The median levels of C3a, C4a, C5a, VEGF, and MCP-1 in the aqueous humor were 2.70 ng/mL (IQR, 2.14–3.35), 1.69 ng/mL (IQR, 1.35–2.09), 0.018 ng/mL (IQR, 0.010–0.033), 40.1 pg/mL (IQR, 27.3–52.6), and 305.8 pg/mL (IQR, 221.4–423.1), respectively, in the eyes with CNV. In the controls, the respective levels were 2.20 ng/mL (IQR, 2.14–3.35), 0.015–0.056), 25.3 pg/mL (IQR, 17.1–36.0), and 201.3 pg/mL (IQR, 122.4–305.5) (Table 3). The concentrations of C3a (*P = 0.004), VEGF (*P = 0.001), and MCP-1 (*P = 0.001) were significantly higher in the eyes with CNV compared to controls. Because the C5a level in the eyes with CNV was lower than in the control group (*P = 0.049), the statistical power was low (0.18).

The post hoc analysis among the subtypes showed that C3a levels were significantly higher in the PCV group (*P = 0.032) and drusen-associated nAMD group (*P = 0.01) than in the control group (Fig. 3). There were no significant differences in C4a levels between the eyes with CNV and the controls (*P = 0.11). There were also no differences in C5a levels among the PCV, PCV, and drusen-associated nAMD groups and the controls. VEGF levels in the PCV (*P = 0.016), PCV (*P = 0.009), and drusen-associated nAMD (*P = 0.043) groups were higher than in the control group. There were no differences in VEGF levels among the PNV, PCV, and drusen-associated nAMD groups. MCP-1 levels in the PCV (*P = 0.039) and drusen-associated nAMD (*P = 0.011) groups were significantly higher than in the controls, but they did not differ significantly between the PNV group and the controls (*P = 0.294).

In the controls, significant correlations were seen among C3a, C4a, C5a, VEGF, and MCP-1, except for C3a versus VEGF (*P = 0.069) and C3a versus VEGF (*P = 0.145) (Table 4). In the PNV, no significant differences were seen among the proteins measured except for C3a versus C4a (rs = 0.578, *P = 0.003). In the PCV group, positive correlations were seen between C3a and VEGF (rs = 0.601, *P = 0.002), C3a and MCP-1 (rs = 0.608, *P = 0.002), C4a and MCP-1 (rs = 0.452, *P = 0.031), and VEGF and MCP-1 (rs = 0.767, *P < 0.001). In the drusen-associated nAMD group, positive correlations were seen between C3a and C4a (rs = 0.664, *P < 0.001), C3a and C5a (rs = 0.501, *P = 0.014), C3a and MCP-1 (rs = 0.619, *P = 0.001), C4a and MCP-1 (rs = 0.452, *P = 0.027), C5a and MCP-1 (rs = 0.444, *P = 0.034), and VEGF and MCP-1 (rs = 0.629, *P = 0.001). VEGF levels were not correlated with C3a, C4a, and C5a levels, but MCP-1 levels were correlated with all of them in the drusen-associated nAMD group.

Similarity

MDS was determined by plotting the mean value of each concentration on a map to visualize the similarities of the complement activation products and cytokines in each
FIGURE 3. Box plot showing median levels of complement activation products and cytokines in aqueous humor of CNV patients and controls. *P < 0.05, Steel–Dwass test. (A) Median levels of C3a were higher in PCV and drusen-associated nAMD than in controls (PCV vs. controls, P = 0.032; drusen-associated nAMD vs. controls, P = 0.010). (B) There were no significant differences in the levels of C4a for each CNV subtype. (C) The levels of C5a were higher in controls than in PNV (P = 0.039). (D) VEGF levels were higher in all CNV subtypes compared to controls (PNV vs. controls, P = 0.016; PCV vs. controls, P = 0.009; drusen-associated nAMD vs. controls, P = 0.043). (E) MCP-1 levels were higher in PCV and drusen-associated nAMD than in controls (PCV vs. controls, P = 0.039; drusen-associated nAMD vs. controls, P = 0.011).

DISCUSSION

C3a, VEGF, and MCP-1 levels in the aqueous humor of the eyes with drusen-associated nAMD and PCV were higher than those of controls. By contrast, C3a and MCP-1 levels in the aqueous humor of the eyes with PNV did not differ from those in the control group, but the VEGF level was significantly higher in the PNV than in the control group. The measurement items showing the correlation were different for each disease type.

Previous genetic studies have suggested an association between the complement system and AMD. Also, elevation of complement activation products (complement factors B a, H, I, C3a, and C4a) in the aqueous humor have been reported. Many cytokines have also been reported as causative factors associated with AMD, VEGF, MCP-1, soluble intercellular adhesion molecule-1, soluble vascular cell

group (Fig. 4). The PNV, PCV, and drusen-associated nAMD mean values were plotted at almost the same distances from the controls. The PCV mean value was plotted between drusen-associated nAMD and PNV. Among the complement activation products and cytokines measured in the current study, all three subtypes had low similarity to the controls; the profiles of PNV and drusen-associated nAMD differed the most.
in nAMD. Macrophages can reportedly be involved in late in the choroidal neovascular membrane and choroid. Histopathologic studies suggest that monocytes accumulate in VEGF production could be mediated by MCP-1. MCP-1 profile of the aqueous humor suggests that an increase in the measurement range of the cytometric bead array. C5a can has various important effects on the inflammatory process, including increased vascular permeability, release of proinflammatory molecules, and monocyte migration. Measurement of C5a levels in the aqueous humor by enzyme-linked immunosorbent assays should be required in the future. Third, all proteins associated with CNV under the retina may not reflect the aqueous humor profiles, although we have previously confirmed correlation of anaphylatoxin levels between the vitreous and aqueous humor. Therefore, we should also consider protein production from other parts of the eye when we interpret cytokine concentrations in the aqueous humor. Fourth, it is ethically difficult to collect aqueous humor from completely healthy eyes. The severity of cataract should be evaluated in future studies. Fifth, there were significant differences in age and gender among the PNV, PCV, and drusen-associated nAMD groups. These differences could have influenced the results and should be taken into account.
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consideration when interpreting differences among the three categories.

In conclusion, PNV, PCV, and drusen-associated nAMD showed significantly distinct profiles of complement activation products and cytokines in the aqueous humor. Our results suggest that differences in the actions of complement systems and cytokines led to differences in the respective pathophysiologies of PNV, PCV, and drusen-associated nAMD. The profiling of complement activation products and cytokines in the aqueous humor can be useful to evaluating the biologic characteristics of CNV.

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