Aqueous humor cytokine profiling in patients with wet AMD

Fang Liu, Xiaoyan Ding, Yu Yang, Jiaqing Li, Miao Tang, Miner Yuan, Andina Hu, Zongyi Zhan, Zijing Li, Lin Lu

(The first two authors contributed equally to this work.)

State Key Laboratory of Ophthalmology, Retina Division, Zhongshan Ophthalmic Center, Sun Yat-sen University, 510060, Guangzhou, Guangdong, China

Purpose: To investigate the chemokine expression profiles in the aqueous humor of wet age-related macular degeneration (wet AMD) patients and to correlate their levels with clinical findings.

Methods: Undiluted aqueous humor samples (100–200 μl) were obtained from 16 wet AMD eyes and 12 control eyes. Forty chemokines were measured using a multiplex method. A 6×6 mm area of the macular region centered on the fovea was examined using spectral domain optical coherence tomography (SD-OCT).

Results: The detection rates were 50% or more for 15 chemokines. Compared with the control group, the aqueous humor in wet AMD patients showed a significantly higher expression of CXCL10 (p=0.004), CCL14 (p=0.002), CXCL16 (p=0.013), CXCL7 (p=0.033), and CCL22 (p=0.037), while growth-related oncogene (GRO) was significantly decreased in the wet AMD patients (p=0.001). When compared with treatment-naïve patients, the recurrent group had significant upregulation of CXCL10 (p=0.012) and CCL22 (p=0.002). CXCL16 was positively correlated with lesion size, and CCL22 was higher in patients whose OCT images showed intraretinal fluid (IRF) or hyperreflective foci (HF).

Conclusions: Elevated levels of inflammation-related chemokines, including CXCL10, CCL14, CXCL16, CXCL7, and CCL22, in the aqueous humor of AMD patients may suggest a pathogenic role for inflammation. CXCL10 and CCL22 were more elevated in eyes with recurrent wet AMD than in treatment-naïve eyes. CXCL16 was positively correlated with lesion size. The increase in CCL22 was correlated with the presence of IRF or HF. These data may be of interest in the search for biomarkers associated with wet AMD and may potentially indicate different treatment strategies.

Age-related macular degeneration (AMD) is the leading cause of irreversible central visual impairment in the elderly population worldwide [1]. Advanced AMD is generally classified into two categories: geographic atrophy AMD (aAMD) or “dry” AMD and exudative (eAMD) or “wet” AMD. While genetic and environmental factors, such as aging, diet, inflammation, and oxidative stress, have been linked to AMD pathogenesis, the etiology of this complicated disease is still unknown.

Consistent with the role of chronic inflammation in AMD pathogenesis, several inflammatory mediators, including complement components, chemokines, and cytokines, are elevated at both the local and systemic levels in AMD patients [1-5]. Chemokines are a superfamily of 8- to 10-kDa soluble chemotactic cytokines that bind to their connoct G protein-coupled receptors to cause a cellular response, such as migration, adhesion, or chemotaxis. They are produced by tissue cells and leukocytes and regulate leukocyte migration in inflammatory and immune processes.

Chemokines are grouped into the CXC, CC, C, and CX3C subfamilies based on the arrangement of the conserved cysteine residues. Several studies have suggested that chemokine signaling plays a role in age-related retinal disease [2,6]. Chemokine expression in retinal pigment epithelial cells has been studied in vitro using a laser-induced choroidal neovascularization model [7]. Increased secretion of CCR2 on systemic pro-inflammatory monocytes and elevated CCL2 have been found in the aqueous humor of wet AMD patients, indicating the role of this chemokine pathway in age-related ocular pathology [4,6]. An improved understanding of the role of chemokines in wet AMD is necessary for successful therapeutic strategies.

To the best of our knowledge, chemokine profiles in the aqueous humor of patients with wet AMD have not been described. The aim of this study was therefore to investigate the chemokine expression profiles of wet AMD patients and to correlate their levels with clinical disease phenotypes.

METHODS

Subjects: This study was conducted in accordance with the World Medical Association’s Declaration of Helsinki and was approved by the Institutional Review Board of Zhongshan Ophthalmic Center, Sun Yat-sen University. Informed consent was obtained from all patients after a full explanation of the study. The project was sponsored by the National Natural Science Foundation of China (81400596) and the Ministry of Science and Technology of China (2015YFC0106302).
consent was obtained from all patients and controls. Sixteen patients with wet AMD who received an intravitreal injection of ranibizumab (Lucentis; Genentech, San Francisco, CA, USA) were recruited, and 12 age-matched patients with age-related cataracts who underwent routine phacoemulsification surgery served as controls. The exclusion criteria were: 1) age below 50; 2) history of any other ocular diseases, apart from age-related cataracts; 3) any previous intraocular surgery or verteporfin photodynamic therapy or intravitreal triamcinolone injection; and 4) previous history of any intravitreal anti-vascular endothelial growth factor (VEGF) treatment within the last six months in the study eye or within the last three months in the fellow eye.

All subjects underwent a complete ophthalmic examination, including Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity testing, slit-lamp examination, intraocular pressure measurements, fundus examinations, fluorescein angiography, and optical coherence tomography (OCT) (Heidelberg HRA SPECTRALIS/HRA2, Heidelberg Engineering, Heidelberg, Germany). The wet AMD patients enrolled in this study were divided into two subgroups according to disease history: seven eyes in the treatment-naive group and nine eyes in the recurrent group (with a history of ranibizumab injection at least six months before).

**SD-OCT measurement:** A 6×6 mm area of the macular region centered on the fovea was examined using spectral domain OCT (SD-OCT). All examinations were performed by one well-trained technician, and only images showing well-defined retinal layers were selected. The OCT images of wet AMD patients were analyzed by two independent masked investigators. The presence of intraretinal fluid (IRF), subretinal fluid (SRF), subretinal hemorrhage (SRH), hyperreflective foci (HF) at the outer retinal layer, and retinal pigment epithelium detachment (PED) was recorded. The greatest linear diameter (GLD) of lesions; maximal retinal thickness (MRT), defined as the maximal vertical distance between the inner limiting membrane (ILM) and the surface of the retinal pigment epithelium (RPE) (including the height of subretinal fluid and subretinal hemorrhages); and maximal neurosensory thickness (MNT), defined as the maximal vertical distance between the ILM and the outer surface of the retinal photoreceptors, were measured.

**Aqueous humor collection and chemokine analysis:** All sample collections were performed using a standard sterilization procedure. Undiluted aqueous humor samples (100–200 μl) were obtained from 16 eyes of 16 wet AMD patients through anterior chamber paracentesis before performing intravitreal ranibizumab injections and from 10 control eyes immediately before cataract surgery. Paracentesis was performed before any conjunctival or intraocular manipulation to avoid breakdown of the blood–aqueous barrier associated with surgical trauma. Samples were snap-frozen and maintained at -80 °C until analysis.

To assess the concentration of chemokines, we used a multiplex method that allows the simultaneous determination of 40 chemokines per sample (Quantibody® Human Chemokine Array, RayBiotech, Inc., Norcross, GA). The sets consisted of the following chemokines: 6Ckine/CCL21, Axl, BTC, CCL28, CTACK/CCL27, CXCL16, ENA-78/CXCL5, Eotaxin-3/CCL26, GCP-2/CXC, GRO (GROα/CXCL1, GROβ/CXCL2, GROγ/CXCL3), HCC-1/CCL14, HCC-4/CCL16, IL-9, IL-17F, IL18-BPα, IL-28A, IL-29, IL-31, IP-10/CXCL10, I-TAC/CXCL11, LIF, LIGHT/TNFSF14, Lymphotactin/XCL1, MCP-2/CCL8, MCP-3/CCL7, MCP-4/CCL13, MDC/CCL22, MIF, MIP-3α/CCL20, MIP-3β/CCL19, MPIF-1/CCL23, NAP-2/CXCL7, MSPα, OPN, PARC/CCL18, PF4, SDF-1/CXCL12, TARC/CCL17, TECK/CCL25, and TSLP. The kit was used according to the manufacturer’s instructions. The results of cytokine concentrations were analyzed using a Quantibody Q-Analyzer (RayBiotech, Inc., Norcross, GA).

**Statistical analysis:** The data were processed and analyzed statistically using SPSS (version 13.0; SPSS, Chicago, IL). For categorical variables, the frequency distribution and percentages were calculated and compared using a chi-square test. For numerical variables, the data were denoted by means ± standard deviations. Chemokine concentrations were compared between wet AMD and control eyes using a Mann–Whitney U test. Correlations between cytokine concentrations and OCT data, including the GLD of lesions, the MRT, and the MNT, were calculated using a Spearman’s correlation test. For the correction of multigroup comparisons, p values of 0.01 for the Spearman’s correlation test were considered statistically significant, with significance levels of 0.05 based on the Bonferroni method. Two-tailed tests or Mann–Whitney U tests were used to compare chemokines between wet AMD patients with and without IRF, SRF, SRH, HF, and PED. p<0.05 was considered statistically significant.

**RESULTS**

The study group included 16 patients (seven females and nine males) with wet AMD, and the control group consisted of 12 patients (five females and seven males) with age-related cataracts only. Age did not vary significantly (p=0.675) between the study group (69.7±7.6 years; range 53–87) and the control group (68.5±8.1 years; range 57–85), and nor did gender (p=0.324). There were no statistical differences in the age and gender of the treatment-naïve wet AMD patients (70.9±10.0 years, three males) and the recurrent wet AMD patients.
(68.8±5.6 years, six males), with p values of 0.758 and 0.341, respectively. The presence of IRF was only detected in recurrent wet AMD patients (8/16, 50%). There were no statistical differences between the two subgroups upon comparison of the GLD of lesions, MRT, MNT, and the presence of SRF, SRH, HF, and PED (Table 1).

The aqueous concentrations of 40 chemokines were measured in both the wet AMD group and the control group (Table 2). The positive expression rates were more than 50% for 15 chemokines: IP-10/CXCL10 (100%), OPN (100%), PARC/CCL18 (100%), GROα (100%), HCC-1/CCL14 (100%), CXCL16 (100%), ENA-78/CXCL5 (89.3%), LIGHT/TNFSF14 (85.7%), GCP-2/CXCL6 (82.1%), CCL28 (64.3%), NAP-2/CXCL7 (78.6%), HCC-4/CCL16 (60.7%), SDF-1/CXCL12 (50%), PF4 (82.1%), and MDC/CCL22 (71.4%). The other 25 chemokines were detected in fewer than 50% of the wet AMD and control group samples and therefore were not included in the statistical analysis (Table 2).

Compared to the control group, the wet AMD group showed a significantly higher expression of the following chemokines: IP-10/CXCL10 (p=0.004), HCC-1/CCL14 (p=0.002), CXCL16 (p=0.013), NAP-2/CXCL7 (p=0.033), and MDC/CCL22 (p=0.037; Table 3). GRO, including GROα/ CXCL1, GROβ/CXCL2, and GROγ/CXCL3, was significantly decreased in the aqueous humor of wet AMD patients compared to the that of the controls (p<0.001). In contrast, there was no significant difference in the expression levels of osteopontin (OPN), pulmonary and activation-regulated chemokines (PARC/CCL18), epithelial neutrophil-activating protein 78(ENA-78/CXCL5), ligand for herpesvirus entry mediator (LIGHT/TNFSF14), granulocyte chemotactic protein 2(GCP-2/CXCL6), chemokine (C-C Motif) ligand 28(CCL28), hemofiltrate CC chemokine 4(HCC-4/ CCL16), stromal cell-derived factor-1(SDF-1/CXCL12), and platelet factor 4(PF4) between patients and controls (Table 3). Further separation of the wet AMD group into recurrent and treatment-naïve groups showed that the recurrent group had significantly elevated concentrations of IP-10/CXCL10 (p=0.012) and MDC/CCL22 (p=0.002) compared to the treatment-naïve group (Table 4). The groups did not differ significantly in the concentrations of OPN, PARC/CCL18, GROα, HCC-1/CCL14, CXCL16, ENA-78/CXCL5, LIGHT/ TNFSF14, GCP-2/CXCL6, CCL28, NAP-2/CXCL7, HCC-4/ CCL16, SDF-1/CXCL12, and PF4.

Table 1. Demographic and clinical data of wet AMD and control.

| Number | Wet AMD | treatment-naïve | recurrent | Control | P † |
|--------|---------|-----------------|-----------|---------|-----|
| Age (yr, mean±SD) | 69.7±7.6 | 70.9±10.0 | 68.8±5.6 | 68.5±7.5 | 0.683², 0.758³ |
| Male/Female (Male %) | 9/7 (56) | 3/4 (43) | 6/3 (67) | 7/5 (58) | 0.912², 0.341³ |
| BCVA | 39.27±16.61 | 39.17±19.47 | 39.33±15.68 | - | 0.689 |
| GLD of lesion | 4833.3±1693.62 | 5110.10±1947.90 | 4618.00±1553.44 | - | 0.681 |
| MRT | 524.12±197.60 | 410.43±180.00 | 612.56±169.72 | - | 0.055 |
| MNT | 345.94±92.28 | 299.86±74.54 | 381.78±92.17 | - | 0.071 |
| Presence of IRF (%) | 8(50) | 0(0) | 8(88.9) | - | 0.001³ |
| Presence of SRF (%) | 10(62.5) | 3(42.9) | 7(77.8) | - | 0.302 |
| Presence of SRH (%) | 10(62.5) | 6(85.7) | 4(44.4) | - | 0.145 |
| Presence of HF (%) | 9(56.3) | 3(42.9) | 6(66.7) | - | 0.615 |
| Presence of PED (%) | 11(68.8) | 5(71.4) | 6(66.7) | - | 1 |

*SD, standard deviation; BCVA, Best Corrected Visual Acuity; GLD, greatest linear diameter; MRT, maximal retinal thickness; MNT, maximal neurosensory thickness; IRF, intraretinal fluid; SRF, subretinal fluid; SRH, subretinal hemorrhage; HF, hyperreflective foci; PED, pigment epithelial detachment. †Significance of differences between groups: χ² test or Mann–Whitney U test.
### Table 2. Summary data for chemokine levels of wet AMD and control.

| Chemokines                        | Levels detected (pg/ml) | Wet AMD (n=16) | Control (n=12) | P * |
|-----------------------------------|-------------------------|----------------|----------------|-----|
| IP-10/CXCL10                      | 6.86                    | 16(100.00)     | 12(100.00)     |     |
| OPN                               | 276.2                   | 16(100.00)     | 12(100.00)     |     |
| PARC/CCL18                        | 20.66                   | 16(100.00)     | 12(100.00)     |     |
| GRO-α/β/γ (CXCL1/CXCL2/ CXCL3)    | 9.61                    | 16(100.00)     | 12(100.00)     |     |
| HCC-1/CCL14                       | 3.55                    | 16(100.00)     | 12(100.00)     |     |
| CXCL16                            | 14.96                   | 16(100.00)     | 12(100.00)     |     |
| ENA-78/CXCL5                      | 11.4                    | 13(81.25)      | 10(83.33)      |     |
| LIGHT/TNFSF14                     | 12.87                   | 13(81.25)      | 11(91.67)      | 0.613|
| GCP-2/CXCL6                       | 30.96                   | 13(81.25)      | 10(83.33)      |     |
| CCL28                             | 110.89                  | 11(68.75)      | 7(58.33)       | 0.698|
| NAP-2/CXCL7                       | 8.13                    | 14(87.50)      | 8(66.67)       | 0.354|
| HCC-4/CCL16                       | 24.85                   | 11(68.75)      | 6(50.00)       | 0.441|
| SDF-1/CXCL12                      | 50.12                   | 10(62.50)      | 4(33.33)       | 0.252|
| PF4                               | 277.41                  | 14(87.50)      | 9(75.00)       | 0.624|
| MDC/CCL22                         | 39.44                   | 12(75.00)      | 8(66.67)       | 0.691|
| 6Ckine                            | 768.9                   | 3(18.75)       | 6(50.00)       | 0.114|
| Eotaxin-3                         | 96.11                   | 3(18.75)       | 2(16.67)       |     |
| MIF                               | 6.28                    | 4(25.00)       | 4(33.33)       | 0.691|
| MIP-3α/CCL20                      | 13.1                    | 5(31.25)       | 3(25.00)       |     |
| MIP-3β/CCL19                      | 15.65                   | 1(6.25)        | 0(0.00)        |     |
| MSPα                              | 125.14                  | 4(25.00)       | 3(25.00)       |     |
| BTC                               | 83.38                   | 4(25.00)       | 2(16.67)       | 0.673|
| Axl                               | 8.67                    | 1(6.25)        | 2(16.67)       | 0.56 |
| CTACK/CCL27                       | 29.52                   | 0(0.00)        | 0(0.00)        |     |
| MCP-2/CCL8                        | 1.6                     | 0(0.00)        | 0(0.00)        |     |
| MCP-3/CCL7                        | 4.08                    | 0(0.00)        | 0(0.00)        |     |
| MCP-4/CCL13                       | 29.5                    | 0(0.00)        | 0(0.00)        |     |
| IL-28A                            | 7.01                    | 0(0.00)        | 0(0.00)        |     |
| I-TAC/CXCL11                      | 15.06                   | 0(0.00)        | 0(0.00)        |     |
| MPIF1/CCL23                       | 21.48                   | 0(0.00)        | 0(0.00)        |     |
| IL-29                             | 282.95                  | 0(0.00)        | 0(0.00)        |     |
| Lymphotactin/XCL1                 | 239.04                  | 0(0.00)        | 0(0.00)        |     |
| Chemokines  | Levels detected (pg/ml) | Wet AMD (n=16) | Control (n=12) | P  |
|-------------|------------------------|----------------|----------------|---|
| IL-18 BPa   | 79.1                   | 0(0.00)        | 0(0.00)        |    |
| IL-31       | 53.26                  | 0(0.00)        | 0(0.00)        |    |
| IL-17F      | 44.91                  | 0(0.00)        | 0(0.00)        |    |
| IL-9        | 44.34                  | 0(0.00)        | 0(0.00)        |    |
| TARC/CCL17  | 4.41                   | 0(0.00)        | 0(0.00)        |    |
| TECK/CCL25  | 83.48                  | 0(0.00)        | 0(0.00)        |    |
| TSLP        | 14.49                  | 0(0.00)        | 0(0.00)        |    |
| LIF         | 58.25                  | 0(0.00)        | 0(0.00)        |    |

*Fisher exact test, p<0.05 represent significance
Optical coherence tomograms were performed for wet AMD patients. The correlations between each cytokine and OCT parameters are shown in Table 5. The GLD of lesions and the MRT were found to correlate positively with CXCL16 ($\rho=0.688$, $p=0.003$) and MDC/CCL22 ($\rho=0.731$, $p=0.005$), respectively. No correlation was found between the aqueous cytokine levels and MNT. Appendix 1 shows the aqueous humor levels of chemokines in wet AMD patients with and without IRF, SRF, SRH, HF, or PED. Patients with SRH had a lower level of IP-10 than patients without SRH (25.06±7.85 versus 38.13±16.74, $p=0.05$), indicating a significant difference. MDC was higher in patients with IRF (143.21±26.13

### Table 3. Aqueous humor levels of chemokines in wet AMD and control.

| Chemokine (pg/ml) | Wet AMD (n=16) | Control (n=12) | P * |
|-------------------|----------------|----------------|-----|
| IP-10/CXCL10      | 29.96±13.16    | 19.67±4.16     | 0.004|
| OPN               | 40.42±23401    | 54.44±19470    | 0.133|
| PARC/CCL18        | 1413.4±1084.7  | 1424.1±810.74  | 0.732|
| GRO-α/β/γ (CXCL1/CXCL2/ CXCL3) | 91.07±51.86     | 330.91±216.31  | 0.001|
| HCC-1/CCL14       | 314.75±75.26   | 236.26±43.72   | 0.002|
| CXCL16            | 1127.7±486.4   | 730.28±156.66  | 0.013|
| ENA-78/CXCL5      | 37.73±23.27    | 26.46±14.06    | 0.708|
| LIGHT/TNFSF14     | 24.88±12.77    | 20.29±7.40     | 0.241|
| GCP-2/CXCL6       | 116.29±84.56   | 94.59±57.92    | 0.732|
| CCL28             | 128.20±37.23   | 146.00±59.59   | 0.478|
| NAP-2/CXCL7       | 17.13±6.71     | 12.38±10.52    | 0.033|
| HCC-4/CCL16       | 39.89±22.37    | 33.68±16.69    | 0.568|
| SDF-1/CXCL12      | 91.71±83.95    | 68.26±23.39    | 0.599|
| PF4               | 773.02±594.14  | 414.83±477.20  | 0.146|
| MDC/CCL22         | 158.66±44.07   | 77.25±36.18    | 0.037|

*Significance of differences between groups: Mann–Whitney U test. $p<0.05$ represent significance.

### Table 4. Chemokines in patients with recurrent and treat-naïve wet AMD.

| Chemokines (pg/ml) | Recurrent AMD (n=9) | Treat-naïve AMD (n=7) | P * |
|--------------------|---------------------|-----------------------|-----|
| IP-10/CXCL10       | 37.19±15.90         | 23.93±7.32            | 0.012|
| OPN                | 35,783±19757.33     | 46,400±28001.29       | 0.408|
| PARC/CCL18         | 1273.4±932.19       | 1593.5±1309.88        | 0.837|
| GRO                | 110.14±55.02        | 66.56±37.98           | 0.174|
| HCC-1/CCL14        | 317.84±91.22        | 310.79±55.08          | 0.918|
| CXCL16             | 1167.8±463.25       | 1076.1±547.57         | 0.837|
| ENA-78/CXCL5       | 32.49±25.01         | 51.22±11.30           | 0.113|
| LIGHT/TNFSF14      | 25.05±15.01         | 24.06±12.63           | 1    |
| GCP-2/CXCL6        | 129.24±92.38        | 115.60±70.52          | 0.864|
| CCL28              | 124.93±45.56        | 132.41±25.68          | 0.918|
| NAP-2/CXCL7        | 15.92±7.10          | 18.71±6.33            | 0.47 |
| HCC-4/CCL16        | 44.51±24.93         | 33.94±18.67           | 0.351|
| SDF-1/CXCL12       | 162.31±88.96        | 98.37±25.70           | 0.257|
| PF4                | 778.14±321.04       | 1020.3±780.25         | 0.95 |
| MDC/CCL22          | 175.39±46.03        | 137.16±37.70          | 0.002|

* Significance of differences between groups: Mann–Whitney U test. $p<0.05$ represent significance.
pg/ml) or HF (125.88±35.37 pg/ml) than in patients without IRF (82.21±28.08 pg/ml) or without HF (73.34±24.74 pg/ml), with p values of 0.006 and 0.015, respectively. However, the levels of other chemokines in the aqueous humor showed no significant difference in wet AMD patients with and without SRF or PED.

**DISCUSSION**

A major limitation of aqueous humor testing is that only small sample volumes (typically 100–200 μl of fluid) can be obtained from human eyes. These amounts are barely sufficient to test for many cytokines or chemokines using traditional enzyme-linked immunosorbent assay techniques. Therefore, in our study, most of the chemokine family members were measured using a multiplex array. Of the chemokines, six (IP-10, OPN, PARC, GROα, HCC-1, and CXCL16) were detectable in all the wet AMD and control samples, and nine (ENA-78, LIGHT, GCP-2, CCL28, NAP-2, HCC-4, SDF-1, PF4, and MDC) were detectable in more than half the wet AMD samples and half the control samples (Table 2). In prior studies, chemokine concentrations in aqueous humor were shown to be significantly increased in active uveitis, which indicates that chemokines are implicated in the development of uveitis [8,9]. However, the total concentration of chemokines in the aqueous humor may be affected by the size and location of ocular lesions. The neovascular lesions in wet AMD are relatively small and more localized compared to the lesions in uveitis, and we speculate that this is at least part of the reason for the low levels of chemokines in the aqueous humor of wet AMD patients.

Among several C-X-C motif chemokines investigated in this study, IP-10/CXCL10, CXCL16, and NAP-2/CXCL7 were found to be significantly increased in the wet AMD patients compared to the controls. CXCL16 is a potent angiogenic factor, is involved in endothelial progenitor cell chemotaxis [10], and has been suggested as a prognostic factor in cancer, bacterial infections, and systemic sclerosis [11-13]. CXCL7 is closely associated with macrophage infiltration, which indicates a potential role in the pathology of AMD [14,15]. However, neither CXCL16 expression nor CXCL7 expression have been reported to date, and further studies are necessary to explore the role of these chemokines in AMD. Moreover, we found that IP-10/CXCL10 is even higher in recurrent wet AMD patients compared to the treatment-naïve ones. IP-10 is secreted by macrophages, endothelial cells, and fibroblasts. It is a chemoattractant for macrophages, dendritic cells, and T-cells and is thought to be an angiogenic regulator in inflammatory microenvironments, such as in neovascularization. Earlier studies have described elevated serum IP-10 concentration as a risk factor for diabetes mellitus, and IP-10 is suggested to be increased in the vitreous of patients with proliferative diabetic retinopathy [16,17]. In addition, IP-10 levels are significantly elevated in the aqueous humor of wet AMD patients [2] and in postmortem eyes with AMD [18]. Several studies have reported that IP-10 mRNA and protein were upregulated in animal eyes with laser-induced choroidal neovascularization [7], experimental autoimmune uveoretinitis [19], and in nude mice after VEGF induction [20]. These data suggest that among the IFN-γ-inducible CXC chemokines, IP-10/CXCL10, CXCL16, and NAP-2/CXCL7 are the predominant chemoattractants in the angiogenesis of wet AMD. We suspect that the upregulation is due in part to the enhanced expression of VEGF in infiltrated inflammatory cells, such as macrophages, and that it may contribute to the initial inflammation, cell migration, and macrophage infiltration in angiogenesis.

Some of our findings confirm those of previous investigations, and some are new. Notably, MDC/CCL22 was found to be increased significantly in the wet AMD patients compared to controls...
to the controls, was even higher in patients with recurrent wet AMD lesions, and was higher in patients with IRF or HF, which usually indicates that the lesion is long-standing. Macrophages are characterized as M1 macrophages (classically activated) and M2 macrophages (alternatively activated) based on their functions, surface markers, and cytokine/chemokine profiles. M1 macrophages, driven by Th1 cytokines, are generally pro-inflammatory and secrete M1 chemokines, such as CXCL11. In contrast, M2 macrophages, driven by Th2 cytokines, facilitate tissue remodeling and secrete M2 chemokines, such as CCL22. Macrophage polarization is highly plastic depending on the tissue microenvironment and has been reported in ocular diseases such as choroidal melanoma and sympathetic ophthalmia [21,22]. Recently, Chan et al. reported increased expression of MDC mRNA in human wet AMD lesions [23,24]. Our study shows enhanced CCL22 in ocular fluid in active wet AMD. M2 macrophages are the main sources of CCL22 [25,26]. Increased CCL22 levels in the bronchoalveolar fluid of idiopathic pulmonary fibrosis patients have been found and positively correlated with the severity of the disease [26]. To our knowledge, this is the first study to suggest MDC evaluation in the aqueous humor of wet AMD eyes. Our findings suggest that M1 and M2 macrophages may undergo phenotype switching during AMD development and progression, which offers important evidence of M2 polarization in active wet AMD, especially in recurrent cases with more fibrosis and in long-standing lesions with IRF or HF. However, further investigations involving more patients with varied phenotypes are required to confirm this and to determine whether it is the cause or the result of the pathological changes in AMD. Future research may suggest different pathomechanisms for pathological angiogenesis.

Interestingly, when we tested the concentration of total GRO, which consisted of three subunits of the total GRO expression, including GROα/CXCL1, GROβ/CXCL2, and GROγ/CXCL3, a significantly lower concentration was found in both naïve and recurrent wet AMD compared to the control patients with cataracts. This is not in agreement with prior studies. GRO is produced in the aqueous humor and can be produced by ciliary epithelial cells [27]. It has been demonstrated that GROα, the main subtype of GRO, is an essential mediator for neutrophil infiltration in rabbit uveitis models [27] and is significantly higher in the aqueous humor of patients with Behcet’s disease or Vogt–Koyanagi–Harada disease [28]. There has also been marked upregulation of GROα after thrombin stimulation in several tumor cell lines and in endothelial cells [29]. However, in one previous study on preterm delivery, the same chemokine kits were employed but lower concentrations of total GRO were found compared to the control group, which was different from other similar studies [30]. It is not known what might cause decreased levels of total GRO; however, GRO is probably increased in the GROα subunit. It is necessary to perform further studies to confirm the expression of total GRO in each subunit, especially GROβ and GROγ, which are poorly understood.

The limitations of our study must be mentioned. First, the number of enrolled patients was relatively low and a limited number of samples were examined because of the nature of the prospective study. The study and control groups were too small for a meaningful multivariate analysis, so it remains inconclusive whether interdependencies between the various cytokines influenced the results. Despite this, the results were statistically significant, so the relatively small number of patients may serve to strengthen the results and conclusions of the study. Second, the concentration of the cytokines was determined from aqueous samples and not from vitreous samples, which usually show higher concentrations and may better reflect the situation of the retina and the choroid [31]. However, obtaining vitreous samples from our patients was not possible as it would have necessitated intravitreal interventions. Third, although AMD is associated with blood–retina barrier breakdown, it remains equivocal as to whether the increased concentrations of the cytokines in the aqueous humor were because of leakage through the macular lesions or because the chemokines were actively produced in the eye and antagonized the development of wet AMD. Fourth, due to the limited volume of the aqueous humor samples, we could not determine all cytokines or examine all molecules that may potentially be associated with wet AMD.

In conclusion, this study demonstrated that the expression of IP-10/CXCL10, HCC-1/CCL14, CXCL16, NAP-2/CXCL7, and MDC/CCL22 was increased in wet AMD eyes, particularly IP-10/CXCL10 and MDC/CCL22 in eyes with recurrent lesions. CXCL16 is positively correlated with the size of lesion, and MDC/CCL22 is correlated with the presence of IRF and HF. These data may be of interest in the search for biomarkers associated with wet AMD. The complexity of macrophage polarization requires further research to be fully understood. As a promising target the results of our study may potentially suggest different treatment strategies.

**APPENDIX 1. SUBGROUP ANALYSIS OF CHEMOKINES IN WET AMD PATIENTS**

To access the data, click or select the words “Appendix 1.” *IRF, intraretinal fluid; SRF, subretinal fluid; SRH, subretinal hemorrhage; HF, hyperreflective foci; PED, pigment
epithelial detachment. † Significance of differences between groups: t test or Mann–Whitney U test. ‡ With significant difference.

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