CCL1, IL-22, and IL-36β Have Higher Expression Levels in Aqueous Humor of Neovascular Age-Related Macular Degeneration Patients in Comparison to Cataract Patients

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Abstract

Purpose: To investigate the aqueous humor cytokine expression profile in neovascular age-related macular degeneration (nAMD) patients before and after ranibizumab treatments in comparison to cataract patients.

Methods: This prospective study included 20 treatment-naïve nAMD eyes of 20 patients who received three consecutive monthly injections of ranibizumab. Aqueous humor samples were collected before the first (baseline), second (1 month later), and third (2 months later) injections. Controls were 20 age- and gender-matched cataract patients without any other ocular disease. The aqueous concentrations of 28 cytokines were measured using a multiplex bead assay. Central macular thickness (CMT) and maximum retinal thickness (MRT)-3mm were measured by spectral domain optical coherence tomography (SD-OCT). The greatest linear diameter (GLD) was measured by fundus fluorescein angiography (FA).

Results: Nine cytokines in aqueous humor, including angiogenin, bone morphogenetic protein-9 (BMP-9), C-C motif chemokine ligand 1 (CCL1), CCL13, interleukin-8 (IL-8), fibroblast growth factor-acidic (FGF-α), IL-22, IL-36β, and vascular endothelial growth factor-C (VEGF-C) were significantly higher in nAMD patients in comparison to cataract patients, whether before or after two consecutive monthly ranibizumab injections. Compared with the nAMD patients' basal levels, two consecutive monthly ranibizumab injections effectively reduced the aqueous concentration of VEGF-A, improved best corrected visual acuity (BCVA), as well as reduced the values of CMT, MRT-3mm, and GLD.

Conclusions: Nine cytokines have higher expression levels in nAMD patients in comparison to cataract patients, whether before or after 2 months of ranibizumab therapy. These cytokines may have correlations with the pathogenesis of nAMD.

Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness in the elderly population. Subfoveal geographic atrophy and choroidal neovascularization (CNV) cause severe visual loss in AMD patients.[1] CNV is the main feature of neovascular AMD (nAMD), in which the formation of new aberrant blood vessels leads to macular edema, hemorrhage, fibrosis, and visual impairment.[2]

Although nAMD accounts for only approximately 20% of the overall incidence of AMD, this subtype of patients is most likely to have severe visual loss among AMD patients.[3] Polypoidal choroidal vasculopathy (PCV) is a common subtype of nAMD, which is characterized by branching choroidal networks with polyp-like aneurysmal dilation.[4]

Drusen deposition, RPE hypertrophy, pigment extrusion, and photoreceptor degeneration followed by the invasion of choroidal blood vessels through the Bruch membrane into the retina all lead to nAMD.[5] Angiogenesis and the development of local inflammatory responses are two major known contributors to nAMD pathogenesis.[2, 6] Vascular endothelial growth factor (VEGF) and many other cytokines are important mediators of inflammatory responses in nAMD patients.[2, 7] Ranibizumab is a kind of anti-VEGF drug that can block VEGF to suppress both excessive angiogenesis and inflammation, improving the clinical appearance of nAMD patients.[8] However, recurrent or persistent CNV is still very common, despite repeated application of anti-VEGF agents, and the efficacy of only using anti-VEGF drugs is still debatable.[9, 10]

It is reported that activated VEGF receptors shed into the vitreous in eyes with nAMD.[11] The aqueous levels of various cytokines (such as VEGF) are significantly correlated with their vitreous fluid levels.[12] Studying cytokines in the aqueous humor of patients with nAMD is feasible and necessary to understand the pathogenesis of nAMD, and could provide ideas to develop new antibody drugs. Previous research has reported that in the aqueous humor of eyes with nAMD, many cytokines are found at higher concentrations than in the eyes of cataract patients. These cytokines include angiogenin,[13] monocyte chemoattractant protein 1 (MCP–1),[14] interleukin–8 (IL–8),[14] macrophage inflammatory protein (MIP)–1α,[15] macrophage-derived chemokine (MDC),[15] and IFN-γ–inducible protein 10 (IP–10/CXCL10).[15] A previous study has reported that cytokines, including inflammatory factors, are involved in the pathogenesis of nAMD, and that these inflammatory factors respond to anti-VEGF agents differently.[14]

However, many cytokines that have different expression in the eyes of nAMD remain unrecognized.

For example, there are few studies about the concentration of C-C motif chemokine ligand (CCL) in aqueous humor of nAMD patients. To fill these gaps in knowledge, we conducted the current analysis. The purpose of the present study was to compare various cytokine levels in the aqueous humor of eyes with nAMD before and after treatments with ranibizumab, in comparison to cataract controls. The differences in aqueous cytokines and clinical appearances caused by ranibizumab treatments were also studied.

Methods

Study design and approval

This study was a prospective observational study. Approval for the collection and research of human aqueous humor was permitted by the Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine. All patients were treated in accordance with the Declaration of Helsinki, and informed consents were obtained from all participants prior to their participation in the study.

Inclusion/Exclusion criteria

In the present study, 20 eyes of 20 consecutive patients with nAMD and 20 eyes of 20 consecutive patients with age-related cataract were included. All eyes were treatment-naïve.

All patients with nAMD or cataract were examined in a full ophthalmological examination, including best corrected visual acuity (BCVA) determined as the logarithm of the minimum angle of resolution (logMAR), slit-lamp biomicroscopy, intraocular pressure (IOP) measurement, dilated fundus examination, color fundus photography, and spectral domain optical coherence tomography (SD-OCT). In addition to the above examinations, participates in nAMD group were examined by fundus fluorescein angiography (FA) and fundus indocyanine green angiography (ICGA). SD-OCT was used to exclude patients with AMD in the cataract group.

The diagnosis of nAMD and the discrimination of type I and II CNV were confirmed by FA. The identification of polypoidal choroidal vasculopathy (PCV) was performed by FA and ICGA. Every patient underwent SD-OCT at every visit. Measured by SD-OCT, retinal thickness (RT) refers to the vertical distance from the inner limiting membrane to the Bruch’s membrane, and central macular thickness (CMT) was defined as the vertical distance from the inner limiting membrane to the Bruch’s membrane, at the central fovea of macula. Maximum retinal thickness (MRT)–3mm was measured by

selecting a point with horizontal distance from the central fovea of macula within 3 mm to maximize the vertical retinal thickness. The greatest linear diameter (GLD) was measured by FA.

The inclusion criteria were: (1) older than 50 years; (2) treatment-naïve nAMD or cataract; (3) absence of concurrent ocular diseases. The exclusion criteria were: (1) pathologic myopia; (2) a history of treatment for nAMD, including intravitreal drug injection, photodynamic therapy, and steroid therapy; (3) previous intraocular surgery, except for cataract surgery (for nAMD patients, the cataract surgery had to have been performed at least 12 months prior to inclusion); (4) active inflammation, diabetes mellitus, use of immunosuppressive drugs and corticosteroids, localized and systemic malignant tumors were all excluded from this study.

Aqueous humor sample collection

All of the nAMD patients received three consecutive monthly intravitreal injections of 0.5 mg ranibizumab. Aqueous samples were collected at baseline (before the first injection), month 1 (before the second injection), and month 2 (before of the third injection). At each injection, approximately 0.15 mL of aqueous humor was collected by anterior chamber limbal paracentesis with a 30-gauge needle, and then the injections of ranibizumab were performed through the pars plana. Immediately after collection, each aqueous humor sample was transferred to sterile plastic tubes and stored at –80°C until analysis.

Measurement of cytokines using multiplex analysis

Twenty-eight cytokines in aqueous humor samples were detected with a multiplex cytokine assay kit (R&D Systems, Minneapolis, MN, USA) using Luminex technology on a Bio-Plex MAGPIX system (Bio-Rad, Waltham, MA, USA) according to manufacturers' instructions. Standard curves for each cytokine were generated (in duplicate) by using the reference set of cytokine concentrations supplied in each kit. To avoid between-run imprecision, we measured cytokines in the samples from all patients in a single run. The sample concentration was calculated using a multi-parameter standard curve for each cytokine.

Statistical Analysis

SPSS software (version 18.0) and R statistical language (version 3.6.0) were used to perform statistical analysis. $P < 0.05$ was deemed to be statistically significant. The statistics were presented

by GraphPad Prism 5.

Patients' age were compared by student's *t*-tests, other quantitative data were analyzed by Mann-Whitney U tests or Wilcoxon signed rank tests. Qualitative data were compared using χ^2 tests. In the analysis of aqueous humor cytokines concentrations, Mann-Whitney U tests were used to compare the aqueous humor levels of cytokines between nAMD and cataract groups. Wilcoxon signed rank tests were used to compare aqueous humor levels of cytokines before the first ranibizumab treatment and before the third ranibizumab treatment in nAMD group. Multiple testing was corrected using the method of Benjamini and Hochberg.

Results

Demographic Characteristics of the Patients

As shown in Table 1, mean ages of nAMD and cataract cases were 74.5 ± 6.9 years (mean ± SD; range, 60–87 years) and 71.4 ± 9.4 years (mean ± SD; range, 54–85 years), respectively ($P = 0.239$). 12 of the 20 nAMD cases (60%) and 10 of the 20 control cases (50%) were men ($P = 0.525$). In the nAMD group, PCV was found in 4 eyes (20%), type I CNV was found in 6 eyes (30%), type II CNV was found in 10 eyes (50%), and there was no retinal angiomatous proliferation.

Variables	**nAMD group**	**Cataract group**	***P*-Value**
n	20	20	/
Male, n (%)	12 (60%)	10 (50%)	0.525 *
Age, years; mean ± SD	74.5 ± 6.9	71.4 ± 9.4	0.239 †
Disease type			
CNV type I, n (%)	6 (30%)	/	/
CNV type II, n (%)	10 (50%)	/	/
PCV, n (%)	4 (20%)	/	/
RAP, n (%)	0 (0)	/	/

*χ^2 test; †Student's *t*-test. nAMD, neovascular age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; RAP, retinal angiomatous proliferation.

Table 1. Baseline characteristics of nAMD patients.

Cytokine Concentrations in the Aqueous Humor of Cataract Patients vs. nAMD Patients Before and After the Treatments with Ranibizumab

The mean ± SD values of 28 cytokines in the aqueous humor of nAMD patients and cataract control patients are summarized in Table 2.

Cytokines	**Cataract (n=20)**	**nAMD, baseline (n=20)**		**nAMD, at month 1 (n=2**	
	Mean ± SD (pg/ml)	**Mean ± SD (pg/ml)**	***P*-Value† (vs. cataract)**	**Mean ± SD (pg/ml)**	***P*-Value† (vs. cataract)**
Angiogenin	24317.3 ± 4154.7	30190.6 ± 3476.4	**0.0010**	29507.4 ± 4350.8	**0.0**
Angiopoietin-1	105.4 ± 35.3	127.1 ± 67.6	0.4659	126.2 ± 76.1	0.8
Angiopoietin-2	84.2 ± 16.8	107.1 ± 56.5	0.1367	104.2 ± 57.9	0.3
BMP-9	3.0 ± 0.2	3.2 ± 0.2	**0.0067**	3.2 ± 0.3	**0.0**
CCL1/I-309	1.0 ± 0.3	1.4 ± 0.24	**0.00199**	1.7 ± 0.8	**0.0**
CCL13/MCP-4	6.6 ± 0.9	7.9 ± 1.5	**0.00199**	8.0 ± 1.9	**0.0**
CCL2/MCP-1	660.3 ± 217.6	1029.6 ± 1214.8	0.2948	1080.2 ± 1392.7	0.2
CCL20/MIP-3 α	20.9 ± 12.1	20.4 ± 10.7	0.9476	17.8 ± 8.9	0.4
CCL27/CTACK	4.5 ± 2.1	5.5 ± 1.9	0.1624	5.5 ± 2.2	0.1
CRP	49709.8 ± 186176.6	7566.9 ± 9756.9	0.4165	7132.9 ± 8391.2	0.4
CXCL10/IP-10	27.3 ± 13.9	48.2 ± 41.2	0.0654	43.8 ± 22.1	**0.0**
CXCL16	609.5 ± 132.1	740.2 ± 314.7	0.2948	759.4 ± 324.7	0.1
CXCL2/GRO β	121.7 ± 24.2	128.5 ± 11.2	**0.0294**	128.6 ± 24.3	0.0
CXCL8/IL-8	61.2 ± 11.0	84.4 ± 45.8	**0.00199**	80.2 ± 30.9	**0.0**
Endocan/ESM-1	113.8 ± 35.3	127.6 ± 46.1	0.4318	132.4 ± 51.0	0.3
Endothelin-1	33.4 ± 2.6	37.1 ± 6.6	**0.0332**	37.8 ± 6.3	**0.0**
FGF acidic	22.7 ± 3.3	26.8 ± 2.3	**0.0004**	28.6 ± 6.7	**0.0**
FGF basic	17.4 ± 5.3	17.6 ± 6.0	0.9476	16.9 ± 6.5	0.4
IL-15	4.9 ± 1.8	6.1 ± 1.7	**0.0226**	6.4 ± 2.5	**0.0**
IL-22	21.0 ± 3.8	23.4 ± 2.9	**0.0194**	24.0 ± 3.6	**0.0**
IL-36 β/IL-1F8	1.1 ± 0.7	1.5 ± 0.3	**0.00199**	1.6 ± 0.7	**0.0**
IL-6	8.9 ± 24.1	3.3 ± 5.5	0.9892	6.1 ± 17.3	0.8
PDGF-AA	37.6 ± 8.8	42.2 ± 10.7	0.2948	41.6 ± 13.5	0.4
PDGF-BB	1.7 ± 0.5	2.4 ± 1.0	**0.0194**	2.3 ± 0.9	**0.0**
PlGF	4.9 ± 0.5	5.3 ± 0.9	0.1367	4.9 ± 1.1	0.7
TNF-α	2.1 ± 0.6	2.4 ± 0.4	**0.0295**	2.8 ± 1.3	**0.0**
VEGF-A	72.0 ± 21.6	70.6 ± 30.5	0.6695	22.5 ± 1.7	**1.8**
VEGF-C	303.3 ± 47.0	327.0 ± 24.0	**0.00199**	329.5 ± 39.0	**0.0**

The concentrations were presented as mean ± SD. †The concentrations of aqueous cytokines were tested by Mann-Whitney *U* tests (or Wilcoxon signed rank tests for nAMD at baseline vs. at month 3) which were used for comparison between two groups, multiple testing was corrected using the method of Benjamini and Hochberg.

Table 2. Aqueous cytokine concentrations in nAMD patients at baseline, month 1, and month 2 versus cataract patients.

Before and after the treatments with ranibizumab, aqueous levels of 9 cytokines in nAMD patients

were significantly higher than in cataract patients (Table 2 and Figure 1). These cytokines (and their *P* values) are: angiogenin ($P = 0.0012$), bone morphogenetic protein–9 (BMP–9) ($P = 0.008$), C-C motif chemokine ligand 1 (CCL1) ($P = 0.014$), CCL13 ($P = 0.012$), interleukin–8 (IL–8) ($P = 0.006$), fibroblast growth factor-acidic (FGF-α) ($P = 0.001$), IL–22 ($P = 0.026$), IL–36β ($P = 0.005$), and VEGF-C ($P = 0.016$). The aqueous concentrations of VEGF-A in nAMD patients were significantly reduced ($P < 0.001$) after two consecutive monthly ranibizumab injections, in comparison to baseline levels.

Functional and Anatomic Parameters of nAMD Patients Before and After the Treatments with Ranibizumab

SD-OCT and FA data of 16 AMD patients were available at all three injections (Supplementary Table S1). After two consecutive monthly ranibizumab injections but before the third ranibizumab injection, CMT ($P < 0.001$), MRT–3mm ($P < 0.001$), and GLD ($P < 0.001$) were all significantly reduced compared to baseline values. Meanwhile, the BCVA (logMAR) of all 20 nAMD patients improved from 0.92 ± 0.38 to 0.85 ± 0.36 ($P = 0.045$) at month 1, and to 0.71 ± 0.30 ($P = 0.002$) at month 2, significantly.

Discussion

Pathologic circumstances, such as ischemia, hypoxia, or inflammation, were proangiogenic factors and could lead to the formation of CNV, corresponding cytokines take part in these processes. The present study compared differences in 28 relevant aqueous cytokines between nAMD patients and cataract patients. Changes in the 28 intraocular cytokines concentrations after the use of ranibizumab were also studied. Before and after two consecutive monthly ranibizumab injections, the aqueous concentrations of nine cytokines, including angiogenin, BMP–9, CCL1, CCL13, IL–8, FGF-α, IL–22, IL–36β, and VEGF-C were significantly higher in eyes with nAMD compared to cataract control eyes. As far as we know, it is the first time that the aqueous concentrations of CCL1, IL–22, and IL–36β in nAMD patients have been studied. These consequences need to be confirmed and further studied. Two consecutive monthly ranibizumab injections effectively reduced the aqueous concentrations of VEGF-A in nAMD patients, compared with their basal levels. Consistent with most previous studies,[8] our study also found that ranibizumab injections improved BCVA, as well as reduced CMT, MRT–3mm, and GLD.

In the current study, higher levels of inflammatory cytokines IL–8, IL–22, and IL–36β in the aqueous

humor in eyes with nAMD were measured, which reflects the inflammation-related parhogenesis of nAMD. Prolonged inflammation is usually detrimental and participates in nAMD.[7] For example, IL–1β and TNF-α secreted by macrophages promote angiogenesis of CNV at least in part by stimulating VEGF production in RPE cells.[16] IL–8 is a potent inflammatory cytokine, a chemokine for migratory immune cells, an activator for neutrophilic granulocytes, and an effective proangiogenic factor. Younger age of nAMD onset is associated with the single nucleotide polymorphism (SNP) rs4073 in the IL–8 promoter region.[17] Secretion of IL–8 is upregulated in response to various stimuli, including inflammatory marker CRP,[18] complement pathways,[19] and oxidative stress.[20, 21] Activation of inflammation, complement pathways, and oxidative stress are all pathogenic factors of nAMD.[6] RPE cells could produce higher levels of IL–8 under chronic oxidative stress, and IL–8 promotes both inflammation and angiogenesis, potentially leading to the development of CNV.[22] IL–8 can induce VEGF-dependent or VEGF-independent angiogenesis, and IL–8 can also increase endothelial permeability.[23, 24] Higher aqueous humor IL–8 levels in nAMD patients than in cataract patients were reported previously.[15, 25, 26] One previous study reported that IL–22 levels were significantly elevated in the serum of exudative AMD patients compared with controls, and C5a induced IL–22 expression in human $CD4^+$ T cells.[27] Elevated expression of IL–36β was found in the aqueous humor of acute uveitis, involved in autoreactive T-cell immune response.[28]

Angiogenin, FGF-α, and BMP–9 take part in angiogenesis. A previous study reported that aqueous angiogenin levels were significantly higher in the exudative AMD group than in the cataract group,[13] but another study found there was no difference in aqueous angiogenin concentrations between cataract patients and AMD patients at different stages.[29] Angiogenin may activate vessel endothelial and smooth muscle cells to promote angiogenesis, and may also facilitate cell invasion, proliferation, and formation of tubular structures.[30] Human choroid and retina cells synthesize and internalize angiogenin, which is localized to normal and pathologic vasculature in eyes with AMD.[31] In conclusion, studies have shown that angiogenin may play an important role in pathologic angiogenesis in nAMD.

Our study also confirms the high expression of angiogenin in the aqueous humor of nAMD patients.

FGF-α takes part in angiogenesis and inflammation.[32, 33] FGF receptor signaling pathway in endothelial cell plays critical role in diseases associated with aberrant vascular proliferation, such as age-related macular degeneration.[34] The FGF/FGF receptor system could be a target for the development of anti-angiogenic therapies.[33] In a previous study, there was no significant difference in the aqueous concentration of FGF-α between nAMD group and control group, but FGF-α concentration increased significantly after intravitreal injection of bevacizumab.[35] In our study, FGF-α concentration was significantly higher in nAMD patients before and after two consecutive monthly ranibizumab injections, in comparison to cataract patients, which deserves further study.

BMP–9 can suppress β-FGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis through ALK1.[36] In a research of spontaneous metastatic breast cancer mouse model, activating BMP–9 pathway could normalize tumor vessel in breast cancer.[37] Another study reported that both VEGF/VEGF receptor and the BMP9/ALK1 pathways are essential for stimulating angiogenesis.[38] In a research based on mouse xenograft model of human pancreatic cancer, simultaneous blockade of VEGF and BMP–9/10 signals significantly inhibited tumor angiogenesis, leading to delay of tumor growth.[39] From the above, the role of BMP–9 in angiogenesis is still undefined. In a previous study, BMP–9 did not show any significant difference between nAMD and cataract group, and there was no difference after intravitreal injection of bevacizumab in nAMD patients.[35] Our study found higher expression of BMP–9 in the aqueous humor of nAMD patients, compared with cataract patients, which needs further research in the future.

CCL1 and CCL 13 belong to a subgroup of chemotactic cytokines (chemokines) characterized by the presence of the CXC motif. CC chemokine receptors are critical mediators of chronic inflammatory response which are expressed by T cells and monocyte-macrophages, predominantly. CC chemokine receptors are potential pharmacological targets of chronic inflammation.[40] The present study found CCL1 and CCL 13 had higher aqueous concentrations in eyes with nAMD compared to cataract,

significantly. Until now, there was only one previous research measured the aqueous concentration of CCL13 in wet AMD patients, but the concentrations were below the detection threshold, and so could not be measured.[41]

In the present study, VEGF-A, VEGF-C, and PIGF in VEGF family were tested. VEGF-A, also called VEGF, is the most important and potent stimulator of angiogenesis[42], which exhibits strong pro-angiogenic effect through binding to VEGFR–1 and VEGFR–2.[43–45] VEGF-A is also the predominant cytokine controlling the formation of neovascularization. VEGF-A could be secreted not only by endothelial cells[43, 44, 46], but also by RPE cells[47] and Müller cells in the retina[48], in response to oxygen deprivation, which is one of the reasons leading to the formation of CNV. Anti-VEGF-A medication for neovascular eyes has revolutionized treatment for nAMD patients and preserved their vision.[49] Some previous studies reported higher VEGF-A levels in the aqueous humor of patients with nAMD than in those with cataract.[14, 50] However, some other studies found no significant difference in the aqueous VEGF-A concentration between cataract patients and active nAMD patients, or between cataract patients and early nAMD patients.[29, 51, 52] In our study, VEGF-A in aqueous humor had no significant difference between nAMD and cataract patients. There were several explications, first, from our data of FA, ICGA, and SD-OCT which were not shown, the activity of CNV in the nAMD group was low, and most of the cases were in inactive chronic state. Active CNV was defined as leakage seen on FA, as well as new macular intraretinal, subretinal hemorrhage, or fluid on SD-OCT. The amounts of visual recovery and retinal thickness improvement were not large, also suggesting that the patients were in prolonged state. Aqueous VEGF-A levels are positively related to the activity of CNV.[29, 53] Consistent with our conclusion, Muether et al. reported that monthly intravitreal injection of ranibizumab effectively reduces VEGF levels in aqueous humor of nAMD patients, the recurrence of CNV activity shown by SD-OCT are always preceded by increase of aqueous VEGF and usually followed by loss of visual acuity in the further course.[54] Second, in the present study, half of our patients were type I CNV and PCV. Tong et al. found that VEGF levels in eyes with PCV were significantly lower than those

in eyes with nAMD.[55] It may reflect that PCV and nAMD are distinct clinical entities and have different angiogenesis courses. Type I CNV, located below the RPE layer, may give rise to the low aqueous VEGF concentration.

On the other hand, in our study, the concentration of VEGF-C in aqueous humor was significantly higher in nAMD than in cataract group. VEGF-C has a high affinity for VEGFR–3, which is expressed on endothelial lymphatic cells, promoting lymphangiogenesis.[45] VEGF-C also have weak affinity for VEGFR–2, which explicates its poor implication in angiogenesis.[44, 45] Otani et al. reported that strong VEGF-C staining was found in most pigment-containing cells in all CNV specimens.[56] VEGF-C was confirmed to be markedly positive in the surgically removed RPE of a nAMD patient.[57] Until now, only one study reported the aqueous levels of VEGF-C in nAMD patients, although there was no significant difference in baseline VEGF-C levels between nAMD and cataract groups, VEGF-C showed a statistically significant increase after intravitreal injection of bevacizumab.[35] In a study of biomarkers related to cancer resistance to bevacizumab, VEGF-C was detected had higher serum concentration in nonresponders compared with responders to bevacizumab.[58] In renal and peritoneal fibrosis processes, TGF-β promotes lymphangiogenesis through the TGF-β/VEGF-C pathway.[59] As previously mentioned, in our study, the activity of CNV in the nAMD group was low, and most of the cases were in chronic state, in which circumstance, elevated VEGF-C may take part in the prolonged fibrotic processes of nAMD.

In conclusion, the present study investigated aqueous humor concentraions of 28 cytokines in eyes with nAMD and cataract controls. Aqueous angiogenin, BMP–9, CCL1, CCL13, IL–8, FGF-α, IL–22, IL–36β, and VEGF-C levels in nAMD eyes were significantly higher than in cataract eyes, both before and after injections of ranibizumab. These results may further confirm the inflammation-related pathogenesis of nAMD. Therefore, these nine cytokines could be novel therapeutic targets for nAMD.

Declarations

The authors declare that there is no competing interests regarding the publication of the present article. The datasets analyzed in the current study are available from the corresponding author for

reasonable request.

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Authors' contributions

Participated in study design: CKL and CZQ. Conduct of the study: CKL, XW, ZJ, SYP, MJ, and CZQ. Performed data analysis: CKL and XW. Wrote or contributed to the writing of the manuscript: CKL and CZQ. All authors have read and approved the final manuscript.

References

1. Schmidt-Erfurth U, Chong V, Loewenstein A, et al. Guidelines for the management of neovascular age-related macular degeneration by the European Society of Retina Specialists (EURETINA). *The British journal of ophthalmology* 2014;98:1144-1167.
2. Ambati J, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. *Nature reviews Immunology* 2013;13:438-451.
3. Balasubramanian SA, Krishna Kumar K, Baird PN. The role of proteases and inflammatory molecules in triggering neovascular age-related macular degeneration: basic science to clinical relevance. *Translational research : the journal of laboratory and clinical medicine* 2014;164:179-192.
4. Spaide RF, Yannuzzi LA, Slakter JS, Sorenson J, Orlach DA. Indocyanine green videoangiography of idiopathic polypoidal choroidal vasculopathy. *Retina (Philadelphia, Pa)* 1995;15:100-110.
5. Ambati J, Ambati BK, Yoo SH, Ianchulev S, Adamis AP. Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. *Survey of ophthalmology* 2003;48:257-293.
6. Nowak JZ. Age-related macular degeneration (AMD): pathogenesis and therapy. *Pharmacological reports : PR* 2006;58:353-363.

7. Kauppinen A, Paterno JJ, Blasiak J, Salminen A, Kaarniranta K. Inflammation and its role in age-related macular degeneration. *Cellular and molecular life sciences : CMLS* 2016;73:1765-1786.
8. Solomon SD, Lindsley K, Vedula SS, Krzystolik MG, Hawkins BS. Anti-vascular endothelial growth factor for neovascular age-related macular degeneration. *The Cochrane database of systematic reviews* 2019;3:Cd005139.
9. Chang AA, Li H, Broadhead GK, et al. Intravitreal aflibercept for treatment-resistant neovascular age-related macular degeneration. *Ophthalmology* 2014;121:188-192.
10. Saito M, Kano M, Itagaki K, Oguchi Y, Sekiryu T. Switching to intravitreal aflibercept injection for polypoidal choroidal vasculopathy refractory to ranibizumab. *Retina (Philadelphia, Pa)* 2014;34:2192-2201.
11. Davuluri G, Espina V, Petricoin EF, 3rd, et al. Activated VEGF receptor shed into the vitreous in eyes with wet AMD: a new class of biomarkers in the vitreous with potential for predicting the treatment timing and monitoring response. *Archives of ophthalmology (Chicago, Ill : 1960)* 2009;127:613-621.
12. Funatsu H, Yamashita H, Noma H, et al. Aqueous humor levels of cytokines are related to vitreous levels and progression of diabetic retinopathy in diabetic patients. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 2005;243:3-8.
13. Agawa T, Usui Y, Wakabayashi Y, et al. Profile of intraocular immune mediators in patients with age-related macular degeneration and the effect of intravitreal bevacizumab injection. *Retina (Philadelphia, Pa)* 2014;34:1811-1818.
14. Motohashi R, Noma H, Yasuda K, Kotake O, Goto H, Shimura M. Dynamics of Inflammatory Factors in Aqueous Humor during Ranibizumab or Aflibercept Treatment for Age-Related Macular Degeneration. *Ophthalmic research* 2017;58:209-216.

15. Agrawal R, Balne PK, Wei X, et al. Cytokine Profiling in Patients With Exudative Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy. *Investigative ophthalmology & visual science* 2019;60:376-382.
16. Oh H, Takagi H, Takagi C, et al. The potential angiogenic role of macrophages in the formation of choroidal neovascular membranes. *Investigative ophthalmology & visual science* 1999;40:1891-1898.
17. Hautamaki A, Seitsonen S, Holopainen JM, et al. The genetic variant rs4073 A-->T of the Interleukin-8 promoter region is associated with the earlier onset of exudative age-related macular degeneration. *Acta ophthalmologica* 2015;93:726-733.
18. Wang Y, Bian ZM, Yu WZ, Yan Z, Chen WC, Li XX. Induction of interleukin-8 gene expression and protein secretion by C-reactive protein in ARPE-19 cells. *Experimental eye research* 2010;91:135-142.
19. van den Berg RH, Faber-Krol MC, Sim RB, Daha MR. The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. *Journal of immunology (Baltimore, Md : 1950)* 1998;161:6924-6930.
20. DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, Remick DG. Regulation of interleukin 8 gene expression by oxidant stress. *The Journal of biological chemistry* 1993;268:25568-25576.
21. Fernandes AF, Zhou J, Zhang X, et al. Oxidative inactivation of the proteasome in retinal pigment epithelial cells. A potential link between oxidative stress and up-regulation of interleukin-8. *The Journal of biological chemistry* 2008;283:20745-20753.
22. Zhu D, Deng X, Xu J, Hinton DR. What determines the switch between atrophic and neovascular forms of age related macular degeneration? - the role of BMP4 induced

senescence. *Aging* 2009;1:740-745.

23. Martin D, Galisteo R, Gutkind JS. CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex. *The Journal of biological chemistry* 2009;284:6038-6042.
24. Mizukami Y, Jo WS, Duerr EM, et al. Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. *Nature medicine* 2005;11:992-997.
25. Mimura T, Funatsu H, Noma H, et al. Aqueous Humor Levels of Cytokines in Patients with Age-Related Macular Degeneration. *Ophthalmologica Journal international d'ophtalmologie International journal of ophthalmology Zeitschrift fur Augenheilkunde* 2019;241:81-89.
26. Ten Berge JC, Fazil Z, van den Born I, et al. Intraocular cytokine profile and autoimmune reactions in retinitis pigmentosa, age-related macular degeneration, glaucoma and cataract. 2019;97:185-192.
27. Liu B, Wei L, Meyerle C, et al. Complement component C5a promotes expression of IL-22 and IL-17 from human T cells and its implication in age-related macular degeneration. *Journal of translational medicine* 2011;9:1-12.
28. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. *Immunity* 2013;39:1003-1018.
29. Muether PS, Neuhann I, Buhl C, Hermann MM, Kirchhof B, Fauser S. Intraocular growth factors and cytokines in patients with dry and neovascular age-related macular degeneration. *Retina (Philadelphia, Pa)* 2013;33:1809-1814.
30. Gao X, Xu Z. Mechanisms of action of angiogenin. *Acta biochimica et biophysica Sinica* 2008;40:619-624.

31. Skeie JM, Zeng S, Faidley EA, Mullins RF. Angiogenin in age-related macular degeneration. *Molecular vision* 2011;17:576-582.

32. Zakrzewska M, Marcinkowska E, Wiedlocha A. FGF-1: from biology through engineering to potential medical applications. *Critical reviews in clinical laboratory sciences* 2008;45:91-135.

33. Rusnati M, Presta M. Fibroblast growth factors/fibroblast growth factor receptors as targets for the development of anti-angiogenesis strategies. *Current pharmaceutical design* 2007;13:2025-2044.

34. Oladipupo SS, Smith C, Santeford A, et al. Endothelial cell FGF signaling is required for injury response but not for vascular homeostasis. *Proceedings of the National Academy of Sciences of the United States of America* 2014;111:13379-13384.

35. Cabral T, Lima LH, Mello LGM, et al. Bevacizumab Injection in Patients with Neovascular Age-Related Macular Degeneration Increases Angiogenic Biomarkers. *Ophthalmology Retina* 2018;2:31-37.

36. Scharpfenecker M, van Dinther M, Liu Z, et al. BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *Journal of cell science* 2007;120:964-972.

37. Ouarne M, Bouvard C, Boneva G, et al. BMP9, but not BMP10, acts as a quiescence factor on tumor growth, vessel normalization and metastasis in a mouse model of breast cancer. *Journal of experimental & clinical cancer research : CR* 2018;37:209.

38. van Meeteren LA, Thorikay M, Bergqvist S, et al. Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. *The Journal of biological chemistry* 2012;287:18551-18561.

39. Akatsu Y, Yoshimatsu Y, Tomizawa T, et al. Dual targeting of vascular endothelial

growth factor and bone morphogenetic protein-9/10 impairs tumor growth through inhibition of angiogenesis. *Cancer science* 2017;108:151-155.

40. White GE, Iqbal AJ, Greaves DR. CC chemokine receptors and chronic inflammation--therapeutic opportunities and pharmacological challenges. *Pharmacological reviews* 2013;65:47-89.

41. Liu F, Ding X, Yang Y, et al. Aqueous humor cytokine profiling in patients with wet AMD. *Molecular vision* 2016;22:352-361.

42. Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacological reviews* 2004;56:549-580.

43. Rosen LS. Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer control : journal of the Moffitt Cancer Center* 2002;9:36-44.

44. Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Molecular diversity* 2006;10:515-527.

45. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes & cancer* 2011;2:1097-1105.

46. Takahashi H, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clinical science (London, England : 1979)* 2005;109:227-241.

47. Klettner A, Westhues D, Lassen J, Bartsch S, Roider J. Regulation of constitutive vascular endothelial growth factor secretion in retinal pigment epithelium/choroid organ cultures: p38, nuclear factor kappaB, and the vascular endothelial growth factor receptor-2/phosphatidylinositol 3 kinase pathway. *Molecular vision*

2013;19:281-291.

48. Wang JJ, Zhu M, Le YZ. Functions of Muller cell-derived vascular endothelial growth factor in diabetic retinopathy. *World journal of diabetes* 2015;6:726-733.

49. Campbell M, Doyle SL. Current perspectives on established and novel therapies for pathological neovascularization in retinal disease. *Biochemical pharmacology* 2019;164:321-325.

50. Sato T, Takeuchi M. Intraocular inflammatory cytokines in patients with neovascular age-related macular degeneration before and after initiation of intravitreal injection of anti-VEGF inhibitor. 2018;8:1098.

51. Ng DS, Yip YW, Bakthavatsalam M, et al. Elevated angiopoietin 2 in aqueous of patients with neovascular age related macular degeneration correlates with disease severity at presentation. *Scientific reports* 2017;7:45081.

52. Rezar-Dreindl S, Sacu S, Eibenberger K, et al. The Intraocular Cytokine Profile and Therapeutic Response in Persistent Neovascular Age-Related Macular Degeneration. *Investigative ophthalmology & visual science* 2016;57:4144-4150.

53. Roh MI, Kim HS, Song JH, Lim JB, Koh HJ, Kwon OW. Concentration of cytokines in the aqueous humor of patients with naive, recurrent and regressed CNV associated with amd after bevacizumab treatment. *Retina (Philadelphia, Pa)* 2009;29:523-529.

54. Muether PS, Hermann MM, Viebahn U, Kirchhof B, Fauser S. Vascular endothelial growth factor in patients with exudative age-related macular degeneration treated with ranibizumab. *Ophthalmology* 2012;119:2082-2086.

55. Tong JP, Chan WM, Liu DT, et al. Aqueous humor levels of vascular endothelial growth factor and pigment epithelium-derived factor in polypoidal choroidal vasculopathy and choroidal neovascularization. *American journal of ophthalmology* 2006;141:456-462.

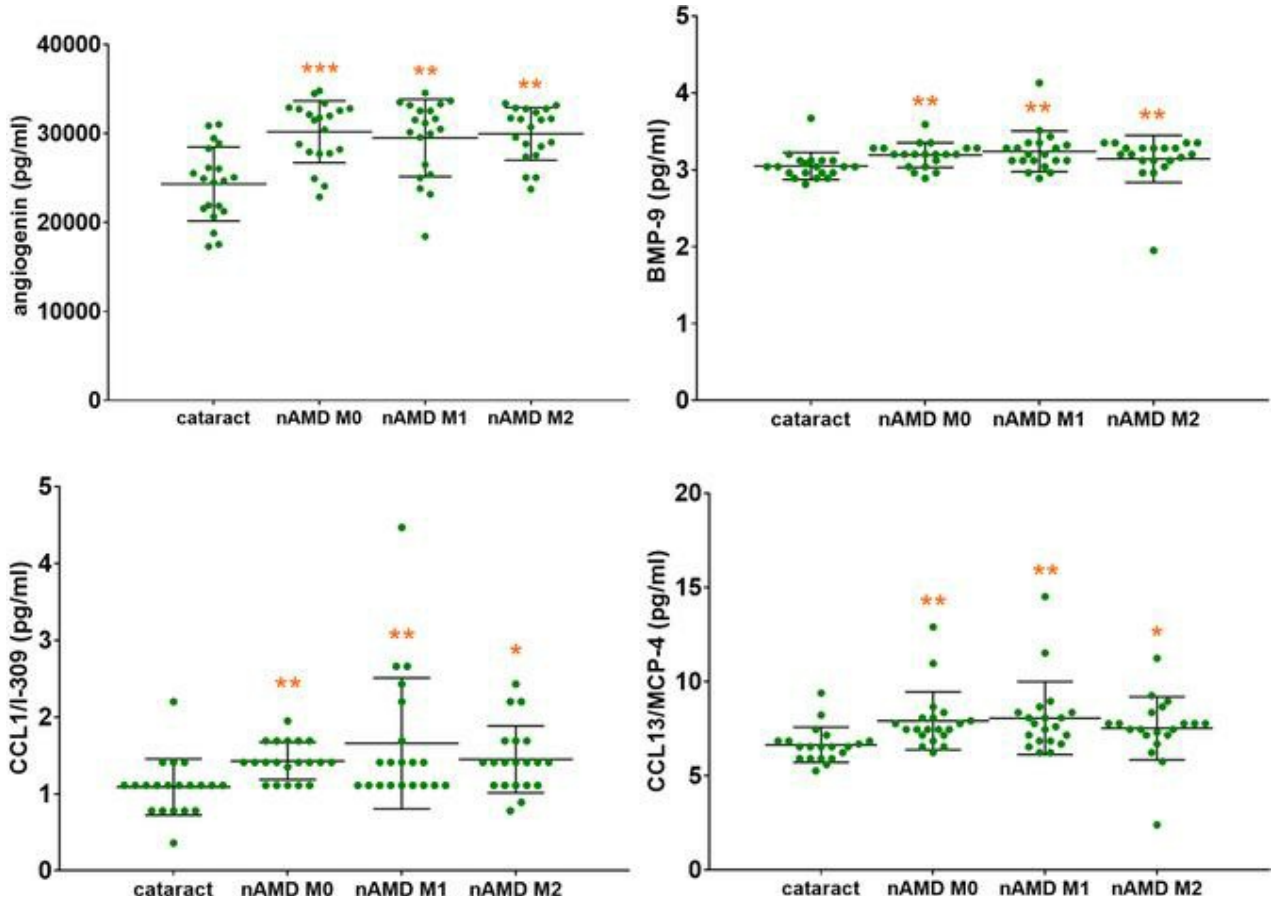
56. Otani A, Takagi H, Oh H, et al. Vascular endothelial growth factor family and receptor expression in human choroidal neovascular membranes. *Microvascular research* 2002;64:162-169.

57. Ikeda Y, Yonemitsu Y, Onimaru M, et al. The regulation of vascular endothelial growth factors (VEGF-A, -C, and -D) expression in the retinal pigment epithelium. *Experimental eye research* 2006;83:1031-1040.

58. Hayashi H, Arao T, Matsumoto K, et al. Biomarkers of reactive resistance and early disease progression during chemotherapy plus bevacizumab treatment for colorectal carcinoma. *Oncotarget* 2014;5:2588-2595.

59. Kinashi H, Ito Y, Sun T, Katsuno T, Takei Y. Roles of the TGF-beta(-)VEGF-C Pathway in Fibrosis-Related Lymphangiogenesis. *International journal of molecular sciences* 2018;19.

Figures



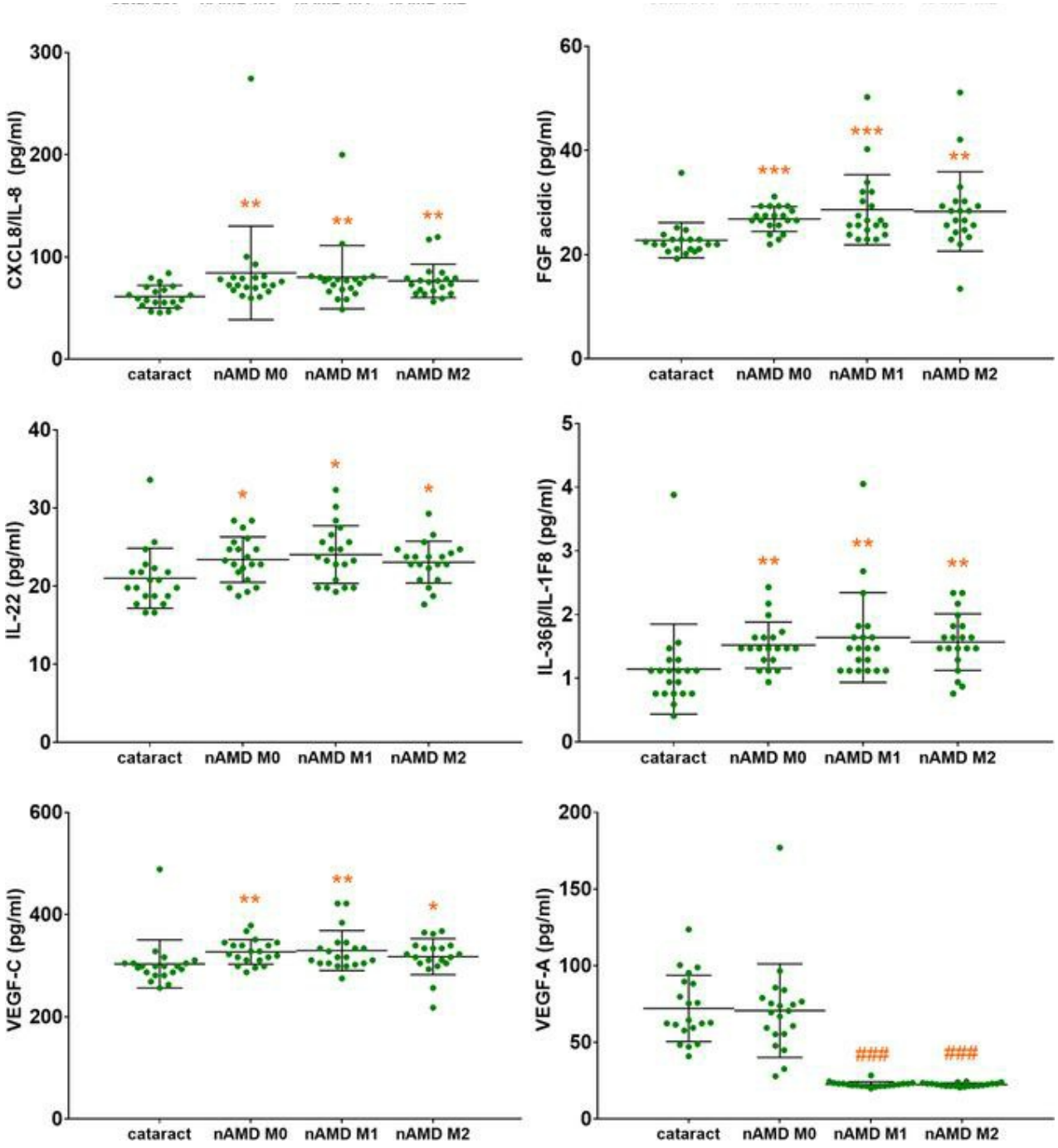


Figure 1

Aqueous humor cytokines concentrations in patients with cataract or nAMD. The nAMD patients received three consecutive monthly ranibizumab injections. Values represent mean ± SD of aqueous levels of cytokines concentrations in 20 cataract or 20 nAMD patients at baseline (M0), month 1 (M1), and month 2 (M2). Aqueous levels of 9 cytokines in nAMD patients were significantly higher than in cataract patients, and aqueous VEGF-A in nAMD patients were significantly reduced after two consecutive monthly ranibizumab injections. *P

< 0.05, **P < 0.01, ***P < 0.001 vs. cataract group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. nAMD baseline group.

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