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Original Article

Cytokine profiling in patients with polypoidal choroidal vasculopathy before and after intravitreal injection of ranibizumab

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Abstract: Objective: This study aims to investigate the cytokines profiling in the aqueous humor of patients with polypoidal choroidal vasculopathy (PCV) before and after intravitreal ranibizumab injection (IVR). Methods: 14 patients clinically diagnosed with PCV and 15 cataract patients of similar age and gender (control group) were included. Throughout the cataract surgery and IVR, aqueous humor samples were collected from the PCV and control groups. Results: The levels of macrophage inflammatory protein 1β (MIP-1β) and normal T cell expressed and secreted (RANTES) in PCV patients were significantly lower than control subjects (P=0.045 and P=0.004, respectively). The concentration of vascular endothelial growth factor-A (VEGF-A) was significantly higher than the control group (P=0.003). The level of MIP-1β was greatly increased in PCV patients compared to prior to IVR (P=0.001). After IVR, the level of VEGF-A in PCV patients were considerably lower compared to before IVR (P=0.001). There was no link between the expression of several cytokines (MCP-1, MIP-1, Eotaxin, G-CSF, IL-8, IL-6, IL-5, IP-10 and IFN-γ) in the aqueous humor of PCV patients before and after intravitreal ranibizumab injection (IVR). The association between IL-5 expression and central macular thickness (CMT) was discovered before IVR (P=0.02), however, the correlation between several cytokines (MCP-1, MIP-1, Eotaxin, G-CSF, IL-8, IL-6, IL-5, IP-10 and IFN-γ) was discovered in PCV patients after IVR. Conclusion: Based on our findings, we discovered that the production of neovascularization in PCV patients is driven by both angiogenic and inflammatory factors, with a correlation seen between several cytokines.

Keywords: Choroidal neovascularization (CNV), polypoidal choroidal vasculopathy (PCV), ranibizumab, cytokine

Introduction

Polypoid choroidal vasculopathy (PCV), first described in 1990, was mostly seen in middle-aged Negroid females [1]. Although it has now been described in people of all races, it was originally thought to be a rare condition. PCV is becoming more prevalent in Asians and African-Americans than in Caucasians [2-5].

The absence of drusen, spatial atrophy, pigment alterations, and disciform scar formation distinguish PCV from conventional age-related macular degeneration [6, 7]. It is made up of subretinal polypoid vascular lesions linking to recurrent serous and hemorrhagic pigment epithelial detachment (PED), which is also prevalently occurs in Asians. According to recent research, PCV is one of a group of disorders defined by pachychoroid [8].

Anti-vascular endothelial growth factor (anti-VEGF) Medication, verteporfin photodynamic therapy (PDT) and thermal laser (TL) photocoagulation were used to treat PCV [9]. Anti-VEGF Medication is currently the most routinely utilized technique. VEGFs have been implicated roles in the development of symptomatic PCV in some pathological studies [10, 11]. Monthly intravitreal injections of an anti-VEGF drug have been shown to prevent vision loss and reduce leakage from CNV [12, 13]. However, this treatment is sometimes not impactful. Furthermore, the levels of several cytokines, including VEGF, were altered before and after IVR. Inflammatory cytokines are thought to be involved in PCV pathogenesis [14, 15].

Therefore, understanding the mechanism causing cytokine changes in the aqueous humor before and after intravitreal injection of ranibi-

zumab (IVR) is an important requirement. Elucidating the cytokines in PCV patients' aqueous humor might be beneficial in determining illness processes and guiding clinical treatment options.

Methods

Study design and participants

This prospective research compared the cytokine profiles of aqueous humor between PCV and cataract patients. This study included 14 PCV patients and 15 cataract patients. Before taking part in the research, each patient signed an informed consent form which had been authorized by the Institutional Review Board. The protocol followed the principles of the Helsinki Declaration. The Shanghai's Tenth People's Hospital Ethics Committee (SHSY-IEC-4.1/21-367/01) approved this study. This clinical study is listed as ChiCTR2000036875 on the website www.chictr.org.cn.

Clinical diagnosis of PCV

By three retina specialists, each patient received a consistent diagnosis. All PCV patients performed Fundus imagings, including colour fundus photography (CFP), indocyanine green angiography (ICGA), optical coherence tomography (OCT) and fundus fluorescein angiography (FFA). The diagnosis of PCV was based on the gold standard of ICGA, which demonstrates early nodular hyperfluorescence, indicating Polypoidal with additional features, such as abnormal vascular network. In the two groups, information on intraocular pressure (IOP) and refractive error were gathered.

Inclusion criteria

The following were the inclusion requirements: 1) Participants older than 50 who signed the informed consent form and agreed to donate an aqueous humor sample; 2) Anti-VEGF medication was necessary for patients who had PCV-related active CNV. Patients undergoing cataract surgery who had no systemic immunological disorder or retinal illness participated as control participants (>50 years old).

Exclusion criteria

The following were the exclusion requirements: 1) Participants who have had several intraocular treatments in last 3 months; 2) People who have systemic or ocular illnesses that are active; 3) People who have pathological myopia, diabetic retinopathy, neovascular age-related macular degeneration and other eye disorders; 4) People who are allergic to indocyanine green and fluorescein sodium; 5) People who have immune system disorders.

IVR operation

Alcaine's eye drop was twice applied before PCV patients reached the surgical room. The patient laid on the operating bed, and the eye was cleaned locally with 0.5 percent povidone-iodine (Shanghai Likang Co., Ltd.), then the eyelashes were cleaned with standard precaution. The eyes were then opened using the eyelid opener, and the conjunctival sac was rinsed with 0.05 percent povidone-iodine. 0.05 mL of Lucentis (Novartis Pharma Schweiz AG) was transfused into the vitreous body. Meanwhile, we placed a syringe at 4 mm beside the corneoscleral junction to collected aqueous humor after 60 seconds of 2 percent lidocaine was utilized. Following the removal of the needle, compression was used to perform hemostasis for 2 minutes. After the procedure, TobraDex ointment was utilised and the operated eye was protected by gauze. The examination was carried again in the following days.

Aqueous humor collection

Approximately 100 μL of aqueous humor was gathered from PCV Participants during intravitreal injection using a needle at 4 mm behind the corneal limbus. Similarly, in cataract operation, aqueous humor was gathered 100 μL. These clinical specimen were saved at -80°C immediately until next research.

Bio-Plex® 200 system cytokine analyzing

The aqueous humor specimens were placed on ice, clarified by centrifugation at 3000 rpm for 5 minutes, and examined using Bio-PlexTM Human Cytokine Standard 27-Plex, Group I (Bio-Rad, Hercules, CA, USA) and Bio-Plex® 200 System, as directed by the manufacturer. The cytokines were chosen based on previous studies and relevant studies.

Interleukin 2-17 (IL2-17), interleukin-1 receptor antagonist (IL-1Ra), platelet-derived growth factor bb (PDGF-bb), basic fibroblast growth factor

Table 1. Baseline demographics of the PCV and control groups

Demographic	PCV group	Control group
Number of subjects	14	15
Age (years)	66.36±5.50	62.33±7.51
Sex (male: female)	6:8	6:9
BCVA (logMAR)	1.02±0.49	0.50±0.20
Refractive error (D)	-3.30±1.95	-2.83±1.25
Mean IOP (mmHg)	13.21±3.04	14.67±1.53
CMT (μm)	266.57±71.43	146.00±14.73
Inner Thickness (ILM-IPL, μm)	45.64±12.40	50.67±10.02#
Hypertension (no. of subjects)	3	2

#PCV vs. control group, all P>0.05.

(basic FGF), granulocyte colony-stimulating factor (G-CSF), Eotaxin, interferon-gamma (IFN-g), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), interferon-gamma-induced protein 10 (IP-10), MIP-1β, monocyte chemoattractant protein 1 (MCP-1), regulated upon activation, interleukin-1 beta (IL-1β), VEGF-A, tumor necrosis factor-alpha (TNF-α) and normal T cell expressed and secreted (RANTES) were analyzed.

Statistical analysis

All data was analyzed using SPSS, version 20. The Shapiro-Wilk test was utilized to determine normality due to the small sample size. Non-paired continuous variables were compared by the Student's t-test if the variables seemed to have a normal distribution; otherwise, the Friedman test was applied. A one-way repeated-measures analysis (ANOVA) was applied to compare the values before and after operation. To investigate correlations between variables, Pearson's correlation coefficient or Spearman's rank-order correlation coefficient was used. P<0.05 marked statistical significance.

Results

Baseline demographics and characteristics

The mean age of the 14 PCV subjects and the control group were 66.36±5.50 years (mean ± SD) and 62.33±7.51 years, respectively. The ratioof male and female of the PCV and control groups was 6:8 and 6:9, respectively. The mean best-corrected visual acuity (BCVA) of the PCV and control groups were 1.02±0.49 and 0.50±0.20 logMAR, respectively. The mean refractive error of the PCV and control groups were -330.43±195.54 and -283.33±125.83, respectively. The average IOP of the control group and PCV were 14.67±1.53 and 13.21±3.04 mmHg, respectively. The mean central macular thickness (CMT) of the PCV and control groups were 266.57±71.43 and 146.00±14.73 μm, respectively. The mean inner thickness of retina from the inner plexiform layer (IPL) to the internal limiting membrane (ILM) in the PCV and control groups were 45.64±12.40 and 50.67±10.02 μm, respectively. 3/14 (21%) PCV and 2/15 (13%) control cases were complicated with hypertension.

Concentrations of cytokines at baseline

14/27 cytokines (MIP-1α, MIP-1β, RANTES, IL-Ra, IL-8, IL-6, IFN-γ, IL-5, G-CSF, IP-10, MCP-1, Eotaxin, basic FGF, and VEGF-A) were detected in the aqueous humor (**Table 1**). RANTES and MIP-1β concentrations in the PCV group were markedly smaller than in the control group (P=0.004 and P=0.045, respectively; **Figure 1**). The concentration of VEGF-A in the controls was considerably lower than in the PCV group (P=0.003; **Figure 2**).

Notably, MIP-1β decreased 0.63-fold in the PCV group compared to the controls. The PCV cohort had a 0.41-fold lower RANTES than the controls. The level of VEGF-A in PCV were 3.34 times higher than controls. However, no substantial differences in cytokines in aqueous humor (G-CSF, Eotaxin, IFN-g, basic FGF, IP-10, IL-1Ra, IL-8, IL-6, IL-5, MIP-1α and MCP-1) were found between case and control groups (**Table 2**).

Fundus imaging changes during therapy

Exudation and subretinal haemorrhage were revealed from CFP and OCT photography. Our research showed that subretinal haemorrhage and exudation were reduced from CFP and OCT images. The neurosensory retina at the macula was disrupted in PCV patients and the RPE layer was elevated with a dome-like structure due to haemorrhage before the operation. When the haemorrhage was absorbed, the neurosensory retina at the macula became more

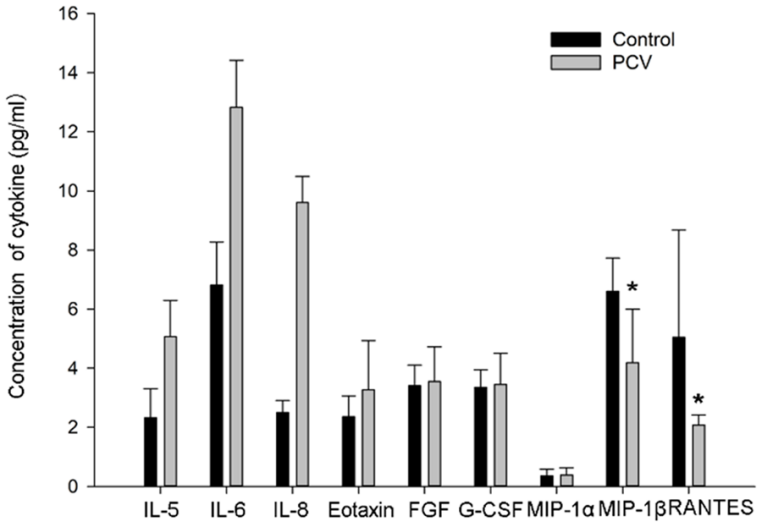

Figure 1. Mean concentrations of aqueous humor cytokines (IL-5, IL-6, IL-8, Eotaxin, FGF, G-CSF, MIP-1α, MIP-1β, RANTES) in the control and PCV patients groups. Data shown are mean ± SD. *Indicates control *vs*. PCV; P<0.05.

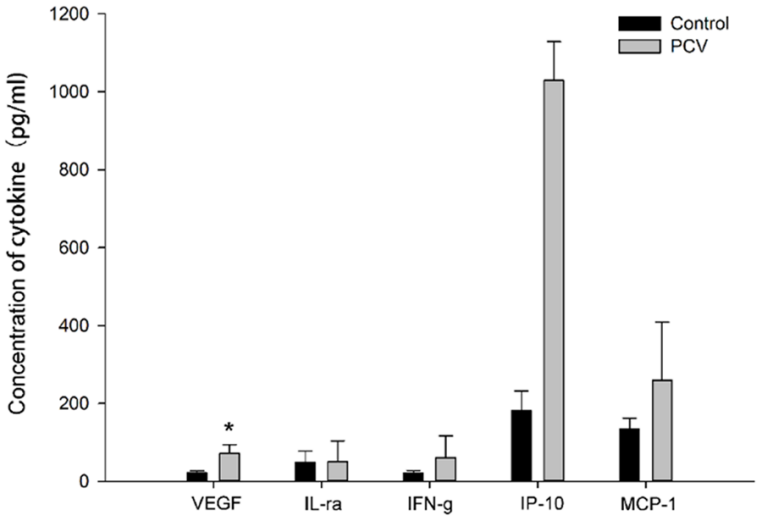

Figure 2. Mean concentrations of aqueous humor cytokines (VEGF, IL-Ra, IFN-γ, IP-10, and MCP-1) in the control and PCV patients groups. Data shown are mean ± SD. *Indicates control *vs*. PCV; P<0.05.

differences were observed in the other cytokines (IP-10, MIP-1α, MCP-1, Eotaxin, G-CSF, basic FGF, IFN-γ, IL-5, IL-1Ra, RANTES, IL-8 and IL-6) in aqueous humor before and after IVR (**Table 3**).

Before IVR, IL-5 expression was linked with that of IL-6 and CMT (P=0.001 and 0.02, respectively). Before IVR, IL-8 expression was connected with Eotaxin, IFN-γ, MCP-1, IP-10, RANTES, and BCVA (P= 0.05). G-CSF, MCP-1, IP-10, IFN-γ, MIP-1β, MIP-1α and RANTES expression were all connected with Eotaxin expression before IVR (P=0.05). Before IVR, G-CSF expression was linked with RANTES and MIP-1β (P=0.042 and P= 0.027, respectively).

Before IVR, IFN-γ expression was related with IP-10, MCP-1, MIP-1β and MIP-1α (P<0.001, P<0.001, P=0.039 and P< 0.001, respectively). Before IVR, IP-10 expression was connected to MCP-1, MIP-1β, MIP-1α, and BCVA (P=0.001, P=0.029, P<0.001 and P= 0.007, respectively). MCP-1 expression was associated with MIP-1β and MIP-1α before IVR (P=0.029 and P= 0.001, respectively). MIP-1α was linked with BCVA and MIP-1β prior to IVR (P=0.038 and P=0.014, respectively) (**Table 4**).

regular, and the RPE layer could be roughly restored to its original position postoperatively (**Figure 3**).

Cytokine levels changes during therapy

MIP-1, an aqueous humor cytokine, was found to be considerably greater in the PCV cohort following IVR (P=0.001). The level of VEGF-A in the PCV group was significantly lesser after IVR than before (P=0.001). However, no significant

The level of different cytokines in the aqueous humor of PCV was interconnected after the first IVR. Following the first administration of IVR, the level of IL-5 was correlated with RANTES and MIP-1β (P=0.007 and P=0.049). After first IVR, IL-6 expression was associated with IL-8, MIP-1β, and RANTES (P=0.036, P=0.020, and P=0.006, respectively). After the first IVR, IL-8 expression was related with IFN-γ, IP-10, MCP-1, MIP-1β, MIP-1α and CMT (P=0.008, P=0.010, P=0.003, P=0.006, P=0.007, and P=0.007,

Table 2. Mean concentrations and fold change in aqueous humor cytokines in the PCV and control groups

Analytes (pg/mL)	Mean concentrations in PCV	Mean concentrations in controls	Fold difference	P
IL-1Ra	50.76±50.03	49.05±27.99	1.03	0.958
IL-5	5.06±4.56	2.32±0.98	2.18	0.328
IL-6	12.81±42.59	6.81±1.46	1.88	0.815
IL-8	9.60±18.53	2.50±0.41	3.84	0.527
Eotaxin	3.27±1.67	2.36±0.70	1.39	0.380
Basic FGF	3.55±1.17	3.41±0.69	1.04	0.849
G-CSF	3.45±1.05	3.35±0.59	1.03	0.882
IFN-γ	60.69±55.98	21.90±5.62	2.77	0.261
IP-10	1029.19±1148.14	181.69±49.99	5.66	0.232
MCP-1	259.27±149.51	134.14±27.95	1.93	0.179
MIP-1α	0.38±0.25	0.36±0.22	1.06	0.875
MIP-1β	4.18±1.81	6.60±1.12	0.63	0.045*
RANTES	2.08±0.34	5.05±3.62	0.41	0.004*
VEGF-A	71.49±22.92	22.31±4.48	3.34	0.003*

Concentration of cytokines between the two groups was compared using an independent t-test. Values are in pg/mL; *P<0.05.

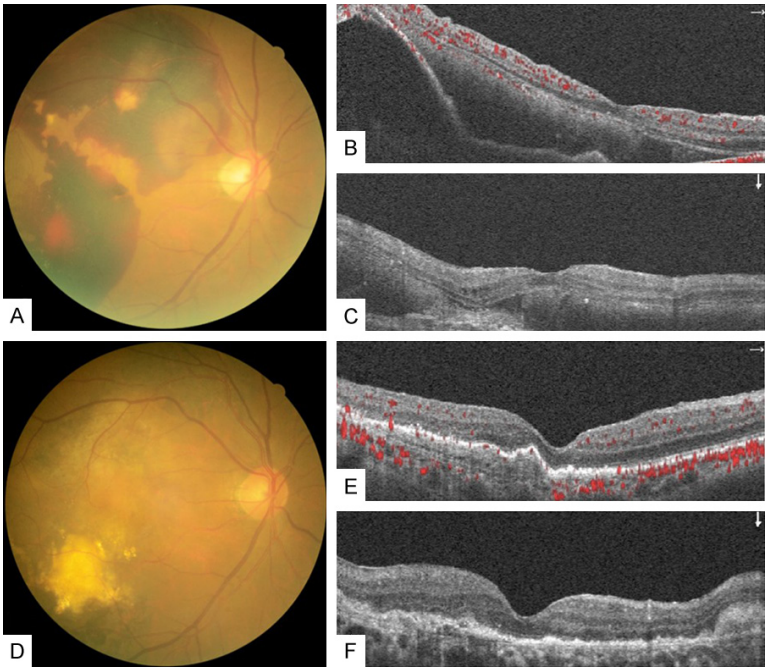

Figure 3. Fundus imaging of PCV patient before and after IVR. Subretinal hemorrhage and exudation on CFP before IVR (A) and optical OCT (B and C). Subretinal hemorrhage and exudation were decreased on CFP after IVR (D) and OCT (E and F).

respectively). After the first IVR, there was a correlation between eotaxin and IFN-γ expression (P=0.035).

After the first administration of IVR, IFN-γ expression was associated with IP-10, MCP-1, MIP-1β, MIP-1α and CMT (P=0.029, P=0.001, P=0.027, P=0.003, and P=0.018, respectively). After the first IVR, IP-10 expression was correlated with MCP-1, CMT, and inner thickness (P=0.016, P=0.003, and P=0.035, respectively). After the first IVR, MCP-1 expression was connected with MIP-1α, CMT and MIP-1β (P=0.004, P=0.006 and P=0.037, respectively). MIP-1α expression was associated with MIP-1β, BCVA, and inner thickness following the first IVR (P=0.001, P=0.028, and P=0.030, respectively) (**Table 5**).

Discussion

In this investigation, a large panel of aqueous humor cytokines were measured in PCV. We discovered considerable differences in the levels of cytokines (RANTES, MIP-1β and VEGF-A) in aqueous humor between the PCV and control groups. In addition, significant differences in the levels of MIP-1β and VEGF-A were observed in patients with PCV after versus before IVR. These findings disprove the assertion that

Table 3. Mean concentrations of aqueous humor cytokines in cases before and after intravitreal injection of ranibizumab

Analytes (pg/ml)	PCV before IVR	PCV after 1-IVR	PCV After 2-IVR	*p*-Value
IL-1ra	50.75±53.03	79.85±117.07	62.24±72.66	0.649
IL-5	5.06±4.56	5.36±6.45	2.49±1.25	0.059
IL-6	12.83±42.58	11.95±41.96	0.76±0.69	0.362
IL-8	9.60±18.53	10.26±25.91	4.31±4.29	0.355
Eotaxin	3.27±1.67	3.07±1.29	3.71±1.35	0.512
basic FGF	3.55±1.17	3.63±3.06	2.95±1.09	0.624
G-CSF	3.45±1.05	4.95±6.91	6.92±2.06	0.128
IFN-γ	60.69±55.98	68.63±126.20	55.24±16.09	0.856
IP-10	1029.19±1148.11	1003.29±940.46	926.40±756.86	0.953
MCP-1	259.27±149.51	305.30±429.09	293.60±47.01	0.854
MIP-1α	0.38±0.25	0.62±1.30	0.92±0.69	0.203
MIP-1β	4.18±1.81	4.68±3.67	8.70±3.36	0.001*
RANTES	2.08±0.34	2.22±0.34	2.46±0.55	0.117
VEGF-A	71.49±22.92	37.67±13.08	27.93±10.81	<0.001*

The concentration of cytokine between the three groups was compared with one-way repeated measures ANOVA. Values are in pg/mL; *the PCV vs. the control group P<0.05.

Table 4. Correlations between aqueous humor factors and the curative effect before IVR in the PCV group

	IL-6	Eotaxin	G-CSF	IFN-γ	IP-10	MCP-1	MIP-1α	MIP-1β	RANTES	BCVA	CMT
	r	r	r	r	r	r	r	r	r	r	r
	p	p	p	p	p	p	p	p	p	p	p
IL-5	0.778**	-	-	-	-	-	-	-	-	-	-0.612**
	0.001										0.02
IL-8	-	0.911**	-	0.899**	0.877**	0.873**	0.857**	0.662**	0.571*	0.641*	
		<0.001		<0.001	<0.001	<0.001	<0.001	0.010	0.033	0.013	
Eotaxin	-	-	0.538*	0.790**	0.744**	0.735**	0.695**	0.623*	0.610*	-	-
			0.047	0.001	0.002	0.003	0.002	0.017	0.021		
G-CSF	-	-	-	-	-	-	-	0.588*	0.550*	-	-
								0.027	0.042		
IFN-γ	-	-	-	-	0.829**	0.969**	0.838**	0.556*	-	-	-
					<0.001	<0.001	<0.001	0.039			
IP-10	-	-	-	-	-	0.789**	0.809**	0.582*	-	0.686**	-
						0.001	<0.001	0.029		0.007	
MCP-1	-	-	-	-	-	-	0.813**	0.582*		-	-
							<0.001	0.029			
MIP-1α	-	-	-	-	-	-	-	0.714**		0.559*	-
								0.004		0.038	

**When the degree of confidence (bilateral) was 0.01, the correlation was significant. *When the degree of confidence (bilateral) was 0.05, the correlation was significant. This table shows only the specific cytokines with a statistically significant difference.

cytokine-mediated inflammation plays a role in PCV pathogenesis.

MIP-1β is a chemokine from the CC family secreted by activated macrophages and leukocytes [16], which is enhanced in ischemic diseases and might be a important predictor of stroke and cardiovascular disease [17, 18]. MIP-1β promotes endothelial cell adhesion via increased intracellular reactive oxygen species [17], inhibits stromal cell-derived factor 1α-induced chemotaxis on B cells [19], and medi-

Table 5. Correlations between aqueous humor factors and the curative effect after IVR in the PCV group

	IL-8 r p	IFN-γ r p	IP-10 r p	MCP-1 r p	MIP-1α r p	MIP-1β r p	RANTES r p	BCVA r p	CMT r p	inner thickness r p
IL-5	-	-	-	-	-	0.535** 0.049	0.681** 0.007	-	-	-
IL-6	0.563* 0.036	-	-	-	-	0.612** 0.020	0.694** 0.006	-	-	-
IL-8	-	0.675** 0.008	0.662** 0.010	0.724** 0.003	0.684** 0.007	0.693** 0.006	-	-	0.688** 0.007	-
Eotaxin	-	0.565** 0.035	-	-	-	-	-	-	-	-
IFN-γ	-	-	0.582* 0.029	0.974** <0.001	0.730** 0.003	0.587* 0.027	-	-	0.622* 0.018	-
IP-10	-	-	-	0.631* 0.016	-	-	-	-	0.736** 0.003	0.565* 0.035
MCP-1	-	-	-	-	0.717** 0.004	0.560** 0.037	-	-	0.697** 0.006	
MIP-1α	-	-	-	-	-	0.770** 0.001	-	0.586* 0.028	-	0.580* 0.030

**When the degree of confidence (bilateral) was 0.01, the correlation was significant. *When the degree of confidence (bilateral) was 0.05, the correlation was significant. This table shows only the specific cytokines with a statistically significant difference.

ates the expression of E-selectin in endothelial cells [20]. This phenomenon illustrates that MIP-1β might play a role in the development of neovasculogenesis in patients with PCV. MIP-1β was found to be upregulated in oxygen-induced retinopathy [21], and another study found that the aqueous humor cytokine MIP-1β expression was dramatically higher in PCV patients than in the control group [22]. The concentration of MIP-1 in aqueous humor was meaningfully lower in the PCV cohort than in the control cohort in this current research. This contradiction could be attributed to differences between patient samples; however, we could only determine that MIP-1β participates in the pathogenesis of PCV.

RANTES (also known as CCL5 or C-C motif ligand 5) is a pro-inflammatory chemokine that attracts plenty of leukocytes, including monocytes, dendritic cells granulocytes, mast cells and T cells to the site of inflammation [23, 24]. The most efficacious arrest chemokine, RANTES, can be created by a variety of cell types, including activated T cells, endothelial cells, smooth muscle, platelets and macrophages [25]. RANTES has been linked to type 2 diabetes mellitus (T2DM), obesity, atherosclerosis, and glucose intolerance in several studies [26, 27]. It is also required for the formation of inflammatory angiogenesis [28]. The concentration of RANTES was meaningfully lower in the aqueous humor in the PCV group than in control group in the current study, implying that inflammation angiogenesis plays little role in the pathological formation of PCV.

There are numerous isoforms of the VEGF protein family, which includes VEGF-D, VEGF-C, VEGF-B, VEGF-A and placental growth factor (PlGF), each of which plays a distinct role in many angiogenesis environments such as lymphatic and embryonic [29, 30]. VEGF-A is a key angiogenesis regulator that can be sheared into four major subtypes of varying lengths (206, 189, 165, and 121 amino acids). The patterns and growth of blood vessels can be regulated by the equilibrium between different subtypes of VEGF-A [31]. The concentration of VEGF-A was meaningfully more in aqueous humor in PCV than in the control group in this study. Strikingly, the concentration of the VEGF-A cytokine was meaningfully lower in the PCV group after IVR than before. These findings showed that VEGF-mediated neovascularization is crucial for the pathological development of PCV. This phenomenon was consistent with the results of our previous study on neovascu-

lar age-related macular degeneration (nAMD), indicating similarities between the pathological mechanism of nAMD and PCV.

Nonetheless, the current study has a number of limitations. First, our study with small samples was insufficient to detect a meaningfully difference in the levels of cytokines between cases and controls. Second, cytokine levels were not measured in the vitreous humor but in the aqueous humor. Although the vitreous humor is nearer to the location of the retinal wound, obtaining vitreous humor is invasive, which is unethical in research.

Conclusion

In conclusion, the current investigation found that while some inflammatory variables play a minor role in the PCV pathogenesis, VEGF has a crucial function. These cytokine profile variations could be useful in discovering new PCV biomarkers and therapeutic targets for the disease's treatment.

Acknowledgements

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Disclosure of conflict of interest

Qing Peng is employed by Shanghai Tenth People's Hospital. There are no conflicts of interest that are directly relevant to the content of this paper for Tingting Sun, Jianhao Bai, Minli Wang, Le Liu, or Qing Peng.

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