

Original Article

Aqueous humor levels of cytokines in polypoidal choroidal vasculopathy and age-related macular degeneration

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Abstract: *Objective:* To investigate the differential aqueous concentrations of interleukin 6 (IL-6), interferon alpha (IFN α), monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor alpha (TNF α), transforming growth factor beta (TGF β), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) in eyes with polypoidal choroidal vasculopathy (PCV) or age-related macular degeneration (AMD). *Methods:* The clinical interventional study included a control group of 8 patients who underwent cataract surgery and a study group of 25 patients with either PCV or AMD. Aqueous humor samples were obtained. Cytokine expression in the aqueous humor samples was measured by Luminex X-MAP technology. *Results:* Significantly higher concentrations of MCP1 and TNF α were found in the aqueous humor of PCV and AMD patients than those in the aqueous humor of control patients. TGF β was significantly higher in the aqueous humor of AMD patients than that of the control group (P=0.004). The level of VEGF was not significantly different among groups. In the neovascular AMD group, aqueous levels of IL6 and MCP1 were significantly associated with retinal thickness at 3 mm and 6 mm (P=0.017, P=0.027, respectively). In the PCV group, aqueous level of VEGF was significantly associated with retinal thickness at 1 mm and 6 mm (P=0.024, P=0.042, respectively). *Conclusion:* Besides VEGF, other inflammatory cytokines and angiogenic factors, like MCP1, TGF β and TNF α may be associated with PCV and AMD, especially with AMD. This finding may have implications for the medical treatment of PCV and AMD.

Keywords: Polypoidal choroidal vasculopathy, age-related macular degeneration, aqueous humor, cytokines

Introduction

Choroidal neovascularization (CNV) is characterized by neovascularization from the choroidal blood vessels into the subpigment epithelial and subretinal spaces when the integrity of Bruch's membrane is disrupted [1, 2]. CNV can cause visual loss because of exudation of intraretinal or subretinal fluid, hemorrhage, or fibrosis at the macula. CNV is associated with a number of disorders, among which the most important one is age-related macular degeneration (AMD).

Polypoidal choroidal vasculopathy (PCV) was first described by Yannuzzi et al [3] as an abnormal vascular network of choroidal vessels with polyp like dilations at the terminals of the branches. It seems to be more prevalent in

Asians and blacks than whites [3-5]. It remains controversial as to whether or not PCV represents a sub-type of neovascular AMD. Several reports suggested that PCV is a type of choroidal neovascularization (CNV) [6] and some ophthalmologists believe that PCV is a type of CNV caused by AMD. While some reports suggested that patients with PCV have different angiographic features, genetic background, prognosis and responses to treatment compared with neovascular AMD [2-5, 7, 8]. Its relationship with AMD is still not fully understood.

The process of CNV can be affected by many cytokines through angiogenic effects, anti-angiogenic effects and inflammatory effects. After the studies by Aiello and colleagues on the neovascularization effect of vascular endothelial growth factor (VEGF) [9], increasing evi-

dence indicates that VEGF is the most important factor that contributes to many neovascular diseases such as proliferative diabetic retinopathy (PDR), central retinal vein occlusion (CRVO) and AMD [10-12]. Besides VEGF, other cytokines were found to be related to the development of neovascular diseases including basic fibroblast growth factor (bFGF) [13], interleukin 1b (IL1b) [14], interleukin 6 (IL6) [15], interleukin 8 (IL8) [16], monocyte chemoattractant protein 1 (MCP1) [10], intercellular adhesion molecule 1 (ICAM1) [10], matrix metalloproteinase 9 (MMP9) [17], tumor necrosis factor alpha (TNF α) [14] and others. However, little is known about the precise roles and differences of these molecules in the patients with PCV and AMD.

Recently, intravitreal anti-inflammatory therapy (triamcinolone acetonide, IVTA), intravitreal anti-VEGF (intravitreal bevacizumab or ranibizumab) therapy, and a combined therapy have been used in the treatment of PCV and AMD, which have been shown to be relatively effective treatments [18-21]. However, few results have been obtained in comparative studies of intravitreal injections, and there have been no exact guidelines for intravitreal injections. We therefore perform this study to examine the concentrations of both angiogenic and inflammatory cytokines in the aqueous humor of patients with PCV and AMD, and we evaluated the potential implications of these cytokines in the pathogenesis of PCV and AMD.

Patients and methods

This study was conducted in accordance with the Declaration of Helsinki, and we received approval from the Investigational Review Board of the People's Hospital affiliated with Peking University. Informed consent for all examinations and procedures was obtained from the subjects. All participants provided their written informed consent to participate in this study.

Undiluted aqueous humor samples were collected from 18 consecutive patients (18 eyes) with exudative age-related macular degeneration (AMD), 7 consecutive patients (7 eyes) with polypoidal choroidal vasculopathy (PCV) who underwent intravitreal injection of bevacizumab (avastin) as the study group and 8 consecutive patients (8 eyes) with cataract who underwent cataract surgery as the control group.

Inclusion criteria in the study group were the presence of exudative AMD or PCV. Exclusion criteria included 1) prior treatment for CNV within 1 year; 2) previous ocular surgery; 3) a history of other neovascular ocular diseases; 4) a history of inflammatory or autoimmune diseases; 5) prior cerebrovascular accident, pulmonary embolus, myocardial infarction or uncompensated coronary artery disease within the past 6 months.

Each patient underwent an ophthalmic and medical examination. The ophthalmic examinations include best corrected visual acuity (BCVA) recording using manifest refraction and logMAR visual acuity chart, non-contact tonometry, slit-lamp biomicroscopy, gonioscopy, ophthalmoscopy, fluorescein angiography, indocyanine green angiography, and optical coherence tomography (OCT; Optovue OCT-IV). All the cases of PCV and CNV were confirmed to be active in disease activity by fluorescein angiography. Indocyanine green angiography was also performed for the diagnosis of PCV. All diagnoses were confirmed by at least 2 doctors (one retina attending and one fellow) independently at the time of admission. OCT was analyzed by one investigator and retinal thickness at 1 mm, 3 mm and 6 mm away from the center of macula were measured using inbuilt software.

The aqueous humour was collected before intravitreal bevacizumab injection or cataract surgery. All injections and sample collections were performed using a standard sterilization procedure that included the use of topical povidone-iodine and levofloxacin drops. Undiluted aqueous samples of 0.05 ml from each eye were collected in sterile tubes, placed immediately on ice, and stored at -80°C until use. The risks and benefits of all therapeutic options were discussed in detail and all patients agreed to the collection and analysis of samples, which would have been otherwise discarded at the time of the surgery.

Cytokine expression in the aqueous humor samples was measured by Luminex X-MAP technology using the Procarta Immunoassay kit (Panomics Inc., Fremont, CA, USA). Procarta Immunoassays use the xMAP technology (multi-analyte profiling beads) to enable the detection and quantitation evaluating multiple protein targets simultaneously in diverse-

Table 1. Composition of the study population (mean ± SD)

Variables	Control group	AMD group	PCV group	P value
Number	8	18	7	-
Age, years old	76.12±5.30	68.78±8.23	73.43±8.01	0.051 ¹
Female sex, n (%)	4 (50)	8 (44.4)	3 (42.9)	0.945 ²
Diabetes, n (%)	2 (25)	2 (11.1)	1 (14.3)	0.897 ²
Hypertension, n (%)	2 (25)	3 (16.7)	3 (42.9)	0.518 ²
BCVA	0.18±0.12	0.12±0.11	0.22±0.13	0.338 ¹
IOP (mmHg)	14.00±3.25	13.29±2.48	12.86±1.57	0.062 ¹

¹Kruskal-Wallis H test, compared among control group, exudative AMD, and PCV groups. ²Fisher's exact t test, compared among control group, exudative AMD, and PCV groups. AMD, age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; BCVA, best corrected visual acuity; IOP, intraocular pressure.

matrices. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (beads), dual laser design and digital signal processing to effectively allow multiplexing of up to 100 unique assays within a single sample. The detailed process of the xMAP technology was reported in similar studies [22].

The following cytokines were tested in each sample: bFGF, IFN α , IL6, MCP1, TNF α , TGF β and VEGF. Based on the information provided by the manufacturer, the multiplex assay kit can quantitatively measure multiple cytokines from as little as 25 μ L of bodily fluids. Aqueous humour samples (50 μ l) were used undiluted and incubated overnight. Each sample had 2 replicates (25 μ l each). Standard curves for each cytokine were generated using the reference cytokine concentrations supplied in this kit. The kit was run according to the manufacturer's instructions.

Statistical method

Statistical analysis of the data was performed using a commercially available statistical software package (SPSS for Windows, version 17.0: SPSS, Inc, Chicago, Illinois, USA). Differences in gender, hypertension proportion and diabetes proportion were analyzed with the chi-square or Fisher exact test when appropriate. Measurement data were presented as the mean ± standard deviation. To assess the normal distribution, the Shapiro-Wilk test was used. Differences between the study group and the control group were estimated with a nonparametric Mann-Whitney rank sum test or t test when appropriate. Correlation coefficients were determined by using the

Pearson correlation test or non-parametric Spearman correlation test when appropriate. Two-tailed probabilities of less than 0.05 were considered to indicate statistical significance.

Results

The study included 33 patients, with 25 patients in the study group and 8 patients in the control group. Gender, age, diabetes proportion, hypertension proportion, BCVA and intraocular pressure (IOP) did not vary significant-

ly between the study group and the control group (all P>0.05) (Table 1).

The aqueous humour level of MCP1 was 2037.36±1660.21 (mean ± SD) pg/ml in eyes with PCV, 1802.54±1787.54 pg/ml in eyes with AMD, and 872.03±209.30 pg/ml in eyes of the control group (Table 2). Concentrations of MCP1 in PCV, AMD were significantly higher than that of the controls (P=0.037, P=0.005, respectively). The concentrations of TNF α in PCV and AMD were significantly higher than that of the controls (P=0.040, P=0.008, respectively). The TGF β levels in AMD patients was significantly higher than that of the controls (P=0.004). The levels of bFGF, IFN α , IL6 and VEGF were not significantly different in neither of AMD or PCV patients compared with the control group (all P>0.05).

The aqueous level of all seven cytokines did not differ between PCV patients and neovascular AMD patients (all P>0.05).

In the neovascular AMD group, the aqueous level of MCP1 was significantly associated with retinal thickness at 3 mm from macula (P=0.027; Table 3, Figure 1A). IL6 level was significantly associated with retinal thickness at 3 mm from macula (P=0.017; Table 3), while VEGF, bFGF, IFN α and TGF β and TNF α showed no significant correlation with retinal thickness at 1 mm, 3 mm and 6 mm from macula (all P>0.05).

In the PCV group, the aqueous level of VEGF was significantly associated with retinal thickness at 1 mm from macula (P=0.024; Table 4,

Cytokines in PCV and AMD

Table 2. Aqueous humor levels of cytokines (pg/ml) in eyes with AMD and PCV (mean \pm SD)

Cytokines	AMD group (n=18)	PCV group (n=7)	Control group (n=8)
bFGF	5.73 \pm 6.55, <i>P</i> =0.160	3.65 \pm 4.47, <i>P</i> =0.907	2.44 \pm 2.32
IFN α	4.06 \pm 2.75, <i>P</i> =0.552	3.90 \pm 1.50, <i>P</i> =0.642	3.37 \pm 2.59
IL6	49.51 \pm 119.18, <i>P</i> =0.062	191.14 \pm 315.93, <i>P</i> =0.563	70.71 \pm 91.50
MCP1	1802.54 \pm 1787.54, <i>P</i> =0.005	2037.36 \pm 1660.21, <i>P</i> =0.037	872.03 \pm 209.30
TGF β	31.62 \pm 20.89, <i>P</i> =0.004	22.50 \pm 14.09, <i>P</i> =0.093	12.15 \pm 7.48
TNF α	5.92 \pm 3.89, <i>P</i> =0.008	4.97 \pm 1.75, <i>P</i> =0.040	2.94 \pm 1.69
VEGF	3.43 \pm 2.52, <i>P</i> =0.694	4.47 \pm 2.05, <i>P</i> =0.504	3.82 \pm 1.62

AMD, age-related macular degeneration; PCV, polypoidal choroidal vasculopathy; bFGF, Basic fibroblast growth factor; IFN α , Interferon alpha; IL6, Interleukin 6; MCP1, Monocyte chemoattractant protein-1; TGF β , Transforming growth factor beta; TNF α , Tumor necrosis factor alpha; VEGF, Vascular endothelia growth factor.

Table 3. *P* values of each correlation test between level of cytokines and OCT parameters in neovascular AMD patients

Cytokine	Retinal thickness (1 mm)		Retinal thickness (3 mm)		Retinal thickness (6 mm)	
	Correlation		Correlation		Correlation	
	Coefficient r	<i>P</i> value	Coefficient r	<i>P</i> value	Coefficient r	<i>P</i> value
bFGF	-0.332	0.178	-0.449	0.062	-0.259	0.299
IFN α	0.171	0.498	0.169	0.504	-0.011	0.966
IL6	0.419	0.083	0.556	0.017	0.245	0.326
MCP1	0.391	0.108	0.521	0.027	0.381	0.119
TGF β	0.018	0.945	0.153	0.545	0.118	0.642
TNF α	0.237	0.344	0.271	0.276	0.225	0.642
VEGF	0.085	0.738	0.048	0.851	0.038	0.880

Spearman correlation test bFGF, Basic fibroblast growth factor; IFN α , Interferon alpha; IL6, Interleukin 6; MCP1, Monocyte chemoattractant protein-1; TGF β , Transforming growth factor beta; TNF α , Tumor necrosis factor alpha; VEGF, Vascular endothelia growth factor.

Table 4. *P* values of each correlation test between level of cytokines and OCT parameters in PCV patients

Cytokine	Retinal thickness (1 mm)		Retinal thickness (3 mm)		Retinal thickness (6 mm)	
	Correlation		Correlation		Correlation	
	Coefficient r	<i>P</i> value	Coefficient r	<i>P</i> value	Coefficient r	<i>P</i> value
bFGF	0.569	0.183	0.382	0.398	0.303	0.969
IFN α	-0.162	0.728	-0.289	0.529	-0.097	0.835
IL6	-0.270	0.558	-0.536	0.215	-0.429	0.337
MCP1	-0.342	0.452	-0.500	0.253	-0.393	0.383
TGF β	-0.261	0.572	-0.125	0.789	-0.023	0.961
TNF α	0.200	0.667	0.246	0.594	-0.102	0.828
VEGF	0.819	0.024	0.718	0.069	0.772	0.042

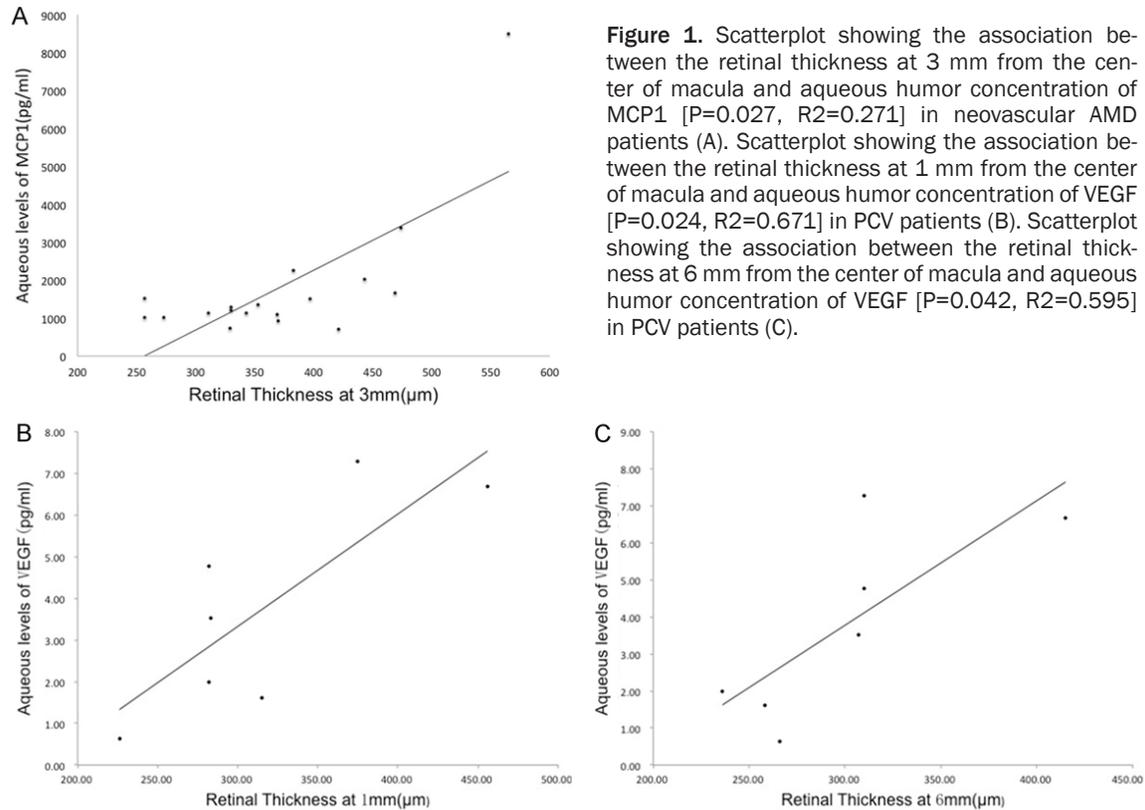
Spearman correlation test bFGF, Basic fibroblast growth factor; IFN α , Interferon alpha; IL6, Interleukin 6; MCP1, Monocyte chemoattractant protein-1; TGF β , Transforming growth factor beta; TNF α , Tumor necrosis factor alpha; VEGF, Vascular endothelia growth factor.

Figure 1B) and 6 mm from macula (*P*=0.042; **Table 4, Figure 1C).** While MCP1, TNF α , IL6, bFGF, IFN α and TGF β showed no significant correlation with retinal thickness at 1 mm, 3 mm and 6 mm from macula (all *P*>0.05) (**Table 4).**

Discussion

CNV is one of important causes of visual impairments and legal blindness in the elderly patients [23, 24]. CNV can be thought of as

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a two-component system [25]. The vascular component is composed of vascular endothelial cells (ECs), pericytes and precursors of EC, whereas the extravascular component is composed of inflammatory cells, glial cells, retinal pigment epithelial cells and fibroblasts. Therefore, inflammation and angiogenesis are both involved in the pathogenesis of CNV. Various cytokines such as VEGF, bFGF, IL1 β , IL6, IL8, MCP1, ICAM1, MMP9, TNF α and others have been implicated in the development of CNV [10, 13-17]. However, little is known about the precise roles and differences of these molecules in the patients with PCV and AMD. The aim of this study was to determine the possible role of inflammation and angiogenesis in PCV and AMD by measuring various cytokine levels in the aqueous humor of patients.

High levels of MCP1 in the aqueous humor with PCV and AMD were detected that were statistically significantly higher than that of the controls. Our results confirm previous investigations [10, 26, 27] and suggests that MCP1 plays an important role in the process of AMD [10], as well as PCV. In our study, the

higher aqueous level of MCP1 indicated that inflammation is probably involved in the formation of choroidal neovascular membranes and may thus contribute to the pathogenesis of CNV [28, 29]. MCP1, as a member of the small inducible gene family, plays an important role in the recruitment of monocytes to sites of injury and infection [30]. MCP1 is constitutively produced from the retinal pigment epithelium [31, 32] and upregulated in cultured RPE cells in response to various stimuli, including other cytokines [31, 33]. MCP1 plays an important role in regulation of the migration and infiltration of monocytes and macrophages, which is an early step in the initiation of the inflammatory and angiogenic processes [34] and it has also been known to facilitate angiogenesis by participating in VEGF-induced angiogenesis and vascular leakage [30, 35], which may be helpful in explaining our result that the aqueous concentration of MCP1 is positively associated with retinal thickness at 3 mm in neovascular AMD patients. We speculate that MCP1 may participate in the angiogenesis process and promote vascular leakage, thus contributing to the increasing thickness of retina in AMD.

TNF α is responsible for a macrophage-derived angiogenic activity [36]. Kobayashi et al suggested an involvement of TNF α in the development of CNV in STZ-diabetic rats [37]. Our results showed that the aqueous humor level of TNF α in PCV and AMD were significantly higher than that of the controls. TNF α was also detected in the macrophages in surgically removed choroidal neovascular membranes [38]. Espinosa-Heidmann et al reported that AMD patients with blood monocytes that express high TNF α mRNA levels demonstrate an almost fivefold increased prevalence of neovascular AMD [39]. These results showed that TNF α participates in the pathogenesis of AMD. It has been reported that TNF α participated in the development of retinal neovascularization during post-ischemic inflammation by triggering bFGF, IL8 and MCP1 [40]. In a laser-induced choroidal neovascularization (CNV) mouse model, anti-TNF α treatment with different inhibitors reduces both the size and the leakage of laser-induced CNV [41]. These results suggest the involvement of TNF α in the development of both AMD and PCV. TNF α may be used as a therapeutic agent.

When CNV develops, the growth of new vascular tissue is followed by a fibrous process with progressive macular destruction. That is to say, both angiogenic and fibrotic processes participate in the pathogenesis of CNV [42]. In CNV, TGF β is not only the main cytokine responsible for scar tissue formation, but also reported as an enhancer effect on VEGF secretion by recent studies in human RPE cultured cells [43]. Anti-TGF β treatment with inhibitor decreases laser-induced CNV in rats [44]. In our investigation, the concentration of TGF β in the aqueous humor was significantly higher in AMD patients, which also supports that TGF β may participate in the pathogenesis of CNV.

In addition, we found no difference in VEGF levels between groups, which was also supported by others [27, 45]. In the study by Jost B Jonas et al the aqueous humor VEGF level in AMD did not vary significant with cataract patients [27]. We speculated that VEGF might be localized to the choroid and there might not be sufficient VEGF distributed throughout the vitreous cavity into the anterior chamber. Although the level of VEGF in the

lesion site and the aqueous humour is higher in CNV, it may not be enough to show statistical difference from the control. Interestingly, in eyes with PDR, aqueous humor concentration of VEGF was markedly elevated [11]. Further investigations could be carried out on whether anti-VEGF drugs such as bevacizumab have a more pronounced anti-angiogenic effect in PDR than in AMD patients. The significant correlation between the level of VEGF in PCV patients and the retinal thickness at 1 mm and 6 mm may indicate that VEGF participates in the pathogenesis of PCV. We speculate that VEGF may contribute to the increasing of retinal thickness by inducing angiogenesis and vascular leakage, which also provided explanations for valid treatment effect by using anti-VEGF therapy [21].

Our results showed no significant differences of various cytokines between the AMD group and PCV group. AMD and PCV could not be distinguished by measuring the cytokine levels in the aqueous humor. It remains controversial as to whether or not PCV represents a sub-type of neovascular AMD. Further studies are warranted to enhance our understanding of PCV.

Certainly, there are still some limitations to our study. First, our findings should be considered preliminary, in part because the number of enrolled patients was not large. However, large, randomized, controlled trials may be prevented by the relatively low prevalence of certain underlying conditions such as PCV. Thus, a large, multicenter, randomized, prospective study is required to clarify the pathogenesis of PCV and AMD as associated with cytokines. This study could serve as the basis for future research that involves a large number of eyes and thus could enable the development of disease-specific treatments. Second, it is inaccurate to assume that a particular cytokine affects pathogenesis on the simple basis of measuring elevated aqueous levels. A particular cytokine is released as a result of the disease process. Thus, it cannot be the cause of a disease process. Third, we have only examined the concentrations of cytokines in the aqueous humor, which may not be able to have reflected the situation of retina and choroid. Vitreous fluid may be more meaningful for the detection of cytokine concentration

for fundus diseases. However, obtaining vitreous samples are not possible without an intravitreal intervention. While previous studies suggested that the levels of cytokines in the aqueous humor may reflect those in the vitreous fluid and the measurement of the aqueous levels of these cytokines may be useful to predict the disease activity and reflect processes that occur in the retina [46].

In conclusion, inflammatory cytokines such as MCP1, TGF β and TNF α may be associated with PCV and AMD. This finding may have implications for the medical treatment of both PCV and AMD. The cytokines examined show no significant difference between the AMD group and PCV group. Further studies are warranted to enhance our understanding of PCV.

Acknowledgements

Informed consent was obtained from each patient after an explanation of the purpose and potential adverse effects of the procedure.

Disclosure of conflict of interest

None.

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