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RETINAL DISORDERS

Expression of angiopoietin-like protein 8 correlates with VEGF in patients with proliferative diabetic retinopathy

Qianyi Lu[1] · Li Lu[2] · Wei Chen[3] · Peirong Lu[1]

Abstract

Purpose To investigate the vitreous and serum levels of angiopoietin-like protein 8 (ANGPTL-8) and vascular endothelial growth factor (VEGF) in patients with proliferative diabetic retinopathy (PDR). The serum levels of these factors were also analyzed in patients with diabetes and no diabetic retinopathy (NDR) and with non-proliferative diabetic retinopathy (NPDR), to detect the possible correlation between the ANGPTL-8 levels and hyperlipidemia.

Methods Vitreous samples were obtained from 28 patients with PDR and from 12 patients without diabetes and with idiopathic macular hole (IMH). Serum samples were also obtained from 26 patients with NDR and 22 patients with NPDR. ANGPTL-8 levels and other factors were determined using an enzyme-linked immunosorbent assay.

Results The ANGPTL-8 and VEGF levels in the vitreous and serum of the patients with PDR were higher than those in the patients with IMH, and were significantly correlated. The vitreous and serum ANGPTL-8 levels were more correlated with the triglyceride and low-density lipoprotein cholesterol levels than with the high-density lipoprotein cholesterol or total cholesterol levels in the patients with PDR.

Conclusions The vitreous and serum ANGPTL-8 levels were both upregulated in patients with PDR. There was an association between the elevation in the ANGPTL-8 levels and

angiogenic and hyperlipidemic factors in the patients with PDR. These results suggest that ANGPTL-8 is a potential new diagnostic marker and therapeutic target for PDR treatment.

Keywords Angiopoietin-like protein 8 (ANGPTL-8) · Vascular endothelial growth factor (VEGF) · Proliferative diabetic retinopathy (PDR) · Hyperlipidemia

Introduction

Diabetic retinopathy (DR) is a microvascular complication that affects approximately 30% of patients with diabetes and is the leading cause of preventable blindness in patients of working age [1–3]; the identification of effective treatments and prevention measures has long been the objective of numerous studies [4, 5]. Several studies have reported the important role of vascular endothelial growth factor (VEGF) in angiogenesis and inflammation due to dysglycemia in DR progression [6–8]. However, the pathogenesis and underlying mechanism of DR, especially proliferative diabetic retinopathy (PDR), are currently unknown.

Increasing evidence from recent studies has suggested that dyslipidemia is closely involved in the progression of PDR [9]; novel adipocytokines, such as angiopoietin-like proteins (ANGPTLs), have been of particular interest [10, 11]. ANGPTL-8, a novel ANGPTL family member, plays a role in both dyslipidemia and dysglycemia [9]. Several recent studies have reported that circulating ANGPTL-8 levels are increased in patients with type 1 and type 2 diabetes mellitus (T1/2DM) [12–15]. Paradoxically, a single-center study has shown that serum ANGPTL-8 levels decreased significantly in T2DM and patients with obesity [16]. Some researchers have attributed these conflicting results to the enzyme-linked

✉ Peirong Lu
  lupeirong@suda.edu.cn

[1] Department of Ophthalmology, The First Affiliated Hospital of Soochow University, Suzhou 215006, China

[2] Department of Ophthalmology, Anhui Provincial Hospital, Anhui Medical University, Hefei 230001, China

[3] Department of Ophthalmology, Affiliated Hospital of Nantong University, Nantong 226001, China

immunosorbent assay (ELISA) kits used in these studies [17]. Nonetheless, ANGPTL-8 has been suggested as a potential biomarker of T2DM. However, there have been few studies on the potential crosstalk between ANGPTL-8 and VEGF, especially considering that the expression levels of ANGPTL-8 and VEGF are both elevated in patients with diabetes.

Recent studies have reported that levels of ANGPTL-8 correlate with low-density lipoprotein cholesterol (LDL-C) [18–20]; furthermore, a substantial amount of data has shown that LDL-C mediates or correlates with VEGF levels [21–24]. Based on all these findings, we examined whether ANGPTL-8 together with the proangiogenic factor, VEGF, play roles in the progression of PDR. If a correlation between ANGPTL-8 and VEGF levels exists, then ANGPTL-8 could possibly be used as a therapeutic target for PDR. In this study, we identified a correlation between ANGPTL-8 and VEGF in the vitreous fluid and serum of patients with PDR. To our knowledge, this is the first study that has reported a novel dyslipidemic role for ANGPTL-8 in dysglycemia-related PDR.

Materials and methods

Participants

The research followed the tenets of the Declaration of Helsinki (as revised in Brazil 2013) and was approved by the First Affiliated Hospital of Soochow University. The nature and possible consequences of the study were explained to all participants, and written informed consent was obtained before participation. After biochemical and physiological tests (medical/health history, vitals, body mass index [BMI], fasting glucose [FG], glycosylated hemoglobin [HbA1c], serum lipid profiles, liver enzymes, and electrocardiography) and careful retinal examination or fluorescein angiography followed by pupillary dilation with tropicamide, 88 patients were divided into the following four groups: the idiopathic macular hole (IMH) group (12 eyes of 12 patients with IMH), nondiabetic retinopathy (NDR) group (26 eyes of 26 patients with T2DM and no diabetic retinopathy), non-proliferative diabetic retinopathy (NPDR) group (22 eyes of 22 patients with NPDR), and PDR group (28 eyes of 28 patients with PDR). The IMH group served as controls, and none of the patients with IMH had DM. The PDR group and IMH group consisted of patients admitted to undergo vitreous surgery. The patients were excluded if they had received treatment for ocular diseases. Weight was measured in light outdoor clothing without shoes, and BMI was calculated by dividing weight (kilograms) by height squared (meters squared). The demographic details of the patients are shown in Table 1.

Vitreous collection and analysis

The vitreous samples were collected from the PDR group and IMH group that underwent vitreous surgery (23-gauge, Alcon Instruments, USA) for the treatment of retinal disorders. In the present study, the patients who had undergone previous laser treatment or a repeat vitrectomy before their current surgery were excluded. After patient consent was obtained, the vitreous fluid (0.3–0.5 ml) was aspirated through a vitreous cutter under the simultaneous inflation of the vitreous cavity with air through an infusion cannula; the fluid was frozen rapidly at −80 °C until the assay was performed. The vitreous ANGPTL-8 measurements were performed using a commercially available ELISA kit (Aviscera Bioscience, CA, USA) according to the manufacturer's protocol. Similarly, the vitreous VEGF measurements were performed using a Quantikine VEGF assay kit (R&D Systems, Inc., Minneapolis, MN, USA).

Serum collection and analysis

Serum samples were collected from all 88 participants. Venous blood was collected from an antecubital vein in a 5-ml K3EDTA Vacutainer using minimum hemostasis after the patients fasted overnight for at least 10 h. The blood was well mixed by gently inverting the vacutainer 10 times. Once centrifuged, the serum was separated and frozen at −80 °C until analysis. The HbA1c measurements were performed by high-pressure liquid chromatography on a Bio-Rad Variant II instrument. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were analyzed using an automatic biochemical analyzer (FUJI DRI-CHEM 4000i, Fuji, Japan).

Statistical analysis

The results are expressed as the means ± standard deviation (SD). Group means were compared by one-way ANOVA using GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA) and the statistical software program SPSS version 17.0 for Windows (SPSS, Chicago, IL, USA). Pearson correlation tests were also performed. For all comparisons, a value of $P < 0.05$ was considered statistically significant.

Results

Table 1 shows the clinical data of all participants in the four groups studied, including the PDR group, IMH group, NDR group, and NPDR group. There was no significant difference in age, BMI, serum level of TC, or medical/health history among the four groups (Table 1). The FG level in the PDR group was

Table 1 Clinical data for controls (IMH) and patients with NDR, NPDR, and PDR

Factors	Control (IMH)	NDR	NPDR	PDR
No. of cases	12	26	22	28
Age (years)	55.28 ± 5.23	54.76 ± 4.78	53.14 ± 4.04	56.57 ± 4.52
Gender (male/female)	6/6	14/12	12/10	13/15
Prior smoking (%)	25.00	11.54	27.27	42.85
Hypertension (%)	16.67	30.77	27.27	32.14
CHD (%)	16.67	7.69	9.09	10.71
BMI (kg/m^2)	23.61 ± 2.17	24.33 ± 1.61	24.52 ± 2.14	24.74 ± 2.35
FG (mmol/l)	5.45 ± 0.32	10.34 ± 1.12[a]	11.21 ± 1.30[a]	11.26 ± 1.41[a]
HbA1c (%)	5.36 ± 0.31	7.25 ± 0.43[a]	7.83 ± 0.65[a]	8.55 ± 0.48[a]
TG (mmol/l)	1.43 ± 0.56	2.48 ± 0.63[a]	2.35 ± 0.58[a]	2.77 ± 0.69[a]
LDL-C (mmol/l)	2.19 ± 0.65	2.18 ± 0.74	2.46 ± 0.82	2.72 ± 0.41[ab]
HDL-C (mmol/l)	1.36 ± 0.25	1.43 ± 0.31	1.28 ± 0.37	1.08 ± 0.33[ab]
TC (mmol/l)	4.79 ± 0.52	4.76 ± 0.68	4.63 ± 0.54	5.37 ± 0.72
Serum ANGPTL-8 (ng/ml)	1.02 ± 0.21	1.12 ± 0.23	1.17 ± 0.11	1.60 ± 0.19[abc]
Serum VEGF (pg/ml)	46.55 ± 8.74	49.28 ± 13.91	64.25 ± 12.53	112.60 ± 19.81[abc]

[a] $P < 0.01$ vs control

[b] $P < 0.01$ vs NDR

[c] $P < 0.01$ vs NPDR

11.26 ± 1.41 mmol/l, which was significantly higher than that in the IMH group (5.45 ± 0.32 mmol/l, $P < 0.01$; Table 1). The HbA1c level in the PDR group was 8.55 ± 0.48%, which was significantly higher than that in the IMH group (5.36 ± 0.31%, $P < 0.01$; Table 1). In addition, the serum LDL-C level in the PDR group was 2.72 ± 0.41 mmol/l, which was higher than that in the IMH group (2.19 ± 0.65 mmol/l, $P < 0.01$; Table 1). In contrast, the serum HDL-C level in the PDR group was 1.08 ± 0.33 mmol/l, which was lower than that in the IMH group (1.36 ± 0.25 mmol/l, $P < 0.01$; Table 1). Furthermore, the serum TG level was significantly higher in the PDR group (2.77 ± 0.69 mmol/l) than that in the IMH group (1.43 ± 0.56 mmol/l, $P < 0.01$; Table 1).

To determine whether the level of ANGPTL-8 was altered in patients with PDR compared with the patients with IMH, the expression levels of ANGPTL-8 in the serum and vitreous samples were measured using ELISA. Serum ANGPTL-8 was detectable in all four groups (Fig. 1b). The mean serum ANGPTL-8 level in the PDR group (1.60 ± 0.19 ng/ml) was significantly higher than that in the IMH group (1.02 ± 0.21 ng/ml, $P < 0.01$; Table 1), the NDR group (1.12 ± 0.23 ng/ml, $P < 0.01$; Table 1), and the NPDR group (1.17 ± 0.11 ng/ml, $P < 0.01$; Table 1). Moreover, the mean vitreous ANGPTL-8 level in patients with PDR (2.10 ± 0.43 ng/ml) was significantly higher than that in the patients with IMH (0.60 ± 0.12 ng/ml, $P < 0.001$; Table 2; Fig. 1a). Accordingly, we sought to determine whether there was a relationship among the elevated biomarkers. A significant correlation was found between serum and vitreous ANGPTL-8 levels ($R = 0.73$, $P < 0.001$; Fig. 2a).

Previous studies over the past few decades have suggested that VEGF is the most quintessential and representative
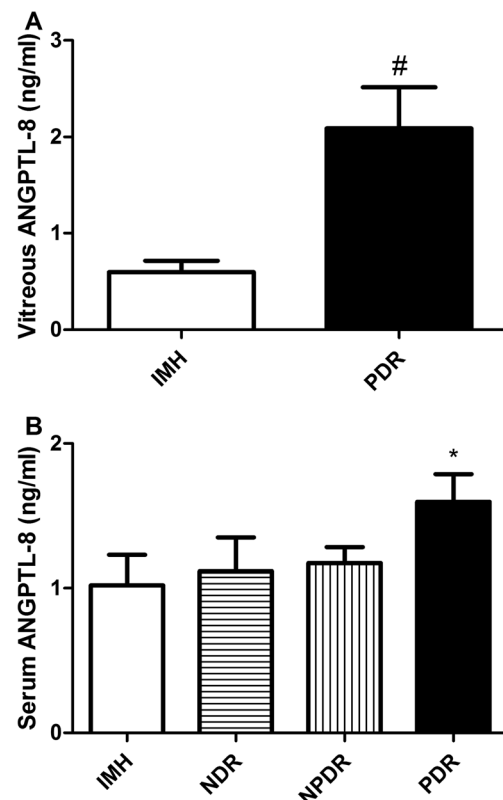

Fig. 1 ANGPTL-8 elevated in the vitreous and serum of patients with PDR. **a** Vitreous ANGPTL-8 expression profiles in IMH and PDR groups (# $P < 0.001$ vs IMH). **b** Serum ANGPTL-8 expression levels in IMH, NDR, NPDR, and PDR groups (* $P < 0.01$ vs others)

Table 2 Intravitreous concentrations of ANGPTL-8 and VEGF in controls (IMH) and patients with PDR

Factors	Control (IMH)	PDR	P value
No. of cases	12	28	-
Age (years)	55.28 ± 5.23	56.57 ± 4.52	-
Gender (male/female)	6/6	13/15	-
IMH	12	-	-
VH	-	14	-
TRD	-	6	-
VH + TRD	-	8	-
ANGPTL-8 (ng/ml)	0.60 ± 0.12	2.10 ± 0.43	0.001
VEGF (pg/ml)	<31.2[a]	1551.00 ± 420.50	0.001

[a] Vitreous level of VEGF regarded as 31.2 pg/ml in control participants

biomarker of angiogenesis, and the most frequent clinical sign induced by PDR. In this study, we evaluated possible correlations between increasing levels of vitreous ANGPTL-8 and vitreous VEGF. In the PDR group, 14 eyes had vitreous hemorrhages (VH), six eyes had tractional retinal detachments (TRD), and eight eyes had VH and TRD simultaneously (Table 2). The mean VEGF level in the vitreous of the patients with PDR (1,551.00 ± 420.50 pg/ml) was significantly higher than that in the patients with IMH (31.2 pg/ml, $P < 0.001$; Table 2). In addition, the mean serum VEGF level in the PDR group (112.60 ± 19.81 pg/ml) was significantly higher than that in the IMH group (46.55 ± 8.74 pg/ml, $P < 0.001$; Table 1), in the NDR group (49.28 ± 13.91 pg/ml, $P < 0.001$; Table 1), and in the NPDR group (64.25 ± 12.53 pg/ml, $P < 0.001$; Table 1). Consistent with our hypothesis, there was a significantly positive correlation between the vitreous ANGPTL-8 and VEGF levels ($R = 0.85$, $P < 0.001$; Fig. 2b). There was also a significant positive correlation between the serum ANGPTL-8 and VEGF levels ($R = 0.72$, $P < 0.001$; Fig. 2c).

The correlation between BMI and the serum ANGPTL-8 level ($R = 0.37$, $P = 0.055$; Fig. 3b) was less than that between BMI and the vitreous ANGPTL-8 level ($R = 0.50$, $P = 0.007$; Fig. 3a) in the patients with PDR. In contrast, the correlation between HbA1c and serum ANGPTL-8 levels ($R = 0.66$, $P < 0.001$; Fig. 3d) was greater than that between the HbA1c and vitreous ANGPTL-8 levels ($R = 0.59$, $P < 0.001$; Fig. 3c) in patients with PDR.

Consistent with the observation that PDR is usually accompanied by hyperlipidemia, there was also a correlation between the ANGPTL-8 level and the serum lipid profile in the patients with PDR. The positive correlation between the serum TG and ANGPTL-8 ($R = 0.63$, $P < 0.001$; Fig. 4b) was less than that between the serum TG and vitreous ANGPTL-8 ($R = 0.73$, $P < 0.001$; Fig. 4a) in the patients with PDR. In contrast, the positive correlation between the serum LDL-C and serum ANGPTL-8 levels ($R = 0.69$, $P < 0.001$; Fig. 4d)
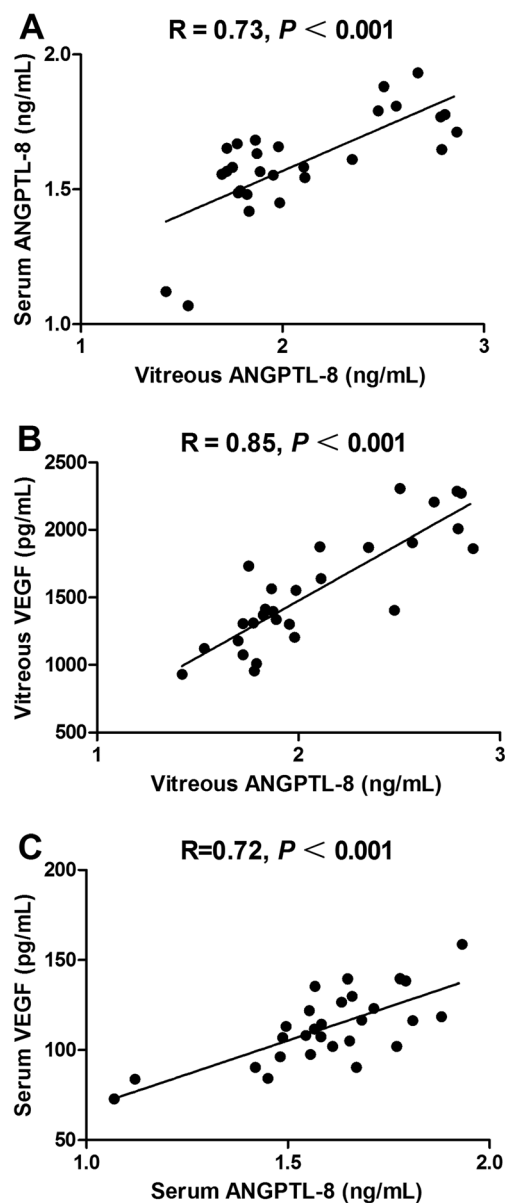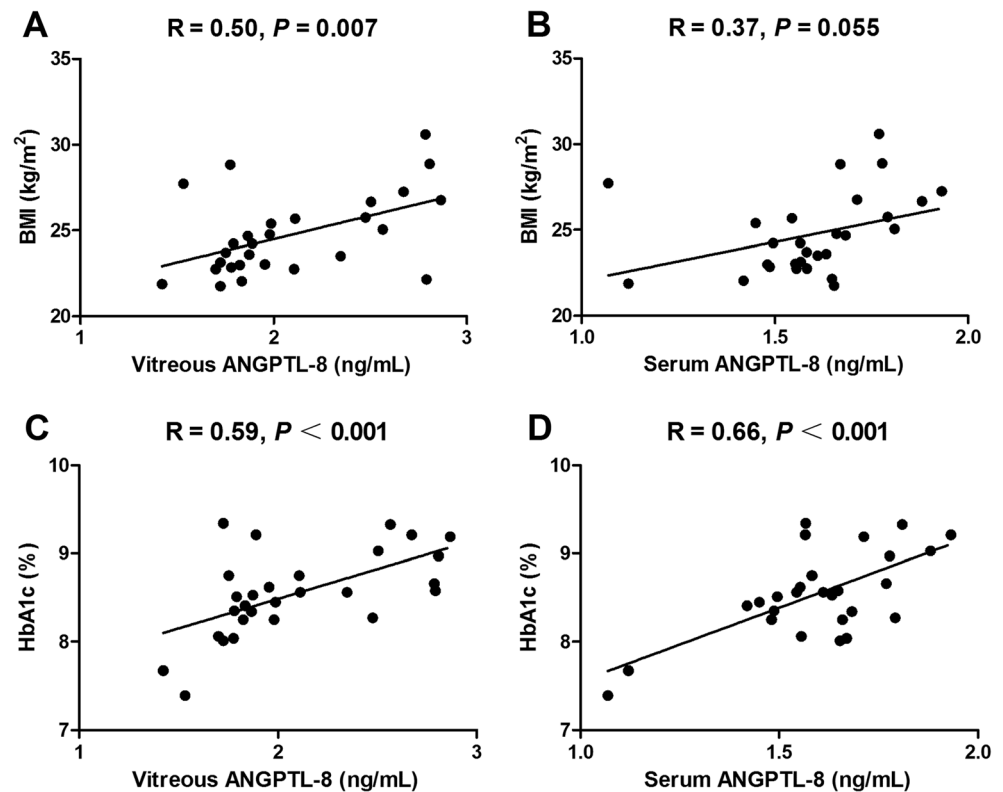
Fig. 2 Correlations between ANGPTL-8 with VEGF in PDR patients. Pearson's correlation tests showed the positively significant correlations **a** between vitreous ANGPTL-8 and serum ANGPTL-8 ($R = 0.73$, $P < 0.001$), **b** between vitreous ANGPTL-8 and vitreous VEGF ($R = 0.85$, $P < 0.001$), and **c** between serum ANGPTL-8 and serum VEGF ($R = 0.72$, $P < 0.001$) in PDR patients

was greater than that between the serum LDL-C and vitreous ANGPTL-8 levels ($R = 0.59$, $P < 0.01$; Fig. 4c) in patients with PDR. However, negative correlations were found between the serum HDL-C and ANGPTL-8 levels ($R = -0.44$, $P = 0.020$; Fig. 4f) and between the serum HDL-C and vitreous ANGPTL-8 levels ($R = -0.41$, $P = 0.028$; Fig. 4e) in the patients with PDR. A lower positive correlation between the serum TC and serum ANGPTL-8 levels was also confirmed ($R = 0.43$, $P = 0.023$; Fig. 4h); however, the correlation between the serum TC and vitreous ANGPTL-8 levels was not

significant in the patients with PDR ($R = 0.33$, $P = 0.091$;
Fig. 4g).

To explore whether ANGPTL-8 is a more representative
adipocytokine than regular lipid molecules in the disease state
of PDR, we analyzed the correlation between VEGF and lipid
molecules, such as TG, LDL-C, HDL-C, and TC. We found
that there were positive correlations only between the vitreous
VEGF and serum TG levels ($R = 0.64$, $P = 0.002$; Fig. 5a) and
between the vitreous VEGF and serum LDL-C levels
($R = 0.67$, $P < 0.001$; Fig. 5b). However, there was no signif-
icant correlation between the VEGF levels and other lipid
molecules ($P > 0.05$; Fig. 5).

Discussion

Recent studies have reported that dyslipidemia may contribute
to DR susceptibility [9]. ANGPTL-8, also known as lipasin
[25, 26], refeeding-induced in fat and liver (RIFL) [27], and
betatrophin [28], is an important adipocytokine in lipid me-
tabolism that contributes to both dyslipidemia and
dysglycemia [29–31]. However, the mechanism by which
dyslipidemia alters retinal homeostasis is unknown; multiple
pathways may be involved in the progression of DR. [1, 32].

In this study, we first determined the ANGPTL-8 levels in
the human vitreous and serum samples, and showed that the
vitreous and serum levels of ANGPTL-8 were increased in the
patients with PDR compared with those in the patients with

IMH. The most destructive damage during PDR is thought to
be closely associated with VEGF overexpression, and we
showed a positive correlation between the VEGF and
ANGPTL-8 levels in the vitreous and serum of the patients
with PDR. To our knowledge, this is the first report to link
ANGPTL-8, a novel adipocytokine, with VEGF in patients
with PDR. Nonetheless, the mechanism underlying the corre-
lation between ANGPTL-8 and VEGF remains unclear.

One possible explanation for the crosstalk between
these two proteins in the vitreous fluid is that leakage
of ANGPTL-8 from the blood to the vitreous body may
occur as a result of the destruction of the blood–retinal
barrier. However, we cannot exclude the possibility that
ANGPTL-8 is produced by cells of the vascular endo-
thelium and other cells within the ischemic retina or
fibrovascular epiretinal membranes.

Recent studies have reported that ANGPTL-8 levels are
correlated with LDL-C [18–20]. Observational and preclinical
studies have reported that elevated LDL-C and TG levels
could be used to predict the progression of DR. [33–35]
Furthermore, Dandapat A et al. have found that small concen-
trations of oxygenized LDL promote capillary tube formation
by inducing the expression of VEGF via lectin-like
oxygenized LDL receptor 1 mediated activation of the
NADPH-oxidase/MAPKs/NF-κB pathway [36]. Thus, we
speculate that the LDL/MAPKs/NF-κB pathway may be a
potential alternative target for future in-vivo and in-vitro re-
search focusing on the correlation between ANGPTL-8 and

Fig. 4 Correlation between ANGPTL-8 with serum lipids in PDR patients. Pearson's correlation tests showed the significant correlations **a** between vitreous ANGPTL-8 and serum TG ($R = 0.73$, $P < 0.001$), **b** between serum ANGPTL-8 and serum TG ($R = 0.63$, $P < 0.001$), **c**) between vitreous ANGPTL-8 and serum LDL-C ($R = 0.59$, $P < 0.001$), **d** between serum ANGPTL-8 and serum LDL-C ($R = 0.69$, $P < 0.001$), **e** between vitreous ANGPTL-8 and serum HDL-C ($R = -0.41$, $P = 0.028$), **f** between serum ANGPTL-8 and serum HDL-C ($R = -0.44$, $P = 0.020$), **g** between vitreous ANGPTL-8 and serum TC ($R = 0.33$, $P = 0.091$), and **h** between serum ANGPTL-8 and serum TC ($R = 0.43$, $P = 0.023$) in PDR patients
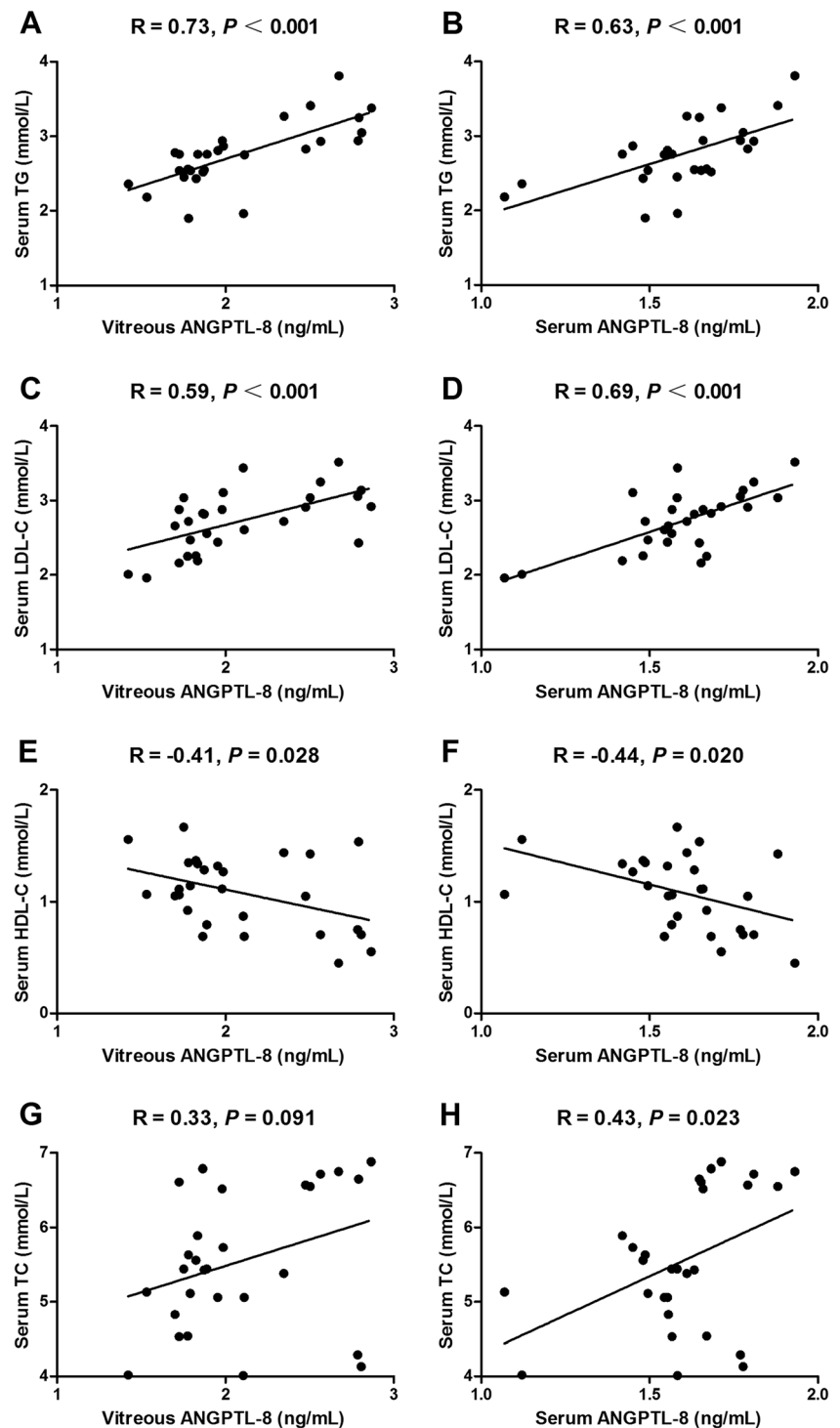

VEGF, especially given the clinical application of anti-VEGF drugs (e.g., ranibizumab and conbercept) [37].

Although the mechanism underlying the increased ANGPTL-8 levels in patients with PDR is currently unknown, we have determined that the increased ANGPTL-8 levels significantly correlated with increased TG and LDL-C levels, more than with TC and HDL-C levels, in patients with PDR.

Moreover, despite the fact that other lipid molecules were simultaneously increased in patients with PDR, analogous correlations were only found between the vitreous VEGF levels and increased TG and LDL-C levels; this finding suggests, at least partly, a greater role for ANGPTL-8 than regular lipid molecules in hyperlipidemia accompanied by PDR in patients. Later, a more significant correlation between the

Fig. 5 Correlation between VEGF with serum lipids in PDR patients. Pearson's correlation tests showed the significant correlations **a** between vitreous VEGF and serum TG ($R = 0.64$, $P = 0.002$), **b** between serum VEGF and serum TG ($R = 0.36$, $P = 0.057$), **c** between vitreous VEGF and serum LDL-C ($R = 0.67$, $P < 0.001$), **d** between serum VEGF and serum LDL-C ($R = 0.31$, $P = 0.111$), **e** between vitreous VEGF and serum HDL-C ($R = −0.32$, $P = 0.093$), **f** between serum VEGF and serum HDL-C ($R = −0.19$, $P = 0.340$), **g** between vitreous VEGF and serum TC ($R = 0.14$, $P = 0.463$), and **h** between serum VEGF and serum TC ($R = 0.27$, $P = 0.165$) in PDR patients
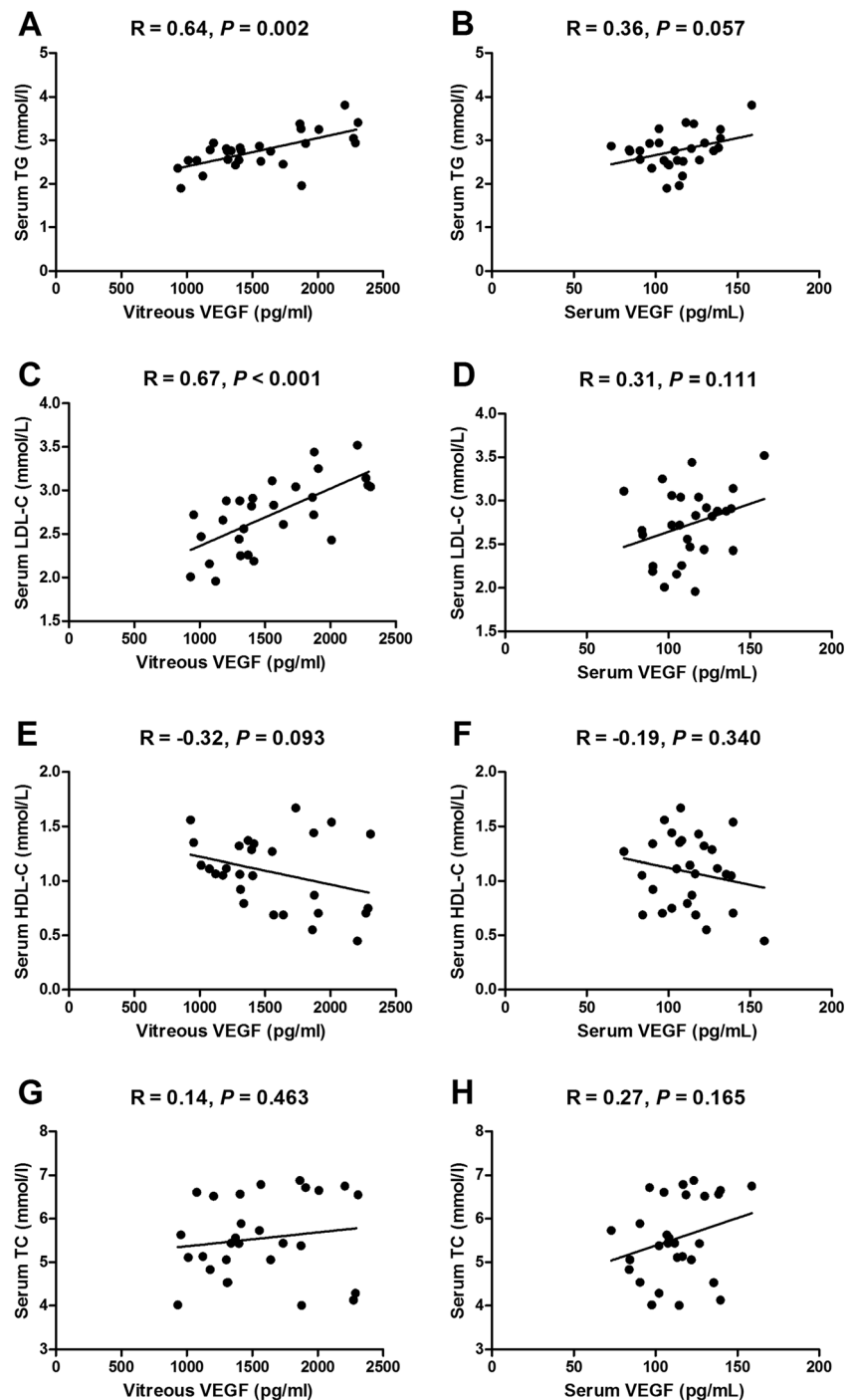
vitreous ANGPTL-8 levels and BMI, as opposed to that between the serum ANGPTL-8 levels and BMI, was found in the patients with PDR. It is widely known that most patients with PDR suffer from hyperlipidemia, which suggests that routine and dietary habits may have contributed, at least in part, to the increase in ANGPTL-8 levels.

We also observed higher serum ANGPTL-8 levels in older patients with IMH, but not in the older patients with PDR. Espes D et al. reported that the ANGPTL-8 levels positively correlated ($P = 0.01$) with age in patients without diabetes [15]. It would therefore be meaningful in future studies to determine possible correlations between ANGPTL-8 levels and age in different groups.

The main limitation of this study was that it was a single-center, small-sample, cross-sectional study; a multicenter, large-sample, prospective study is needed to confirm these results. Additionally, the molecular mechanism(s) and underlying pathway(s) between ANGPTL-8 and VEGF require

further in-vivo and in-vitro studies. Finally, the potential feedback between ANGPTL-8 and dyslipidemia in patients with PDR requires a more comprehensive investigation.

ANGPTL-8, which is representative of lipid metabolism, may contribute to glucose homeostasis and facilitate future targeted synergistic therapies for hyperglycemia and hyperlipidemia [29–31]. After all, PDR is much more than simple chronic hyperglycemia in one eye and may involve novel factors, which suggests that future studies are needed to determine how to control ANGPTL-8 regulation of VEGF expression in hyperglycemia homeostasis. Due to the importance of VEGF in the treatment of PDR, the current results suggest the importance of future studies regarding the roles of ANGPTL-8 in diabetic retinal complications.

Compliance with ethical standards

Conflict of interest   All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval   We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers/animals were followed during this research. Our research adhered to the tenets of the Declaration of Helsinki and its later amendments or comparable ethical standards. This article does not contain any studies with human participants or animals performed by any of the authors.

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