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Changes in expression of inflammatory cytokines and ocular indicators in pre-diabetic patients with cataract

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Abstract

Pre-diabetes is the preceding condition of diabetes, and in some cases, fundus changes have been seen in pre-diabetes. The inflammatory response is widely recognized as being involved in the pathophysiologic process of diabetic eye disease. Therefore, we aimed to acquire understanding of the role of early altered blood glucose levels in the development and etiology of diabetic ocular disorders from the perspective of inflammation. In this study, serum, tear, aqueous humor and vitreous fluid samples were collected from patients undergoing cataract surgery. VEGF, IL-6, TNF-a, MCP-1, APOA-1, ICAM-1, VCAM-1, PEDF, TSP-1 were measured by ELISA. The quantity of hyperreflective retinal spots (HRS) was counted by optical coherence tomography OCT images. We found that the levels of inflammatory cytokines are already altered in pre-diabetes. Levels of pro-inflammatory cytokine expression and quantity of HRS can reflect the disease process to some extent.

Keywords Pre-diabetes, Diabetic retinopathy, Inflammatory cytokines, Hyperreflective foci

Introduction

The prevalence of diabetes mellitus (DM), an endocrine metabolic disease that poses a major threat to human health, is rising globally. Pre-diabetes is seen as the critical phase, a state in which the glycemia is abnormal but does not meet the diagnostic criteria for diabetes. A better understanding of the pathogenesis of pre-diabetes will help develop new and more potent strategies for early prevention or intervention [1]. Based on observational data, pre-diabetes may raise the risk of macrovascular disease, diabetic retinopathy, small fiber neuropathy, chronic kidney disease, and early nephropathy. Elevated blood glucose itself is a major cause of retinal and microvascular dysfunction associated with pre-diabetes and T2DM, and it has been suggested that microvascular endothelial dysfunction may be combined with neuronal dysfunction [2]. In addition to blood glucose values, multifactorial risk scores can be used to optimize estimates of diabetes risk using non-invasive parameters and blood-based metabolic profiles [3]. Thankfully, the stage of pre-diabetes is still reversible and could serve as a potential route to combat diabetes [4].

In recent studies, inflammation in particular has been identified as an important factor in the emergence and advancement of problems associated with diabetes [5]. Biomarkers are chemicals that can be discovered in bodily fluids and tissues, such as blood, that can be used to monitor the progression of a disease or the effectiveness of a treatment. Variations in the concentrations of different biomarkers related to the inflammatory

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response have been found and recognized in body circulation and ocular samples of diabetic complications, namely vitreous fluid, aqueous humor, and tear [6]. Inflammatory cytokines such as IL6 and TNF-α, for example, are involved in pathological processes such as immune regulation, inflammation, increased permeability of the vessels and stimulation of cell proliferation in diabetic eye disease. It mainly exerts its effects through the classical signaling pathway and the trans-signaling pathway [7]. Chemokines have also been shown to be involved in the pathogenesis of diabetic eye disease by regulating the attraction and activation of immune cells. Patients with diabetes have higher levels of several chemokines, including MIP-1β, MIP-1α, and monocyte chemotactic protein-1 (MCP-1). It has been demonstrated that in diabetic mice, MCP-1 knockdown reduces retinal vascular leakage [8]. Adhesion molecule-mediated leukocyte-endothelial adhesion is associated with leukocyte aggregation seen in diabetic eye disease. Furthermore, it has been discovered that genetic flaws in endothelial cell adhesion molecules, such as CD18, ICAM-1, and selectin (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1), significantly lower the number of adherent leukocytes in diabetic patients and animals. Inhibition of CD18 or ICAM-1 using antibodies reduced retinal leukocyte stasis and vasculopathy in diabetic rats [9]. However, whether the aforementioned inflammatory cytokines are also altered early in pre-diabetes remains inconclusive.

Therefore, we measured the levels of inflammatory cytokines (VEGF, IL6, TNF-α), chemokines (MCP-1), adhesion molecules (ICAM-1, VCAM-1), PEDF, TSP-1 and APOA-1 in blood and ocular samples of prediabetic patients for a comprehensive and complete study of ocular changes in pre-diabetes.

Materials and methods

Patient recruitment

The character of this clinical study is a cross-sectional study. Following receipt of participant informed consent and institutional ethics committee approval, 69 pre-diabetic (Pre-DM) (70 eyes),66 type 2 diabetic mellitus (T2DM) (70 eyes),54 diabetic retinopathy (DR) (60 eyes), nonpredaibetic and nondiabetic patients (Control) (60 eyes) undergoing phacoemulsification combined with IOL implantation were enlisted for the research. The four groups were closely matched in terms of the grade of cataract. The study was conducted in accordance with the Declaration of Helsinki principles. The inclusion criteria were as follows: (1) The diagnosis of Pre-DM, T2DM and DR met the DM diagnosis guidelines and classification standards [10, 11]; Pre-diabetes: A state in which the glycemia is abnormal but does not meet the diagnostic criteria for diabetes. Non-diabetes: Fasting blood glucose less than 5.6 Mmol/L, glycated hemoglobin ratio less than 5.7% and blood glucose less than 7.8 Mmol/L after 2-hour OGTT. All three need to be met at the same time. (2) Cataract was defined as any Lens Opacities Classification System III (LOCS III) grading of 2 or higher (LOCS III based on slitlamp imaging).The exclusion criteria were as follows: (1) Patients with other ocular pathologies, such as high myopia, ocular surface disease, uveitis, glaucoma, and any retinopathy other than diabetic retinopathy; (2) With ocular surface disease in the past; (3) Having taken topical or systemic ant-inflammatory medication within three months of being recruited; (4) Having recovered from ophthalmic procedures (such as vitreous injections, cataract surgeries, retinal detachment surgeries, etc.) no more than six months before being recruited; (5) With a systemic disease upregulating inflammatory cytokines, like chronic obstructive pulmonary disease, asthma, immune disorders, blood disorders, malignancy.

Data collections

Every patient received a thorough ophthalmologic examination that included slit-lamp examination, fundus photography, dilated fundus examination and optical coherence tomography (OCT). We collected and recorded the participants' age, gender, duration of DM, blood pressure, blood lipids, BMI, fasting blood glucose (FPG), glycated hemoglobin (HbAlc), oral glucose tolerance test 2 h (OGTT 2 h), smoking status (active, non-smoker and ex-smoker), drinking status (active, non-drinker and ex-drinker), classifications of diabetic retinopathy, quantity of hyperreflective retinal spots (HRS) and other laboratory indicators.

OCT

All patients underwent SDOCT using Spectralis (cirrus HD-5000, ZEISS). For every patient, a single 180∞SD-OCT line scan (6 mm length) oriented into the fovea was examined to check for HRS. The HRS, which are minute, punctiform, white lesions, were manually counted.

Sample collection

Following a thorough explanation of the process to the patients, a venepuncture was performed and 5 mL of blood were obtained. The ophthalmology lab received the blood samples. After centrifuging the blood samples for 15 min at 5000 rpm, the serum was extracted and kept at -80˚C.

Using 20 µl glass capillary tubes (Coman Medical Devices, China), tears were extracted from the temporal inferior tear meniscus on the eyes. A low-light slit lamp biomicroscope was used to observe the inferior tear meniscus. From each eye, 50 µl of basal tear fluid that had been mildly aroused was extracted. Care was taken to avoid touching the eye to minimize reflex tearing. Tears

were pooled to reach a final volume of 100μL-150μL. After being put on ice right away, the samples were kept at -80˚C until needed.

The same operator collected vitreous fluid and aqueous humor from each patient. Before surgery, 0.5% compound tropicamide (Santen, Japan) was administered for pupil dilation and 0.5% proparacaine hydrochloride (Novartis, Switzerland) for topical anesthesia. The centrurion phacoemulsification device (Alcon, TX, erica) was utilized for all surgical procedures.

Aqueous humor collection: Before intraoperative surgery, a 1.0 ml syringe was used to extract 0.1–0.15 ml of atrial fluid from the central pupil of the pupil, avoiding contact with the cornea, lens, and iris, and then placed in a refrigerator at -80 °C for storage and testing.

Vitreous sample collection: After implantation of the IOL, the tip of a 1.0 ml syringe was inserted into the pars plana (about 3.5 mm behind the scleral limbus) to obtain an undiluted vitreous sample of 0.3-0.5 ml, which was stored in a refrigerator at -80℃ for measurement.

All written informed consent was obtained from the patient(s) (or relative/guardian) for the publication of any/all images, clinical data and other data included in the manuscript. We Confirm that the study complies with all regulations.

Sample detection

Concentrations of vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-a), monocyte chemotactic protein-1(MCP-1), apolipoprotein A-1 (APOA-1), intercellular cell adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1(VCAM-1), pigment epithelium-derived factor (PEDF) and Human thrombospondin-1(TSP-1) were measured using human enzyme-linked immunosorbent assay (ELISA) kits (EK183, EK106HS, EK182, EK187, EK1204, EK189, EK190, MultiSciences (Lianke) Biotech Company, China; MK0383A, MK0951A, MK4419A, MEI KE Biotechnology Company, China) according to the manufacturers' protocols. A microplate reader was used to measure each sample's optical density at 450 nm, with a correction wavelength set at 630 nm. While the concentrations of VEGF, IL-6, TNF-a, MCP-1, APOA-1, ICAM-1, and VCAM-1 were expressed in picograms (pg)/ml, the concentrations of PEDF and TSP-1 were determined using standards and expressed in nanograms (ng)/mL.

Statistical analysis

The statistical analysis was performed using SPSS statistical software (version 27.0; IBM SPSS Statistic, Armonk, NY, USA). Mean ± standard deviation (M ± SD) was used to express measurement data when they followed a normal distribution. One-way analysis of variance was used for comparison between multiple groups. The group t test was employed to compare the two groups. A p-value of less than 0.05 was considered significant. The interquartile range (IQR) and median were used to express measurement data when they did not fit into a normal distribution. Shapiro-Wilk test was used for normality of the sample distribution. Group difference was compared using nonparametric Mann-Whitney or Kruskal-Wallis test. The Bonferroni method was used for post hoc tests. A p-value of less than 0.05 was considered significant. The chi-square test was employed to compare the count data sets. We counted twice the patients who were included twice to correct for demographic analysis. Blood samples were collected twice. There was a minimum of 15 days between each eye surgery.

Results

General clinical characteristics

In total, 247 patients with 260 eyes with cataracts (control group: $n = 60$; Pre-DM group: $n = 70$; DM group: $n = 70$; DR group: $n = 60$) were enrolled in this study. Table 1 displays the study group's demographic and clinical characteristics. Apart from blood glucose-related readings, there were no discernible differences between the various study groups. In Pre-DM group and DM group, patients were further divided into normal fundus and altered fundus group.

Differences in immune cells within the blood routine in four groups of patients

As shown in Fig. 1 (negative results not shown), we counted the difference in the number of white blood cells, neutrophils, lymphocytes, monocytes, eosinophils and basophils in the serum of the four groups of patients and found that only the number of leukocytes (Fig. 1A) and neutrophils (Fig. 1B) were statistically different between the control and DR groups.

Levels of inflammatory cytokines in serum and ocular samples

The levels of the nine inflammatory cytokines in the serum were shown in Table 2, and the levels of the factors were different and statistically significant in all groups except APOA-1. Except for PEDF and TSP-1 decreased with elevated blood glucose, the rest of the inflammatory cytokines increased with elevated blood glucose. The inflammatory cytokines changed with statistical significance were VEGF (from 213.13 ± 18.06 to 238.3 ± 24.22 pg/mL, $p = 0.02$), IL-6 (from 2.51 ± 0.11 to 2.66 ± 0.11 pg/mL, $p<0.001$), TNF-a (from 12.18 ± 4.18 to 16.04 ± 3.55 pg/mL, $p<0.001$), MCP-1 (from 60.68 ± 25.61 to 75.18 ± 24.07 pg/mL, $p<0.001$), ICAM-1 (from 320.92 ± 56.83 to 406.47 ± 79.04 pg/mL, $p<0.001$), VCAM-1 (from 462.30 ± 78.09 to 514.87 ± 51.00 pg/mL, $p<0.001$), PEDF (from 85.94 ± 30.38 to 106.62 ± 21.63 ng/

Table 1 Demographic characteristics and systemic profile among patients

Variables	CON (n=60)	Pre-DM (n=70)	DM (n=70)	DR (n=60)	*P*-value
Age (years)					
Mean±SD	65.28±9.06	66.96±9.47	68.21±8.17	64.58±8.58	0.08
Gender					
Male	24	29	28	33	0.264
Female	36	41	42	27	
BMI (kg/m^2)					
Mean±SD	23.04±3.03	24.28±3.43	24.63±3.83	24.26±3.47	0.06
Smoking Status					
Non-smoker	50	60	60	44	
Active smoker	7	7	6	7	0.267
Ex-Smoker	3	3	4	9	
Drinking Status					
Non-drinker	50	62	58	45	
Active drinker	9	6	8	13	0.340
Ex-drinker	1	2	4	2	
Co-morbidity					
Hypertension	19	27	36	30	0.07
Dyslipidaemia	15	16	26	19	0.242
Glycemic testing					
FPG (mmol/L)	4.98±0.37	5.77±0.57	7.30±1.86	7.66±1.86	<0.001
HbA1c (%)	5.31±0.34	5.75±0.44	7.16±1.10	8.15±1.68	<0.001
OGTT 2h PG (mmol/L)	6.72±0.65	8.35±1.27	-	-	<0.001

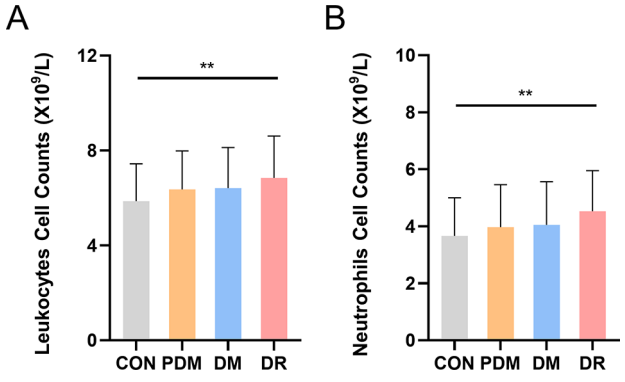

Fig. 1 Immune cells are elevated in the peripheral blood of patients with diabetic retinopathy. (**A**) Leukocyte cell counts are elevated in the peripheral blood of patients with diabetic retinopathy. (**B**) Neutrophils cell counts are elevated in the peripheral blood of patients with diabetic retinopathy. *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. The results are presented as mean ± SD

mL, p<0.001), TSP-1 (from 64.16 ± 6.45 to 59.61 ± 4.79 ng/mL, p<0.001). The levels of factors in the tear are shown in Table 3, in which the levels of APOA-1 and TSP-1 did not differ among the four groups. Among the other inflammatory cytokines, the inflammatory cytokines changed with statistical significance were VEGF (from 51.29 ± 5.26 to 69.18 ± 7.85 pg/mL, p<0.001), IL-6 (from 5.57 ± 3.55 to 14.03 ± 8.88pg/mL, p<0.001), TNF-a (from 13.35 ± 6.84 to 22.07 ± 6.20 pg/mL, p<0.001), MCP-1 (from 190.35 ± 5.88 to 197.39 ± 9.80 pg/mL, p = 0.004), VCAM-1 (from 141.49 ± 6.23 to 145.75 ± 8.41 pg/mL, p = 0.028).The levels of factors in the aqueous humor are shown in Table 4, and the levels of all factors except TSP-1 were different and statistically significant in all groups. The inflammatory cytokines changed with statistical significance were VEGF (from 107.81 ± 18.69 to 158.91 ± 23.89 pg/mL, p<0.001), IL-6 (from 13.14 ± 4.10 to 30.55 ± 6.16 pg/mL, p<0.001), TNF-a (from 11.22 ± 5.27 to 19.74 ± 7.60 pg/mL, p<0.001), MCP-1 (from 207.89 ± 40.92 to 261.03 ± 95.77 pg/mL, p<0.001), APOA-1 (from 167.14 ± 9.24 to 185.37 ± 8.20 ng/mL, p<0.001), ICAM-1 (from 311.79 ± 18.64 to 333.22 ± 37.12 pg/mL, p<0.001), VCAM-1 (from 514.26 ± 114.35 to 715.47 ± 128.4 pg/mL, p<0.001). The levels of factors in the vitreous fluid are shown in Table 5, and the levels of all factors except TSP-1 and TNF-a were different and statistically significant in all groups. The inflammatory cytokines changed with statistical significance were VEGF (from 357.36 ± 28.36 to 559.43 ± 53.79 pg/mL, p<0.001), IL-6 (from 13.78 ± 3.80 to 14.63 ± 3.34 pg/mL, p<0.001), MCP-1 (from 233.68 ± 30.78 to 339.29 ± 45.54 pg/mL, p<0.001), APOA-1 (from 219.71 ± 25.45 to 239.00 ± 28.85 ng/mL, p = 0.033), ICAM-1 (from 798.16 ± 70.37 to 992.26 ± 78.83 pg/mL, p<0.001), VCAM-1 (from 1651.33 ± 280.6 to 2245.65 ± 254.91 pg/mL, p<0.001), PEDF (from 157.44 ± 30.95 to 127.33 ± 25.27 ng/mL, p<0.001).

Table 2 Comparison of inflammatory cytokines levels in serum between the groups

Cytokine	CON (n=30)	Pre-DM (n=35)	DM (n=35)	DR (n=30)	P^a	P^b	P^c	P^d
VEGF (pg/ml)	213.13±18.06	238.3±24.22*	270.36±31.75*	304.37±37.84*	< 0.001	0.02	< 0.001	0.002
IL-6 (pg/ml)	2.51±0.11	2.66±0.11*	2.68±0.14*	2.84±0.20*	< 0.001	< 0.001	0.960	0.002
TNF-a (pg/ml)	12.18±4.18	16.04±3.55*	17.91±1.80*	27.90±3.93*	< 0.001	< 0.001	0.044	< 0.001
MCP-1 (pg/ml)	60.68±25.61	75.18±24.07*	89.90±27.21*	135.73±33.75*	< 0.001	0.038	0.028	< 0.001
APOA-1 (ng/ml)	142.90±24.01	138.72±22.69	146.80±25.56	136.02±21.40	0.269	-	-	-
ICAM-1 (pg/ml)	320.92±56.83	406.47±79.04*	425.68±83.81*	537.24±90.39*	< 0.001	< 0.001	0.310	< 0.001
VCAM-1 (pg/ml)	462.30±78.09	514.87±51.00*	625.16±104.89*	749.16±49.61*	< 0.001	0.016	< 0.001	< 0.001
PEDF (ng/ml)	85.94±30.38	106.62±21.63*	189.70±38.54*	221.55±44.08*	< 0.001	0.018	< 0.001	0.019
TSP-1 (ng/ml)	64.16±6.45	59.61±4.79*	57.55±5.62*	55.53±6.11*	< 0.001	0.011	0.815	0.960

*: Comparison with control group, t test, $P<0.05$, significant

P^a: ANOVA

P^b: Control VS Pre-DM

P^c: Pre-DM VS DM

P^d: DM VS DR, $P<0.05$, significant

Differences in inflammatory cytokine levels in different fundus conditions between patients in the Pre-DM and DM groups

We divided the fundus condition of patients in the Pre-DM group into normal fundus group and DR questionable (DR questionable defines a group of patients with soft exudates, hard exudates, intraretinal microvascular abnormalities or hemorrhages definite but microaneurysms absent) group (Figs. 2 and 3) according to ETDRS Final Retinopathy Severity Scale [11]. The levels of factors in different samples were shown in Figs. 4 and 5. There were statistically significant differences in the levels of VCAM-1 in the Pre-DM group in the serum. About the levels of factors in the aqueous humor, only MCP-1 and PEDF in the Pre-DM group and MCP-1, TSP-1 in the DM group showed statistically significant differences in the different disease stages in the fundus. Except for IL-6, TNF-a, MCP-1, APOA-1, TSP-1 in the Pre-DM group and TNF-a, MCP-1, APOA-1, TSP-1 in the DM group, there were statistically significant differences in the other factors in the vitreous fluid.

Differences in the levels of inflammatory cytokines in different numbers of HRS

A manual count of the retina's HRS was conducted along a 6-mm length (Fig. 6). We divided the number of HRS into 0–5, 6–10, 11–15 three groups and compared the changes of inflammatory cytokines in different groups. As shown in Fig. 6, with the exception of PEDF and TSP-1, the levels of inflammatory cytokines in serum, tear, aqueous humor, and vitreous fluid increased as the number of HRS increased in sum. The differences in IL-6 were statistically significant between the different HRS groups in all four samples. Only in serum, the level of VCAM-1 was not statistically different between the three groups. VCAM-1, PEDF and TSP-1 were not statistically significant mainly in the tear. Only TSP-1 was not statistically insignificant between three groups in the aqueous humor. The difference in TNF-a and TSP-1 was not statistically significant mainly in the vitreous fluid.

Table 3 Comparison of inflammatory cytokines levels in tear between the groups

Cytokine	CON (n=30)	Pre-DM (n=35)	DM (n=35)	DR (n=30)	P^a	P^b	P^c	P^d
VEGF (pg/ml)	51.29±5.26	69.18±7.85*	86.55±17.18*	112.63±24.64*	< 0.001	< 0.001	< 0.001	< 0.001
IL-6 (pg/ml)	5.57±3.55	14.03±8.88*	20.20±8.08*	21.51±8.82*	< 0.001	< 0.001	0.02	0.989
TNF-a (pg/ml)	13.35±6.84	22.07±6.20*	27.67±3.71*	30.05±2.79*	< 0.001	< 0.001	< 0.001	0.066
MCP-1 (pg/ml)	190.35±5.88	197.39±9.80*	217.14±9.43*	246.19±16.36*	< 0.001	0.004	< 0.001	< 0.001
APOA-1 (ng/ml)	148.48±26.21	151.76±30.86	158.83±24.78	160.09±30.95	0.306	-	-	-
ICAM-1 (pg/ml)	141.49±6.23	145.75±8.41*	153.37±8.26*	157.97±7.55*	< 0.001	0.028	< 0.001	0.018
VCAM-1 (pg/ml)	218.83±46.55	236.25±41.49	239.92±42.55	285.83±43.36*	< 0.001	0.109	0.724	< 0.001
PEDF (ng/ml)	22.74±4.27	22.40±3.40	18.43±3.51*	18.01±3.61*	< 0.001	0.706	< 0.001	0.644
TSP-1 (ng/ml)	8.64±2.09	9.11±2.13	8.17±2.09	8.24±1.75	0.370	-	-	-

*: Comparison with control group, t test, $P<0.05$, significant

P^a: ANOVA

P^b: Control VS Pre-DM

P^c: Pre-DM VS DM

P^d: DM VS DR, $P<0.05$, significant

Discussion

Pre-diabetes is a state of elevated blood glucose in which the threshold for diabetes has not been reached and may lead to the development of type 2 diabetes and diabetic eye disease. Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are usually already present in pre-diabetes [12]. Current research suggests that in the pre-diabetic period, elevated blood glucose induces a widespread, chronic, non-specific inflammatory process in the body, which ultimately leads to type 2 diabetes and diabetic eye disease. Rational modulation of the inflammatory response induced by hyperglycemia is essential to delay the onset of type 2 diabetes as well as diabetic eye disease. Therefore, this paper focuses on a comprehensive insight into the inflammatory state of the pre-diabetic eye by comparing the expression of inflammatory cytokines in serum, tear, aqueous humor and vitreous samples.

In fact, according to our study, as blood glucose rose, the number of white blood cells and neutrophils have been elevated in blood. This is consistent with the findings of A Klisic et al. [13] Elevated white blood cells mostly indicate inflammation and neutrophils which is a sign of a destructive nonspecific inflammatory response can secrete pro-inflammatory cytokines [14]. This is strong evidence that elevated glycemia is inextricably linked to inflammation. It likewise validates our hypothesis that chronic inflammatory responses are widely involved even in pre-diabetes.

We found that typical DR lesions can also be found in prediabetic patients, and according to Ramachandran Rajalakshmi a et al., compared to patients with IFG, those with IGT experience DR more frequently. Wong et al. found that 6.7% of patients with IGT or IFG showed symptoms of retinopathy [15]. In the diabetes prevention program, 7.9% of patients with pre-diabetes developed diabetic retinopathy [16]. In fact, in the population included in this study, this percentage may be higher (17.1%). Macular thinning, one of the early and subtle changes associated with pre-diabetes, was found to have a considerable causal mediation effect on the retinal vasculature. Chronic hyperglycemia is thought to lead to increased glutamate release and loss of vascular

Table 4 Comparison of inflammatory cytokines levels in aqueous humor between the groups

Cytokine	CON (n=30)	Pre-DM (n=35)	DM (n=35)	DR (n=30)	P^a	P^b	P^c	P^d
VEGF (pg/ml)	107.81±18.69	158.91±23.89*	182.18±39.24*	212.21±45.14*	< 0.001	< 0.001	0.024	0.036
IL-6 (pg/ml)	13.14±4.10	30.55±6.16*	35.00±7.74*	41.24±9.73*	< 0.001	< 0.001	0.057	0.038
TNF-a (pg/ml)	11.22±5.27	19.74±7.60*	25.50±10.34*	32.80±10.71*	< 0.001	< 0.001	0.059	0.041
MCP-1 (pg/ml)	207.89±40.92	261.03±95.77*	389.55±98.95*	632.98±122.16*	< 0.001	0.027	< 0.001	< 0.001
APOA-1 (ng/ml)	167.14±9.24	185.37±8.20*	187.28±9.46*	192.45±7.08*	< 0.001	< 0.001	0.351	0.017
ICAM-1 (pg/ml)	311.79±18.64	333.22±37.12*	375.51±38.31*	404.98±34.98*	< 0.001	0.012	< 0.001	< 0.001
VCAM-1 (pg/ml)	514.26±114.35	715.47±128.48*	918.72±267.62*	1079.06±311.7*	< 0.001	0.002	0.001	0.171
PEDF (ng/ml)	150.73±31.21	133.66±34.35	92.28±23.40*	58.32±21.74*	< 0.001	0.213	< 0.001	< 0.001
TSP-1 (ng/ml)	21.54±8.21	21.05±9.06	20.74±9.23	17.09±7.17	0.161	-	-	-

*: Comparison with control group, t test, $P<0.05$, significant

P^a: ANOVA

P^b: Control VS Pre-DM

P^c: Pre-DM VS DM

P^d: DM VS DR, $P<0.05$, significant

protective factors due to oxidative stress and accumulation of glycolytic waste products [17]. Many studies have linked different inflammatory indicators to the presence of DR, including TNF-a, VEGF, intracellular adhesion molecules, soluble gp130, soluble tumor necrosis factor receptor 1 and so on. Increased levels of inflammatory markers may be the earliest determinant of diabetic eye complications in pre-diabetes [18]. To address this mechanism of inflammatory response, we measured the levels of inflammatory cytokines in serum, tear, aqueous humor, and vitreous fluid of pre-diabetic patients.

We selected DR-related inflammatory cytokines VEGF, IL-6, TNF-a, MCP-1, APOA-1, ICAM-1, VCAM-1, PEDF and TSP-1. VEGF is a dimeric glycoprotein that binds to heparin and is mostly released by retinal Muller cells. Its primary function is to stimulate the creation of neointima and increase the proliferation of endothelial cells [19]. IL-6, which is elevated primarily in retinal astrocytes, can induce VEGF production and thus promote neovascularization [20]. As part of the repair process, blood stimulates an inflammatory response in the extravascular space, and acute hemolysis increases the level of TNF-a [21]. Sequencing results showed that TNF-a and MCP-1 were highly enriched in microglia [22]. Additionally, it has been demonstrated that the activation of endothelial cells by cytokines causes inflammation, and that this activation raises the production of cell adhesion molecules including VCAM-1 and ICAM-1 [23, 24]. Furthermore, human retinal pigment epithelium was shown to have APOA-1 mRNA, and the vitreous of PDR patients had much greater levels of APOA-1 than that of non-diabetic patients [25]. PEDF is a popular protease inhibitor used to treat ocular angiogenesis [26], as with TSP-1, both of them can antagonize VEGF [27]. In the four groups of samples, the expression of most pro-inflammatory cytokines was up-regulated and statistically significant in the Pre-DM group compared to the control group, suggesting that the inflammatory response in the patients has started to gradually increase in the pre-diabetes period. Interestingly, when the Pre-DM group was compared with the DM group, the differences in the levels of some inflammatory cytokines were not statistically significant.

Table 5 Comparison of inflammatory cytokines levels in vitreous fluid between the groups

Cytokine	CON (n=30)	Pre-DM (n=35)	DM (n=35)	DR (n=30)	P^a	P^b	P^c	P^d
VEGF (pg/ml)	357.36±28.36	559.43±53.79*	588.65±54.20*	651.24±57.52*	< 0.001	< 0.001	0.148	< 0.001
IL-6 (pg/ml)	77.88±2.53	92.62±7.41*	103.51±9.51*	110.00±12.53*	< 0.001	< 0.001	< 0.001	0.133
TNF-a (pg/ml)	13.78±3.80	14.63±3.34	15.24±3.63	17.07±4.16*	0.007	0.361	0.495	0.052
MCP-1 (pg/ml)	233.68±30.78	339.29±45.54*	401.71±44.43*	774.43±47.15*	< 0.001	< 0.001	< 0.001	< 0.001
APOA-1 (ng/ml)	219.71±25.45	239.00±28.85*	245.91±14.49*	260.61±17.01*	< 0.001	0.033	0.749	0.001
ICAM-1 (pg/ml)	798.16±70.37	992.26±78.83*	1053.89±93.04*	1104.32±106.18*	< 0.001	< 0.001	0.004	0.023
VCAM-1 (pg/ml)	1651.33±280.6	2245.65±254.91*	2469.67±275.83*	2752.96±336.78*	< 0.001	< 0.001	0.001	< 0.001
PEDF (ng/ml)	157.44±30.95	127.33±25.27*	101.04±33.94*	84.67±31.35*	< 0.001	< 0.001	< 0.001	0.033
TSP-1 (ng/ml)	8.93±4.28	7.27±4.48	7.11±4.12	6.50±4.36	0.155	-	-	-

*: Comparison with control group, t test, $P<0.05$, significant

P^a: ANOVA

P^b: Control VS Pre-DM

P^c: Pre-DM VS DM

P^d: DM VS DR, $P<0.05$, significant

The possible reason for this is that in the diagnostic criteria for pre-diabetes, Pre-DM can be diagnosed as if one of FPG, HbAlc and OGTT 2 h meets the criteria at a time, so the patients in the control group had normal levels of blood glucose in their bodies. Therefore, the levels of inflammatory cytokines in the patients of DM group are often statistically significant. Meanwhile, some diabetic patients control their blood glucose level by medication or insulin injection close to pre-diabetes, or pre-diabetic patients' blood glucose level has risen to almost diabetic diagnostic criteria, so there is no statistically significant difference between the Pre-DM group and DM group. These results show that the ocular microenvironment is already abnormal which is predicting progressive retinal disease in the pre-diabetic period. To further explore the relationship between inflammatory cytokines and fundus disease process, we analyzed the DR questionable group with normal fundus. We found that the differences in the levels of only a few factors in serum and aqueous humor were statistically significant, while the levels of all factors in tear were not statistically significant. This may be due to the number of people in the DR questionable group is too small. In the vitreous fluid which is the most reflective of the fundus, most of the factors can reflect the disease process of DR, which indicates that the inflammatory response is also quietly progressing step by step in the early stage before the diagnosis of DR. Further research is necessary to determine if blood glucose-lowering therapies or other metabolic abnormalities associated with pre-diabetes affect how long-term problems develop. It is also regrettable that we did not explore and intervene in the causes of differential expression of inflammatory cytokines, which will be the focus of our next work.

HRS was first described by Coscas et al. among patients with age-related Macular degeneration through spectral domain OCT [28]. HRS is defined as a high signal point with clear boundary, no rear projection, and diameter less than 30 μm in the retina and Choroid. In recent years, the HRS in the retina detected by OCT are considered as new biomarkers to predict the progress, curative effect and prognosis of fundus diseases. There

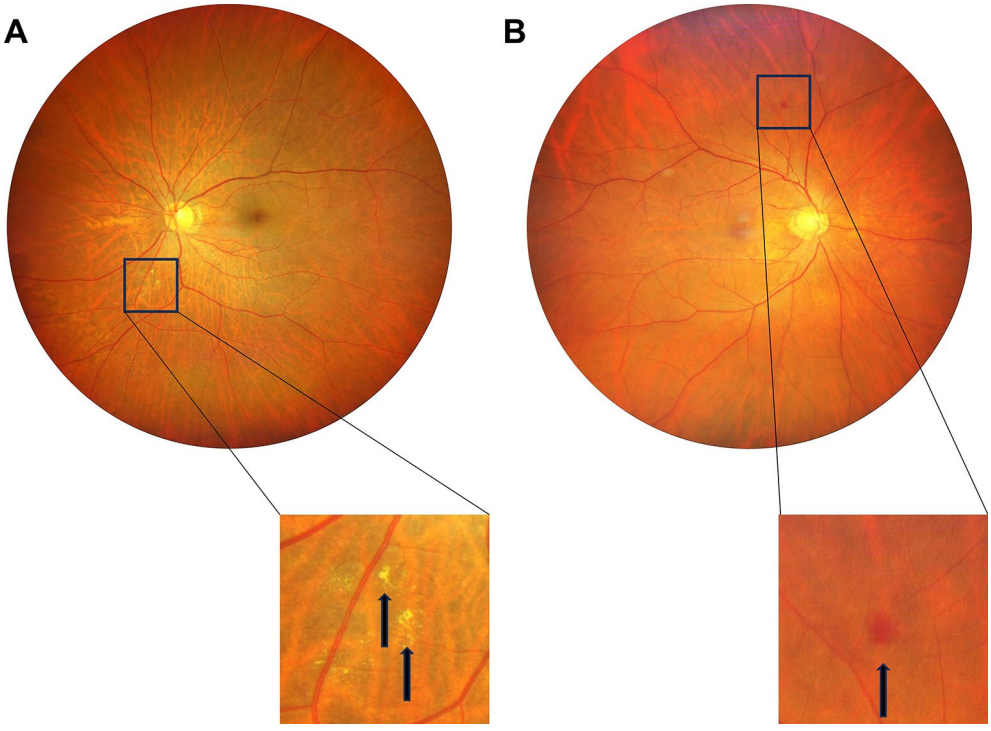

Fig. 2 Representative fundus photography in patients with questionable diabetic retinopathy. (**A**) Representative fundus photography of hard exudates. (**B**) Representative fundus photography of hemorrhage

are still some controversies regarding the origin of HRS, with some studies suggesting that HRS originates from the deposition of retinal extravasation proteins and/or lipids, and is considered a precursor to hard exudation [29]. Other studies believe that HRS is the result of the destruction of the blood retinal barrier, which is, lipid containing macrophages migrate into the retina. With the wide application of OCT, in recent years, HRS is thought to be derived from activated inflammatory cells in the retina, especially microglia and macrophages. The activated Microglia cell body became larger, and the cell morphology was like "amoeba", showing HRS in OCT. HRS can be seen in a variety of retinal diseases, including DR, DME, retinal vein occlusion, non-choroid disease, and other retinal Degenerative disease [28, 30]. In our study, we grouped all the patients by 0–5 h, 6–10 h, and 11–15 h. We learned that there were HRS in OCT images of patients in Pre-DM group and the number of HRS was basically 0–5. We speculate that this is related to macrophage recruitment and microglia activation induced by chronic hyperglycemia and low-grade inflammation [31]. As a medium of inflammation and angiogenesis, MCP-1 activates retinal microglia, which is also mainly secreted by microglia. In our study, with the increase of the number of HRS, the level of MCP-1 in serum, tear, aqueous humor, and vitreous fluid increased. Therefore, there is a certain relationship between blood glucose changes and HRS levels.

In addition, we found the most unstable correlations between tears and other samples, which may be related to the difficulty of sampling tears and repeated freezing and dilution [32]. Another limitation of this study is that intraoperative collection of samples from cataract patients, which may be affected by cataract pathologic changes and surgical operations.

In conclusion, ocular lesions have already occurred in the pre-diabetic period. The inflammatory response, quantity of HRS can reflect the disease process to some extent. Timely intervention of altered cytokines in the pre-diabetes stage may delay the onset of diabetes and diabetic eye disease, which will also provide clinicians with new ideas and rationale for the selection of therapeutic targets and the development of novel drugs.

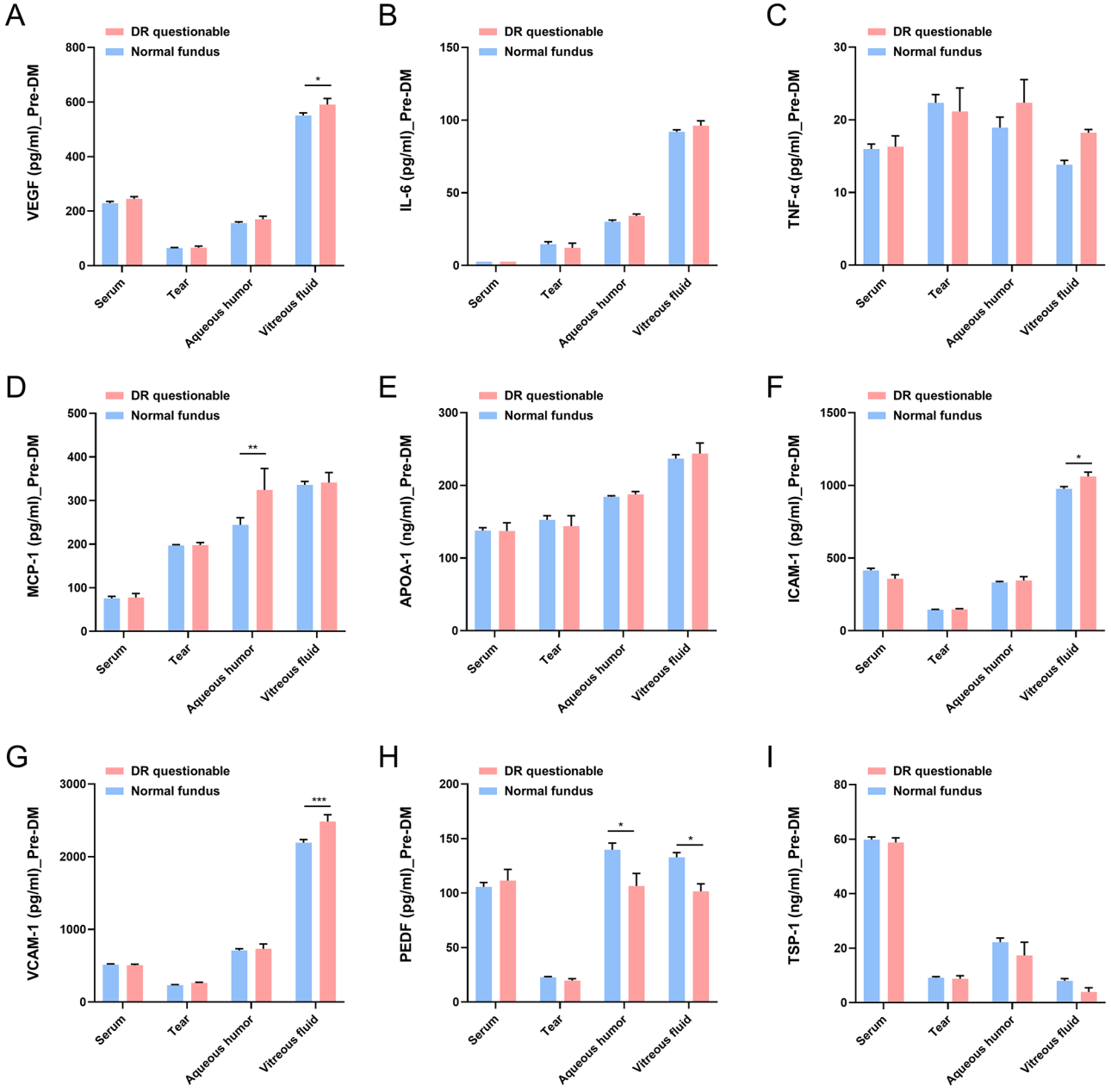

Fig. 3 Expression of inflammatory cytokines in serum, tears, aqueous humor and vitreous fluid in pre-diabetes mellitus. (**A**-**I**) Elisa detection and quantification of inflammatory cytokines expression in serum, tears, aqueous humor, and vitreous fluid in a group of patients with questionable diabetic retinopathy and a group of patients with a normal fundus, (**A**) Elisa detection and quantification of VEGF expression; (**B**) Elisa detection and quantification of IL6 expression; (**C**) Elisa detection and quantification of TNF-α expression; (**D**) Elisa detection and quantification of MCP-1 expression; (**E**) Elisa detection and quantification of APOA-1 expression; (**F**) Elisa detection and quantification of ICAM-1 expression; (**G**) Elisa detection and quantification of VCAM-1 expression; (**H**) Elisa detection and quantification of PEDF expression; (**I**) Elisa detection and quantification of TSP-1 expression. *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. The results are presented as mean ± SD

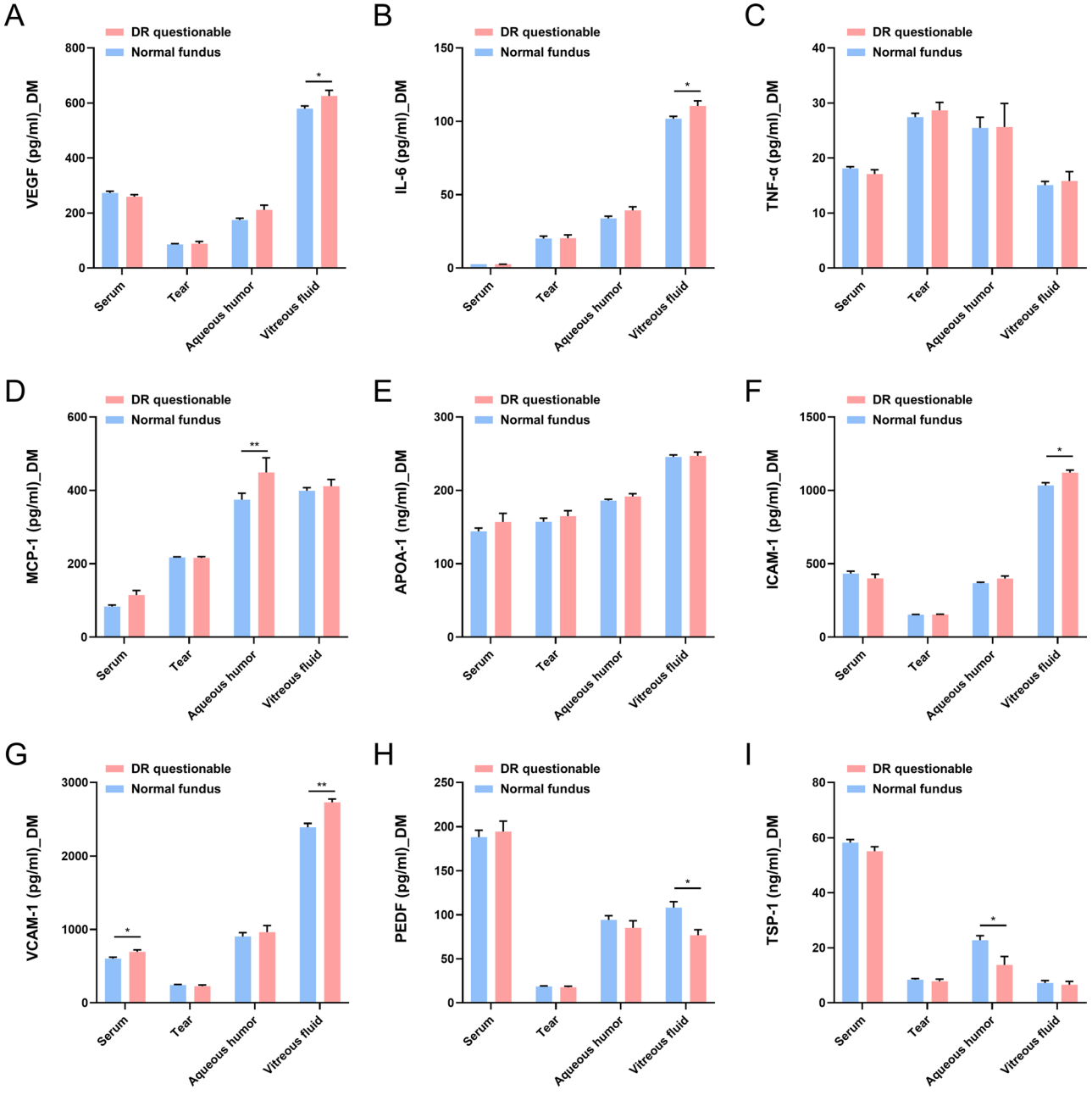

Fig. 4 Expression of inflammatory cytokines in serum, tears, aqueous humor and vitreous fluid in diabetes mellitus. (**A**-**I**) Elisa detection and quantification of inflammatory cytokines expression in serum, tears, aqueous humor, and vitreous fluid in a group of patients with questionable diabetic retinopathy and a group of patients with a normal fundus, (**A**) Elisa detection and quantification of VEGF expression; (**B**) Elisa detection and quantification of IL6 expression; (**C**) Elisa detection and quantification of TNF-α expression; (**D**) Elisa detection and quantification of MCP-1 expression; (**E**) Elisa detection and quantification of APOA-1 expression; (**F**) Elisa detection and quantification of ICAM-1 expression; (**G**) Elisa detection and quantification of VCAM-1 expression; (**H**) Elisa detection and quantification of PEDF expression; (**I**) Elisa detection and quantification of TSP-1 expression. *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. The results are presented as mean ± SD

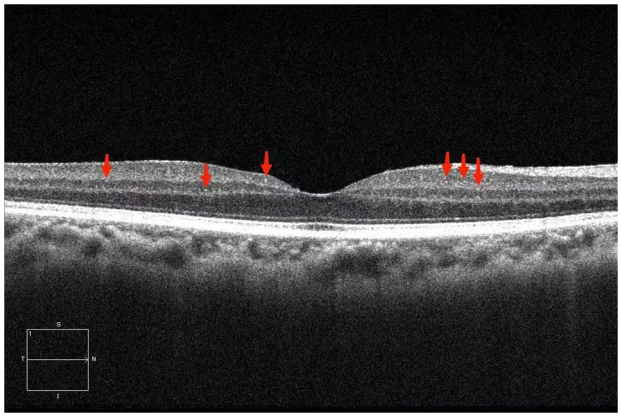

Fig. 5 Representative images of the number of HRS in the retina

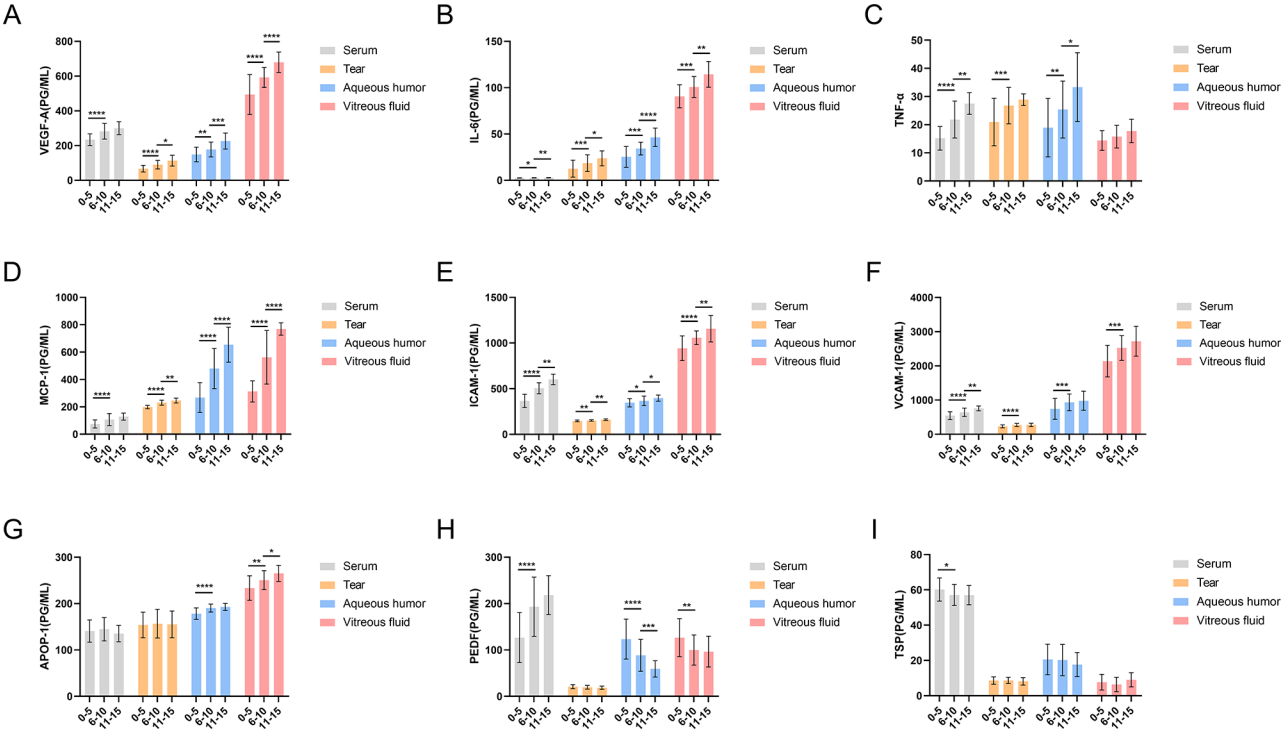

Fig. 6 Expression of inflammatory cytokines in serum, tears, aqueous humor and vitreous fluid in different numbers of HRS groups. (**A**-**I**) Elisa detection and quantification of inflammatory cytokines expression in serum, tears, aqueous humor, and vitreous fluid in different numbers of HRS groups, (**A**) Elisa detection and quantification of VEGF expression; (**B**) Elisa detection and quantification of IL6 expression; (**C**) Elisa detection and quantification of TNF-α expression; (**D**) Elisa detection and quantification of MCP-1 expression; (**E**) Elisa detection and quantification of APOA-1 expression; (**F**) Elisa detection and quantification of ICAM-1 expression; (**G**) Elisa detection and quantification of VCAM-1 expression; (**H**) Elisa detection and quantification of PEDF expression; (**I**) Elisa detection and quantification of TSP-1 expression. *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. The results are presented as mean ± SD

Abbreviations

DM	Diabetes mellitus
HRS	Hyperreflective retinal spots
MCP-1	Monocyte chemotactic protein-1
MIP-1α	Macrophage inflammatory protein-1α
HRS	Hyperreflective retinal spots
Pre-DM	Pre-diabetic
T2DM	Type 2 diabetic mellitus
DR	Diabetic retinopathy
OCT	Optical coherence tomography
FPG	Fasting blood glucose
HbAlc	Glycated hemoglobin
OGTT 2 h	Oral glucose tolerance test 2 h
LOCS III	Lens Opacities Classification System III
VEGF	Vascular endothelial growth factor
IL-6	Interleukin 6
TNF-a	Tumor necrosis factor alpha
MCP-1	Monocyte chemotactic protein-1
APOA-1	Apolipoprotein A-1
ICAM-1	Intercellular cell adhesion molecule-1
VCAM-1	Vascular cell adhesion molecule-1
PEDF	Pigment epithelium-derived factor

TSP-1	Human thrombospondin-1
IGT	Impaired glucose tolerance
IFG	Impaired fasting glucose

Author contributions
YXC, YM, MJT, JM, JZ, MJ, and HJG conceived and designed the study. YXC, YM, MJT and JM acquired the data. YXC, YM and JZ analyzed and interpreted the data. All authors drafted and/or revised the manuscript for content. All authors read and approved the final manuscript.

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Data availability
The datasets generated and/or analysed during the current study are not publicly available due laboratory regulations on data confidentiality but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Written informed consent was obtained from each participant to participate in the study. The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration. Ethical permissions were granted by the Human Ethics Committee at Affiliated Hospital of Nantong University (2023-L044).

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

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