Increased expression of TSPO-VDAC complex is correlated with NLRP3 inflammasome activation in diabetic retinopathy

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Abstract. The present study aimed to investigate the level of translocator protein (TSPO) and its correlation with different inflammatory cytokines in diabetic retinopathy (DR) patients. Peripheral blood samples were obtained from 54 DR patients and 22 age-related cataract (ARC) patients. The mRNA expression of TSPO, voltage-dependent anion channel (VDAC), apoptosis-associated speck like protein with a caspase recruitment domain (ASC), NOD-like receptors pyrin domain-containing 3 (NLRP3) and caspase-1 were examined by reverse transcription-quantitative PCR. Interleukin-1β and interleukin-18 levels were detected by enzyme-linked immunosorbent assay. The mRNA levels of TSPO, VDAC, ASC, NLRP3 and capase-1, the protein levels of IL-β and IL-18 were all significantly higher in the DR group compared with those in the ARC group. The expression levels of those aforementioned cytokines/proteins were more significantly higher in the subgroup of active proliferative DR (PDR) compared with those in the inactive PDR group ($P<0.05$). Significant positive correlations between TSPO/VDAC complex and ASC, NLRP3, capase-1, IL-β and IL-18 were found in DR patients. These outcomes suggested that TSPO/VDAC complex and NLRP3 inflammasomes may play an important role in the development and progression of DR.

Introduction

Diabetic retinopathy (DR) is the most commonly observed microvascular complication associated with diabetes and is the major cause of blindness among working-age population (1). The disturbed energy homeostasis following chronic hyperglycemia (2,3), and the subsequent activation of innate immune signaling are the most significant mechanisms contributing to microvascular impairment found in diabetes (4,5).

In relationship to this, an enhanced level of glucose induces oxidative phosphorylation, therefore depolarizing the plasma membrane and leading to enhanced mitochondrial ion flux levels (6,7). In particular, mitochondria are the main source of reactive oxygen species (ROS) generation under oxidative stress (8). Translocator protein (TSPO) is a transporting protein located at the outer mitochondrial membrane (OMM) and contributes to multiple process including cholesterol import (9), Ca^{2+} signaling and mitochondrial metabolism. TSPO forms a complex with VDAC to facilitate mitochondrial ion flux and metabolite transport across the OMM (10). TSPO-VDAC complex selectively promotes the mitochondrial Ca^{2+} channel, therefore precipitates the activation of the Ca^{2+}-dependent NADPH oxidase, further mediates ATP production and transportation, ROS generation and mitochondrial membrane potential ($\Delta\psi m$) transitions (11).

Those overly produced ATP and disturbed ROS can be recognized as self-derived damage-associated molecular patterns by pattern recognition receptors of the innate immune system. The priming igniter of innate immune system, the NOD-like receptors pyrin domain-containing 3 (NLRP3) inflammasome, is proven to be located to cytoplasmic granular structures, in close vicinity to mitochondria. More direct evidence is that the NLRP3 may be activated by VDAC through its capturing function targeting ROS (12,13). Notably, blockade of TSPO-VDAC ion channels results in highly diminished mitochondrial ROS generation and therefore significantly impairs NLRP3 inflammasome activation, suggesting the crucial role of TSPO-VDAC complex in innate immune response ignition (14).

The significance of how the TSPO-VDAC-NLRP3 signaling cascade contributes to the pathogenesis of DR is notably apparent. However, its expression and possible function in the PDR are still not well understood. Therefore, it is important to gain insights into the clinical implications of TSPO-VDAC-NLRP3 signaling in the pathogenesis and development of DR.

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Materials and methods

Participants. Age- and sex-paired 54 DR and 22 ARC patients were consecutively recruited from the outpatient clinic of Eye & ENT Hospital of Fudan University during February 2018 to July 2019 (Table I). The study followed the guidelines of the Declaration of Helsinki, and all experimental protocols were approved (approval no. 2020142) by the Human Ethics Committee of Eye & ENT Hospital of Fudan University (Shanghai, China). Written informed consent was obtained from the enrolled participants. The detailed explanations of the study purpose were given to all the participants and the signatures of the informed consent form were obtained correspondingly.

Patients with known systemic inflammatory, autoimmune, immunosuppressive disease or other diabetic complications such as nephropathy were excluded. Patients were also excluded if they had been subjected to intraocular procedures, intravitreal treatments, uveitis, trauma, or immunosuppressive drug administration.

All the selected DR patients were assessed by ultra-wide fluorescein fundus angiography (Optos 200Tx; Optos PLC). Body mass index was determined using the weight (kg)/height (m^2) formula. Based on the DR Disease Severity Scale, diabetics were placed into one of the following two groups: i) Non-proliferative DR (NPDR) and ii) proliferative DR (PDR) (Table II). According to the standard set by Aiello *et al* (15), patients with PDR are further divided into active neovascularization group and inactive neovascularization group by their presentation in fundus image and fluorescein angiography. Patients with age-related cataract were selected as control group. All eyes of the subjects underwent an overall ophthalmic evaluation, including slit-lamp biomicroscopy, IOP measurement (Goldmann applanation tonometry), fundus examination and ultrasound B-scanning.

Whole blood sample preparation. Whole blood samples (12 ml) were collected from each participant using sterile tubes containing lithium heparin prior to mRNA and protein extraction. The remaining blood samples were utilized for glycated hemoglobin and fasting plasma glucose determination. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Hypaque density-gradient centrifugation (Lymphoprep; Takeda Pharmaceutical Company, Ltd.). They were then cultured under room temperature in RPMI-1640 medium (cat. no. #11875093) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.; cat. #26010074) and 1% penicillin/streptomycin and seeded at a concentration of 10^6 cells/ml into 24-well plates for overnight. To determine the effect of cytokine production, PBMCs from ARC and PDR patients were stimulated for 4 h with 100 ng/ml lipopolysaccharides (Sigma-Aldrich; Merck KGaA) and then incubated for an additional 15 min with RPMI-1640 containing 1 mM adenosine 5-triphosphate (lipopolysaccharide/ATP; Sigma-Aldrich; Merck KGaA).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from isolated PBMCs of DR patients and ARC patients using the RNA easy Mini kit (Qiagen) according to the manufacturer's instructions. The high-capacity cDNA reverse transcription kit (cat. #4368813. Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for to cDNA synthesis according to the manufacturer's instructions. Reactions were performed for 10 min at 25˚C, 2 h at 37˚C, and 5 sec at 85˚C. cDNA was subsequently kept at -20˚C until it was used for qPCR amplification. TSPO, VDAC, NLRP3, apoptosis-associated speck like protein with a caspase recruitment domain (ASC) and caspase-1 gene expression levels were measured by qPCR using specific TaqMan FAM/MGB assays (Applied Biosystems; assay ID Hs00559362_m1 for TSPO, assay ID Hs01631624_gH for VDAC1, assay ID Hs00918082_m1 for NLRP3, assay ID Hs01547324_gH for ASC and assay ID Hs00354836_m1 for caspase-1).

Reactions were performed in an Applied Biosystems 7500 real-time PCR system. Gene expression levels were normalized using a TaqMan VIC/MGB eukaryotic 18S rRNA endogenous control assay (Applied Biosystems, cat. #4319413E). Reaction system consists of 6 μl 2x TaqMan Fast Advanced Master Mix (Applied Biosystems; cat. # A44360), 0.6 μl of 20x TaqMan gene expression assay, 0.6 μl 20x TaqMan endogenous control, 3.8 μl of water, and 1 μl of cDNA sample solution.

The thermal cycling parameters were 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The following primer sequences were used: TSPO, 5'-CAT GGGGTATGGCTCCTA-3' (forward) and 5'-AGACCCAAG GGAACCATA-3' (reverse); VDAC1, 5'-GGTGCTCTGGTG CTAGGTTA-3' (forward) and 5'-CAGCGGTCTCCAACT TCTTG-3' (reverse); NLRP3, 5'-GTAGGTGTGGAAGCA GGACT-3' (forward) and 5'-CTTGCTGACTGAGGACCT GA-3' (reverse); ASC, 5'-AACCCAAGCAAGATGCGG AAG-3' (forward) and 5'-TTAGGGCCTGGAGGAGCAAG-3' (reverse); caspase-1, 5'-CTCAGGCTCAGAAGGGAATG-3' (forward) and 5'-CGCTGTACCCCAGATTTTGT-3' (reverse). Relative expression levels were determined by the $2^{-\Delta\Delta Cq}$ method (16). All reactions were performed in triplicate.

ELISA for IL-1β and IL-18. The concentration of IL-1β and IL-18 in the culture supernatants of PBMCs from DR and ARC patients was measured by ELISA following the manufacturer's instructions (R&D Systems, Inc.; cat. nos. QK201 and DL180.). The minimal detectable concentration of IL-1β was 3.9 pg/ml and the minimal detectable concentrations of IL-18 was 26.6 pg/ml. All the samples were measured in replication for two times.

Statistical analysis. Statistical Package for the Social Sciences Statistical Software (SPSS v18.0; SPSS, Inc.) was used for statistical evaluations. Non-parametric Kruskal-Wallis test, one-way ANOVA analysis of variance and Bonferroni correction were used to evaluate group variations between diabetic patients and cataract patients according to normality assumption and homogeneity of variances. Unpaired Student's t-tests and Mann-Whitney U tests were employed to examine variations between all groups. Spearman's correction test was used to examine study parameters between groups. Graphs were drawn using Prism version 5 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Clinical characteristics and laboratory results of patients.

Characteristic	DR group (n=54)	Control group (n=22)	P-value
Age, years	59.11±1.188	61.00±1.484	0.2563[a]
Female sex, n (%)	30 (55.6)	12 (54.5)	>0.05[b]
Hypertension	37/17	10/12	>0.05[b]
Fasting blood glucose, mmol/l	11.09±0.4479	6.323±0.1091	<0.0001
IOP	14.63±0.3608	14.68±0.5481	0.9085
HbA1c	7.748±0.2018	5.809±0.1369	0.0001

Data are expressed as the mean ± standard deviation. Indicates statistically significant result compared with the corresponding data in the control group as shown in Table I. P<0.05. [a]Independent sample t-test. [b]χ^2 test. DR, diabetic retinopathy.

Table II. Demographic, clinical and laboratory data of DR patient characteristics.

Characteristic	NPDR group (n=18)	PDR group (n=35)	P-value
Age, years	60.77±1.462	56.05±1.888	0.8446[a]
Female, n (%)	9 (50)	21 (60)	>0.0500[b]
Active PDR, n (%)	0/18	18/17	NA
Duration of diabetes, years	8.971±0.7328	6.947±0.8110	0.3604
Hypertension	12/6	25/10	>0.0500[b]
Fasting blood glucose, mmol/l	8.92±0.3948	12.26±0.5682	0.0039
IOP	14.32±0.6625	14.80±0.4296	0.507
HbA1c	7.17±0.2440	8.060±0.2696	0.0708
Vitreoretinal condition			
Vitreous hemorrhage	0/18	18/17	
Diabetic macular edema	0/18	20/15	
Traction retinal detachment	0/18	21/14	

Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. [a]Independent sample t-test. [b]χ^2 test. NA, not applicable.

Results

Patient clinical features. The clinical characteristics and laboratory results of patients were summarized in Table I. No significant deviations among groups in age and sex were found (P>0.05). No significant difference was found in the mean duration of diabetes between PDR and NPDR groups (Table II). HbA1c and fasting glucose levels were also found to be significantly elevated in the PDR group than in the cataract group (P<0.0001). Fasting glucose levels were significantly higher in PDR group than that in NPDR group (Table II), while no significant difference was found regarding HbA1c between two groups.

Expression levels of TSPO/VDAC Complex and NLPR3 inflammasomes. The mRNA expression levels of TSPO, VDAC, NLRP3, ASC and caspase-1 were evaluated in the PBMCs of patients with DR and controls by using RT-qPCR. VDAC expression was found to be associated with increasing levels of TSPO. The mRNA expression levels of TSPO and VDAC in PDR and NPDR patients were significantly higher than that in controls (Fig. 1A and B). Among DR patients, the expression of TSPO and VDAC were significantly higher in PDR group compared with that in NPDR group (Fig. 1C and D). TSPO/VDAC expression levels were also significantly higher in the active PDR subgroup than that in the inactive PDR subgroup (Fig. 1E and F).

The expression levels of NLRP3 and its key molecules ASC and caspase-1 were significantly upregulated in patients with DR compared with ARC patients (Fig. 2A-C). The expression levels of NLRP3, ASC and caspase-1 were significantly higher in PDR patients compared with NPDR patients (Fig. 2D-F). Meanwhile, higher levels of NLRP3, ASC and caspase-1 were detected in patients with active PDR than that of those with inactive PDR (Fig. 2G-I). As expected, there was a strong positive correlation between NLRP3-related inflammasome activation proteins (NLRP3, ASC, caspase-1, IL-1β, and IL-18) and TSPO-related proteins (TSPO and VDAC), respectively (Fig. 3).

Concentration levels of IL-1β and IL-18 in PBMC of patients with DR and ARC. One of the features of activated inflammasomes is to promote IL-1β and IL-18 production (17). Therefore, the protein levels of IL-1β and IL-18 were investigated by

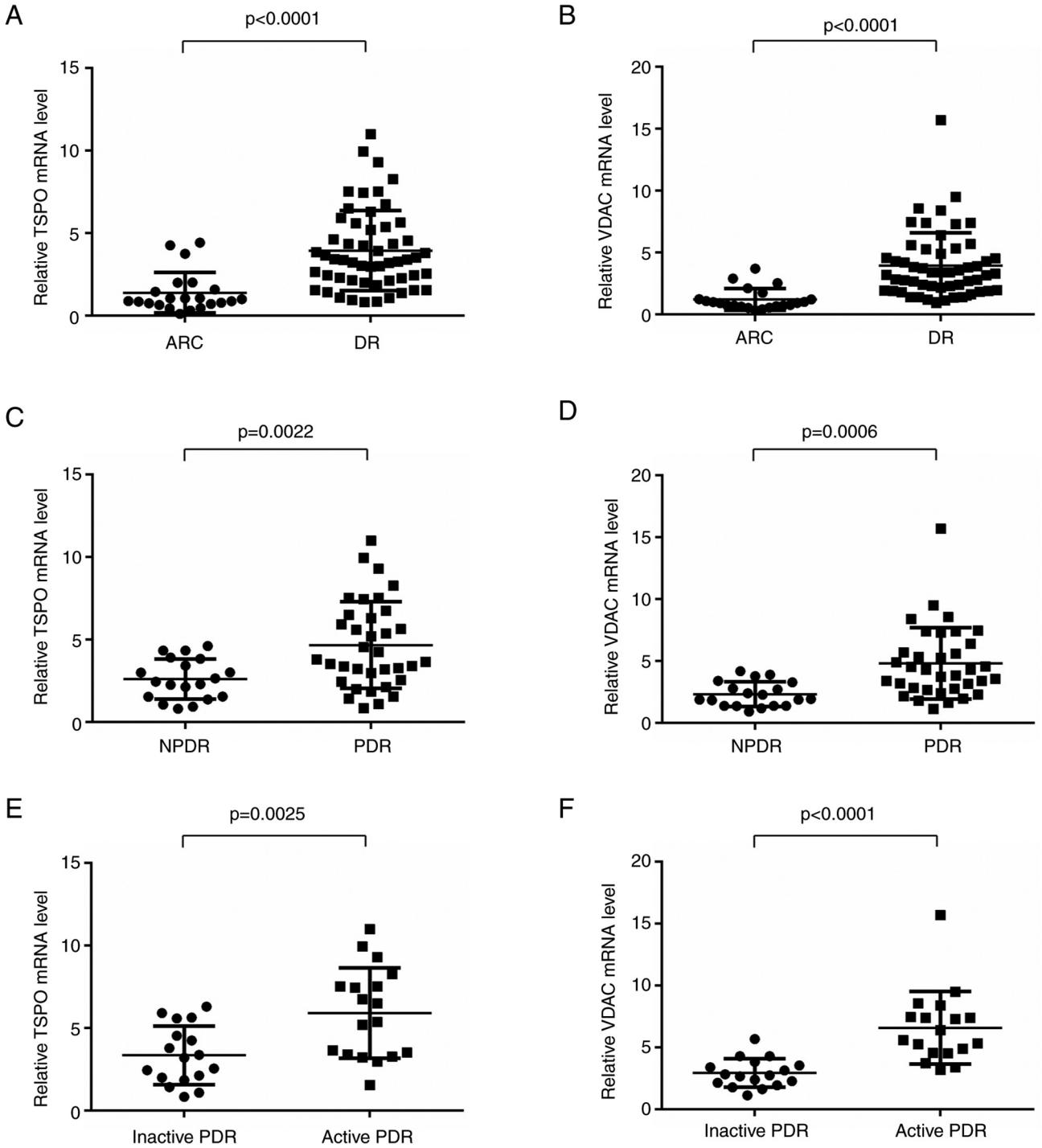

Figure 1. Expression levels of TSPO, VDAC and NLPR3 in PDR and NPDR patients (A and B) mRNA expression levels of TSPO and VDAC in DR patients were significantly higher than that in controls (DR, n=54; ARC, n=22). (C and D) The expression of TSPO and VDAC was significantly higher in the PDR group compared with that in the NPDR group (PDR, n=35; NPDR, n=18). (E and F) Relative TSPO and VDAC mRNA levels in PBMCs from active PDR patients were significantly higher than that in inactive PDR cohorts (active PDR, n=18; inactive PDR, n=35). Data are presented as the median and interquartile range. TSPO, translocator protein; VDAC, voltage-dependent anion channel; DR, diabetic retinopathy; PDR, proliferative DR; NPDR, non-proliferative DR.

using ELISA. The expression levels of IL-1β and IL-18 were significantly higher in DR patients compared with those in the ARC group (Fig. 4A and B). In PDR patients, the expression levels of IL-1β and IL-18 were significantly elevated compared with those in NPDR patients (Fig. 4C and D). Meanwhile, DR patients with active neovascularization showed significantly higher levels of IL-1β and IL-18 expression in comparison with active DR patients (Fig. 4E and F).

Positive correlations between TPSO, VDAC and NLRP3 cascade levels and IL-1β and IL-18 expression levels. Accumulating evidence has linked the role of TSPO and VDAC with the NLRP3 activation in numerous pathologic conditions (18-20). In accordance with those studies, positive correlations were found among NLRP3, ASC and caspase-1 levels and TSPO (Fig. 3A-C) and VDAC expression levels (Fig. 3D-F). As expected, positive correlations were also

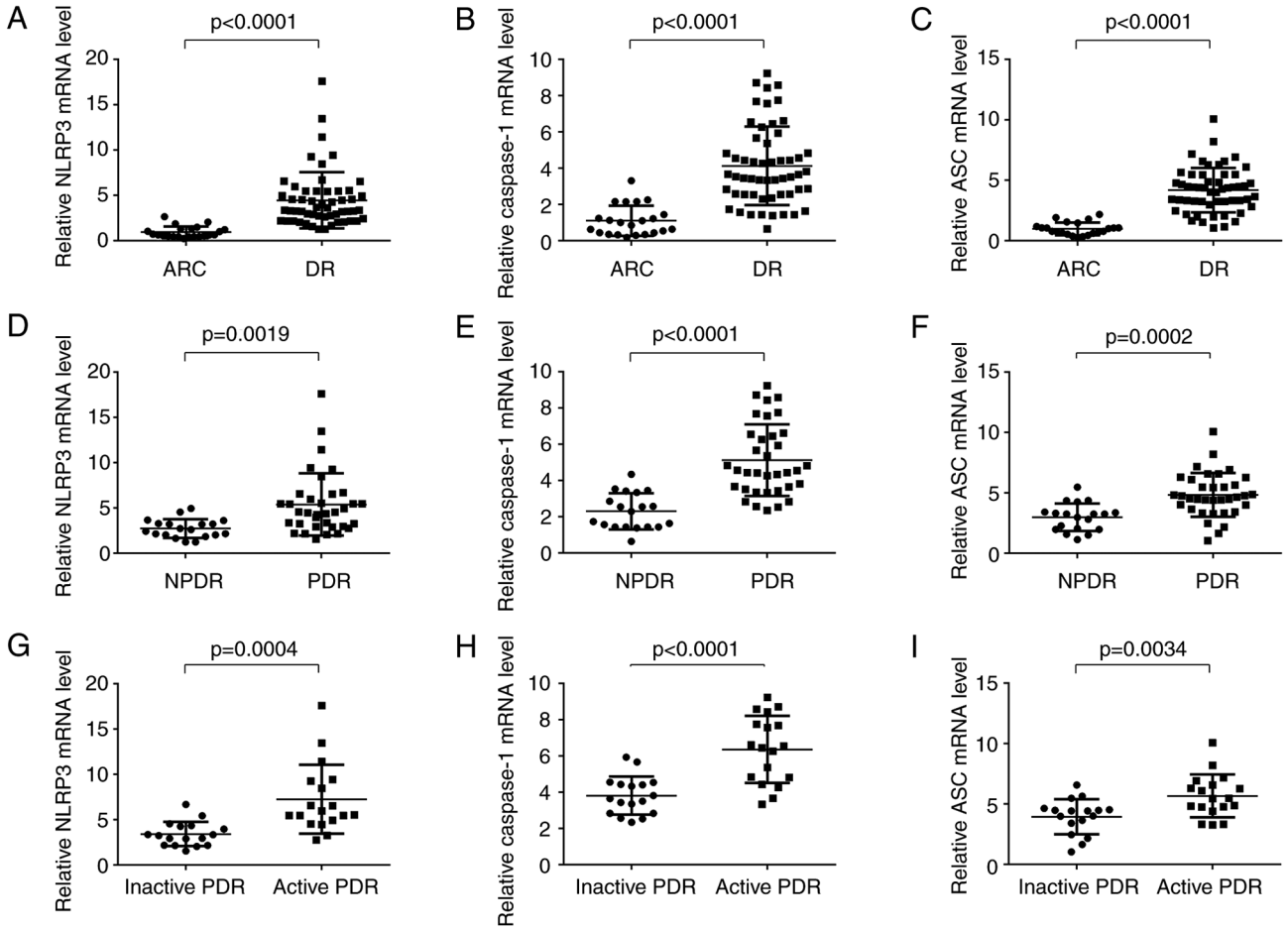

Figure 2. Expression levels of NLRP3, ASC and caspase-1 are significantly higher in DR patients compared with ARC patients. (A) NLRP3, (B) caspase-1 and (C) ASC mRNA level in ARC (n=22) and DR (n=54) patients. (D-F) NLRP3, caspase-1 and ASC mRNA levels in PDR (n=35) patients were elevated compared with that in NPDR (n=18) patients. (G-I) NLRP3, caspase-1 and ASC mRNA levels in active PDR (n=18) patients were significantly higher compared with that in inactive PDR (n=35) patients. Data are presented as the median and interquartile range. NLRP3, NOD-like receptors pyrin domain-containing 3; ASC, apoptosis-associated speck like protein with a caspase recruitment domain; DR, diabetic retinopathy; PDR, proliferative DR; NPDR, non-proliferative DR.

found between IL-1β and IL-18 protein levels and TSPO (Fig. 5A and B) and VDAC expression levels (Fig. 5C and D).

Discussion

DR is considered a para-inflammation entity triggered by metabolic and biochemical disorder. Investigating the interface between metabolic dysregulation and inflammatory activities is essential for understanding the pathogenesis of DR. In the present study, it was found that the elevated expression levels of TSPO-VDAC complex are associated with the activation of NLRP3 inflammasome, demonstrating the inflammatory crosstalk in the development of DR.

Hyperglycemia induces various stress conditions including disorderly metabolic rates, mitochondrial respiratory chain overreaction and accumulation of cytosolic damage signals such as NADPH oxidase and ROS (21). TSPO is the most abundant channel protein in the OMM to exchange metabolites between cellular compartments including mitochondria. It is proven that TSPO and its coordinating protein VDAC are crucial components in the process of mitochondrial metabolism activity and are essential for cytosolic ROS production (11), therefore initiates innate immune responses and pathological angiogenesis in retinal microglia (22). In this aspect, the mRNA expression of TSPO and VDAC were examined in PBMCs of patients with DR and controls. The present results revealed that the mRNA expression levels of TSPO and VDAC were significantly greater in DR patients in comparison with those in controls. Moreover, the levels of TSPO and VDAC were significantly higher in PDR patients compared with those in DR patients in earlier stage, indicating that TSPO and VDAC are positively correlated with the severity of DR. Notably, DR patients with active neovascularization showed higher expression levels of TSPO and VDAC compared with those in inactive DR patients. This phenomenon is in parallel with a recent study conducted by Wolf *et al* (22), which demonstrated that TSPO plays important role in promoting the secretion of IL-1β, and subsequently mediates neovascular formation in retinal microglia. Those results suggested that TSPO and VDAC may participate in the pathogenic angiogenesis in DR through activating the innate immune responses.

A previous study conducted by Chen *et al* (23) demonstrated that NLRP3 inflammasome contributes to the inflammatory responses in different stages of DR, but how TSPO-VDAC exert their roles in DR and whether they are connected with

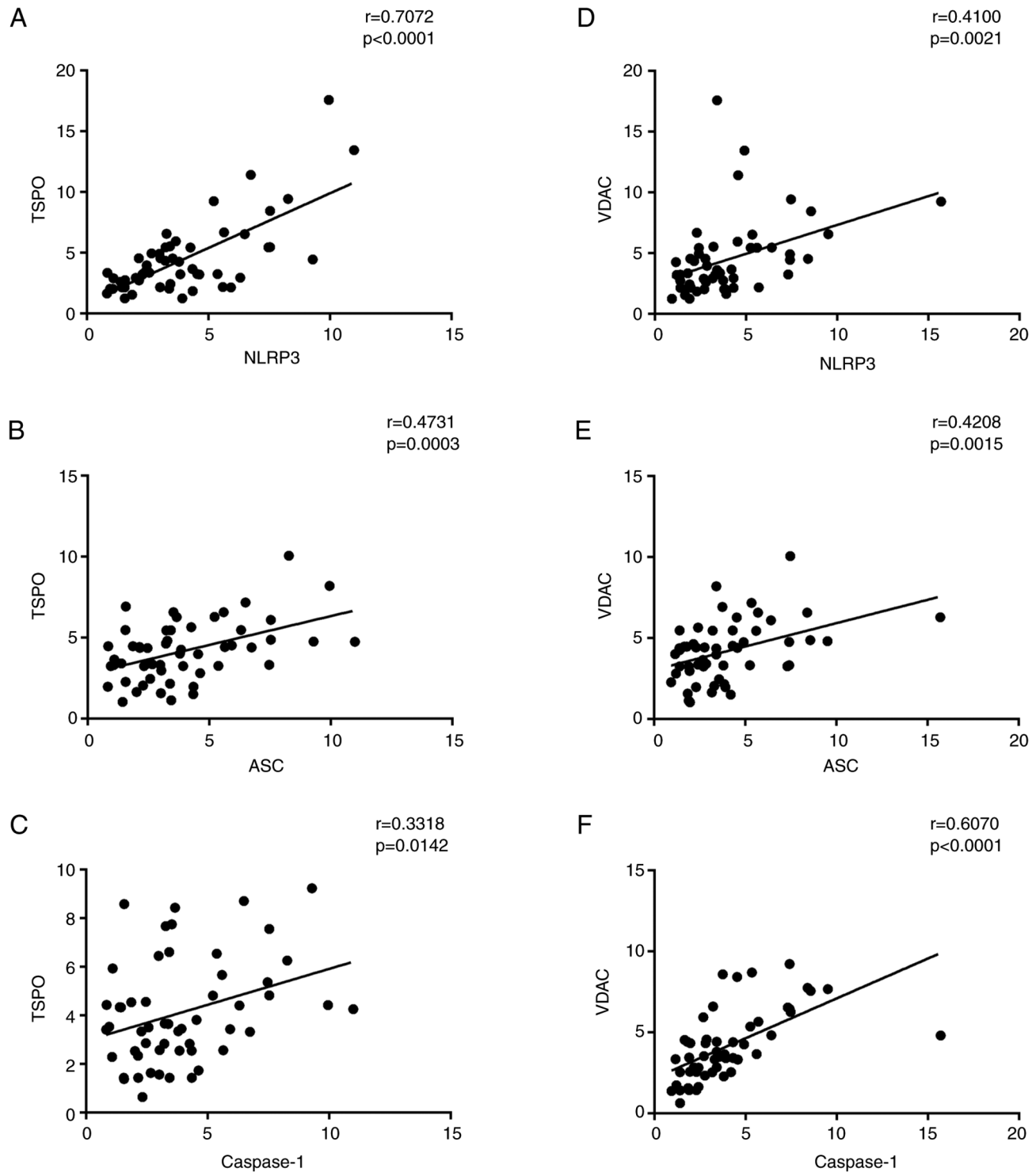

Figure 3. Changes in NLRP3, ASC and caspase-1mRNA levels after TSPO and VDAC relative mRNA expression levels. A positive correlation coefficient was observed between NLRP3, ASC and caspase-1 with (A-C) TSPO and (D-F) VDAC relative mRNA expression levels. Data are presented as the median and interquartile range. The results were assessed using Spearman's correlation test. NLRP3, NOD-like receptors pyrin domain-containing 3; ASC, apoptosis-associated speck like protein with a caspase recruitment domain; TSPO, translocator protein; VDAC, voltage-dependent anion channel.

NLRP3 inflammasomes in DR, and whether their expression changes upon the severity of DR remain unclear. The NLRP3 inflammasome is activated upon 'cellular damage signal' and works as a molecular platform to integrate and ripen pro-inflammatory cytokines, but the underlying mechanism of NLRP3 activation remains unclear. One of the models proposes that NLRP3 is activated by a common pathway of ROS (18). NLRP3 was proven to be localized in close vicinity to mitochondria for efficient sensing of the presence of the fast-fading ROS (17). Zhou *et al* provided more direct evidence by demonstrating that knocking down VDAC significantly diminishes mitochondrial ROS generation and therefore considerably impairs NLRP3 inflammasome activation (12). In the present study, the mRNA expression

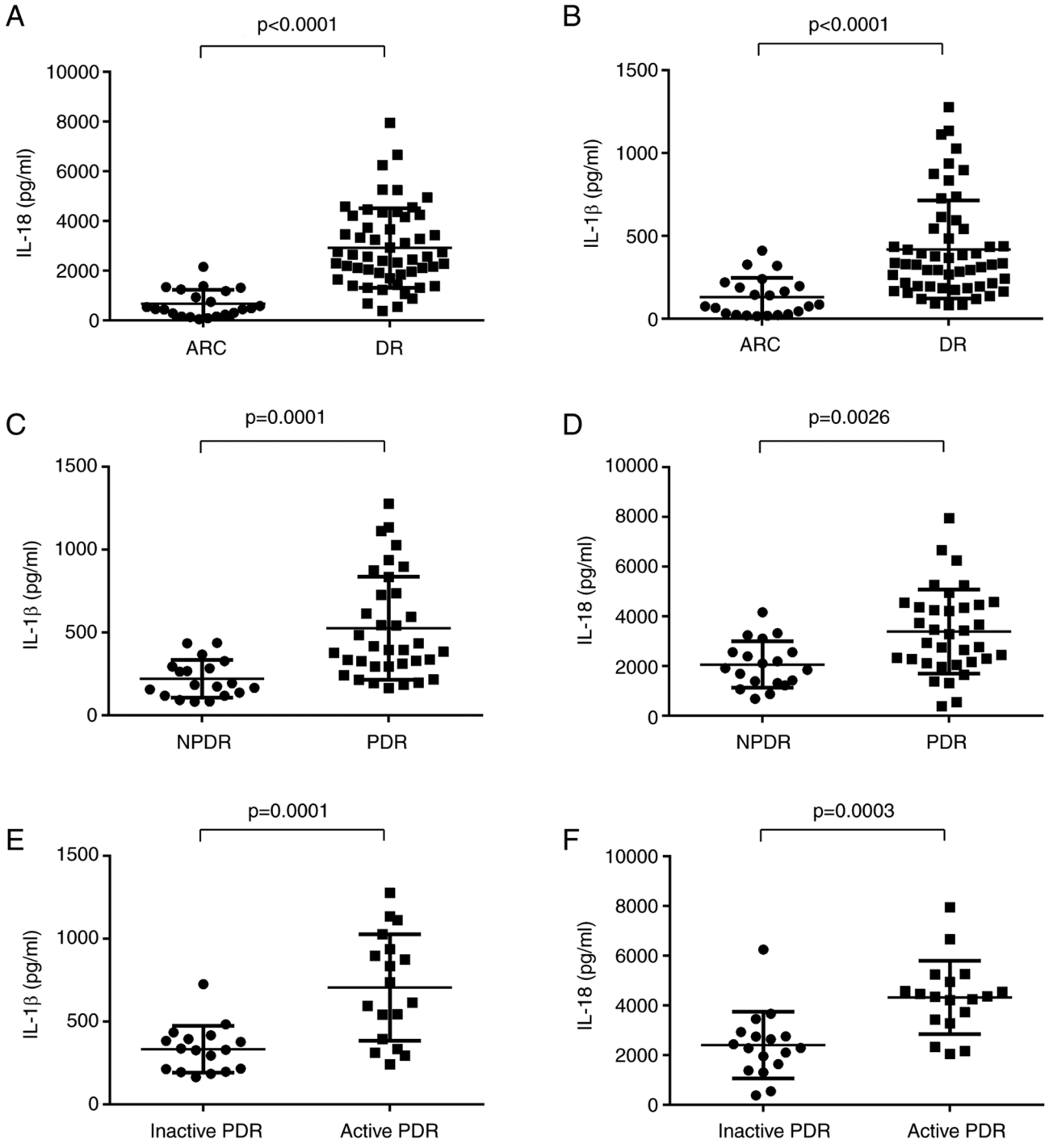

Figure 4. Expression levels of IL-1β and IL-18 in ARC, DR and NPDR. (A and B) Expression levels of IL-1β and IL-18 were significantly higher in DR patients compared with those in ARC group (DR, n=54; ARC, n=22). (C and D) Relative IL-18 and IL-1β protein levels in PBMCs from PDR patients were significantly elevated compared with that in NPDR cohorts (PDR, n=35; NPDR, n=18). (E and F) Relative IL-18 and IL-1β protein levels in PBMCs from active PDR patients were higher than that in inactive PDR cohorts (active PDR, n=18; inactive PDR, n=35). Data are presented as the median and interquartile range. DR, diabetic retinopathy; PBMCs, peripheral blood mononuclear cells; PDR, proliferative DR; NPDR, non-proliferative DR.

of NLRP3 inflammasome adaption proteins (NLRP3, ASC and caspase-1) and protein levels of IL-1β and IL-18 were examined. Our data revealed that the expression levels of NLRP3 inflammasome activation proteins (NLRP3, ASC, caspase-1, IL-1β and IL-18) were significantly greater in DR patients than those in ARC patients. Furthermore, the TSPO and VDAC levels were found to be positively correlated with NLRP3, ASC, caspase-1, IL-1β and IL-18 expression. The present results were in consistency with a previous study conducted by Nakahira *et al* (17), who found that TSPO ligands inhibited NLRP3 inflammasome activation through mitochondrial disturbance in BMDM cells.

In addition, it was revealed that the levels of NLRP3, ASC, caspase-1, IL-1β and IL-18 expression were significantly higher in PDR patients compared with those in NPDR patients. Meanwhile, active DR patients showed significantly

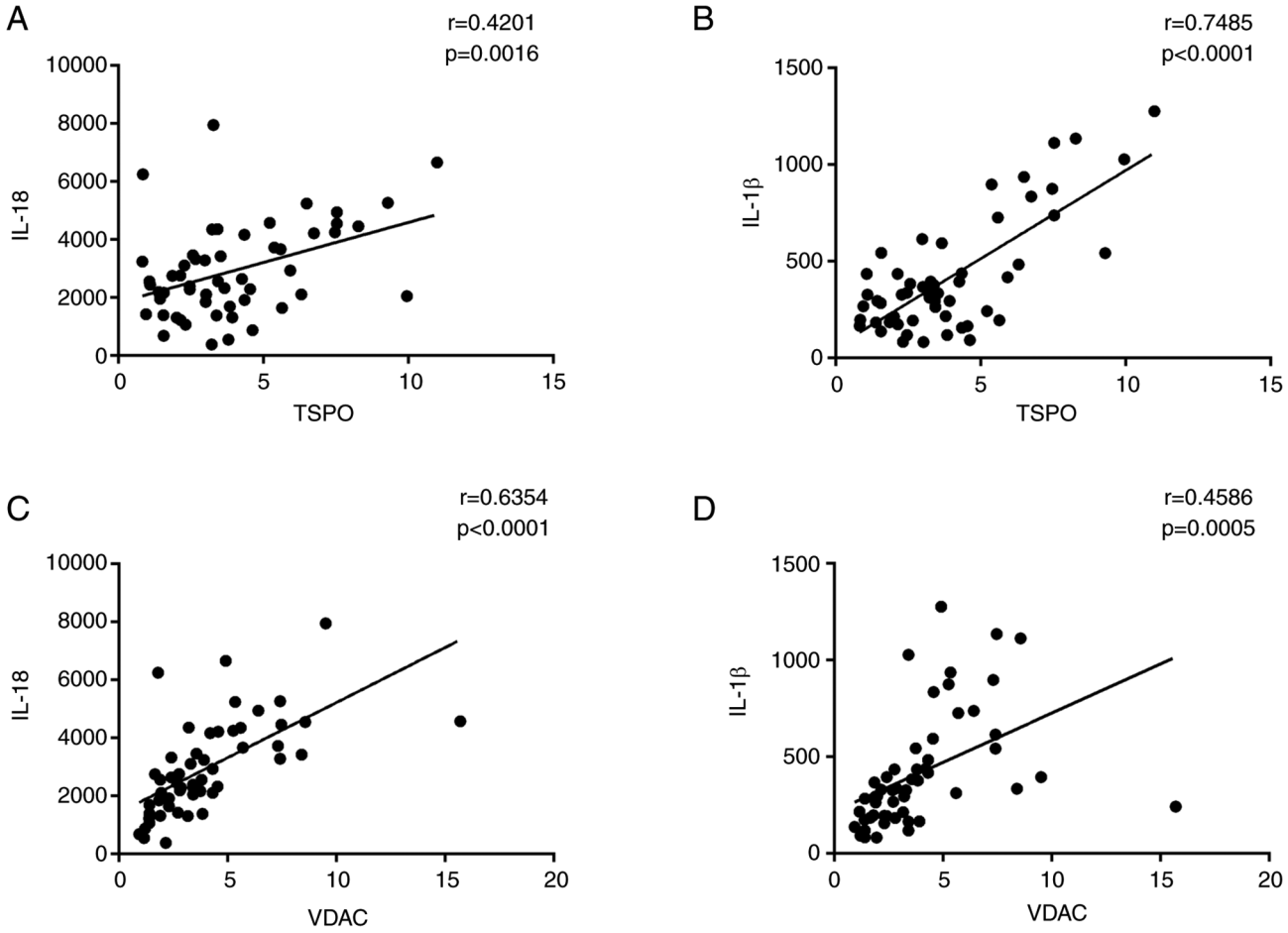

Figure 5. Positive correlation coefficient between inflammatory cytokine protein levels (IL-18 and IL-1β) and relative mRNA expression levels of (A and B) TSPO and (C and D) VDAC. Data are presented as the median and interquartile range. The results were assessed using Spearman's correlation test. TSPO, translocator protein; VDAC, voltage-dependent anion channel.

more elevated expression levels of NLRP3, ASC, caspase-1, IL-1β and IL-18 compared with those in inactive DR patients. ASC is the adaptor protein of NLRP3 and plays critical role in the formation of inflammasome (24). After being recruited by NLRP3, ASC interacts with cleaved caspase-1, and in turn permits the maturation and secretion of IL-1β and IL-18 (25); the latter ones are potent proinflammatory cytokines that can drive downstream molecular cascades such as IL-6 and VEGF (26).

The aforementioned evidence is in consistency with the present findings that the expression levels of TSPO-VDAC and NLRP3 activation proteins (NLRP3, ASC, caspase-1, IL-1β and IL-18) develop in parallel with the severity of DR and are related to the clinical complications of DR such as neovascularization.

There was a limitation to the present study; the cytokines were only partially examined in protein level due to inadequate blood sample volumes as the count of PBMCs was not enough for sufficient protein extraction for western blotting. Therefore, RT-qPCR and ELISA were used to evaluate the expression of related cytokines.

In summary, the present study demonstrated for the first time that metabolic and inflammatory markers measured in peripheral blood are associated with DR. The present findings emphasized the critical need for further characterization of the TSPO and VDAC pathways in DR patients, as changes in their expression may result in activation of NLRP3 pathways, resulting in inflammation and neovascularization. Future longitudinal studies examining the relationship between mitochondrial markers and the progression of DR, as well as the assessment of inflammatory markers in other tissues relevant to the pathophysiology of DR, are necessary.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YG collected samples and performed the experiments and data analyses. ZS contributed significantly to analysis and manuscript preparation. QS contributed to the conception of the study, performed the data analyses and wrote the manuscript. YG, ZS and QS confirm the authenticity of all the raw data. LW, RJ and GX helped in performing the analysis with constructive discussions. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study followed the guidelines of the Declaration of Helsinki, and all experimental protocols were approved (approval no. 2020142) by the Human Ethics Committee of Eye & ENT Hospital of Fudan University (Shanghai, China). Written informed consent was obtained from the enrolled participants. The detailed explanations of the study purpose have been given to all the participants, and the signatures of the informed consent form have been obtained correspondingly.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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