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Downregulation of SIRT6 and NMNAT2 is associated with proliferative diabetic retinopathy

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Purpose: To determine the expression levels of *SIRT6* and *NMNAT2* in diabetic retinopathy (DR).
Methods: We obtained peripheral blood mononuclear cells (PBMCs) and vitreous samples from 77 patients with type 2 diabetes mellitus: 52 with DR and 25 without DR, and 27 healthy control subjects. Western blot analysis and qRT-PCR were performed to evaluate the expression of *SIRT6* and *NMNAT2* in their PBMCs. The levels of *IL-1β*, *IL-6*, and *TNF-α* in the vitreous fluid were determined by ELISA. Immunohistochemistry was performed to detect the expression of *SIRT6* and *NMNAT*2 in proliferative DR (PDR) and the control subjects.
Results: The expression of *SIRT6* and *NMNAT2* was markedly downregulated in DR patients, which was negatively correlated with the increased expression of *IL-1β, IL-6* and *TNF-α*. Additionally, we observed decreased expression of *SIRT6* and *NMNAT2* in the fibrovascular membranes of PDR patients.
Conclusions: The downregulated expression of *SIRT6* and *NMNAT2* in PDR patients reveals a potential pathogenic association; more extended studies could verify them as potential therapeutic targets.

Diabetic retinopathy (DR) is among the common microvascular complications of diabetes mellitus that develop after chronic hyperglycemia. Irreversible vision loss occurs in up to 80% of patients who have been affected with diabetes for 20 years or more [1,2]. While chronic hyperglycemia increases inflammation and causes neuronal and vascular injuries, such as loss of ganglion cells and generation of degenerative capillaries, the mechanism underlying the pathogenesis of DR remains elusive.

Sirtuins (SIRTs) are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases that play a role in inflammation, energy metabolism, stress resistance, and cancer [3]. Among them, *SIRT6* is localized in the nuclei and regulates a variety of biologic processes, including transcription, inflammation, carcinogenesis, metabolism, and so forth, while affecting numerous pathophysiological conditions such as diabetes mellitus and cardiovascular disorders [4,5]. A recent study demonstrated that *SIRT6* was downregulated in human endothelial cells [6]. To date, the precise function of *SIRT6* in DR and the mechanism underlying the regulation of type 2 diabetes mellitus (T2DM)-related metabolism by *SIRT*6 has yet to be determined.

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Nicotinamide mononucleotide adenylyltransferase 2 (*NMNAT2*) catalyzes NAD synthesis [7]. *NMNAT2* can serve as a sensor for monitoring intracellular redox equilibrium as well as the energy state in cells with high energy demand, such as retinal ganglion cells [8]. It has been proven that *NMNAT2* participates in the regulation of *SIRT6* and its downstream signaling pathways related to neuroinflammation [9-12]. Hence, it is hypothesized that *NMNAT2/SIRT6* could exert a regulatory effect on inflammatory reactions in DR. Herein, we comprehensively examined the expression of *SIRT6*, *NMNAT2*, and inflammatory cytokines in various categories of DR patients and then determined their relationships.

METHODS

Patients: In this study, we enrolled consecutive T2DM patients as well as nondiabetic subjects who presented to the outpatient department of the Zhongshan Ophthalmic Centre, China, from January 2021 to July 2022 (Table 1). Diagnosis was confirmed in accordance with American Diabetes Association standards (2002) [13].

The study's exclusion criteria for participation were: 1) cases complicated by infectious diseases or other disorders such as nephropathy (including stage 3 chronic kidney disease, macroalbuminuria, proteinuria, and hemodialysis patients); 2) patients who had undergone intraocular procedures, intravitreal treatments, or photocoagulation within 3 months before the study; 3) patients with a history of uveitis, trauma, vitreous hemorrhage, or retinal detachment; and 4)

Table 1. Clinical and biochemical characteristics of type 2 diabetic patients and healthy control subjects.

Variables	Control (n=27)	NDR (n=25)	NPDR (n=26)	PDR (n=26)	p
Sex (m/f)	14/13	13/12	14/12	12/14	0.951
Age (years)	65.7±7.6	63.3±7.0	62.0±6.2	64.7±6.8	0.249
BMI (kg/m^2)	22.2±2.5	22.5±2.2	23.6±1.9	25.3±2.6	<0.001*
Diabetes Duration (years)	-	7.8±3.5	9.7±3.0	14.3±1.9	<0.001*
FPG (mmol/l)	5.3±0.6	7.8±1.8	9.9±2.1	12.8±1.8	<0.001*
HbAlc (%)	5.1±0.6	7.2±1.6	9.0±1.8	11.6±1.7	<0.001*

DR: diabetic retinopathy; NDR: no apparent retinopathy; NPDR: non-proliferative retinopathy; PDR: proliferative diabetic retinopathy;BMI, Body mass index; FPG: fasting plasma glucose; HbA1c, glycated hemoglobin Data are expressed as mean±SD * p≤0.05

patients taking immuno-suppressive drugs. The assessment of DR was performed using fluorescein fundus angiography (FF450 fundus camera, Carl Zeiss Meditec AG, Germany). Body mass index (BMI) was calculated using the standard formula, weight (kg)/height (m^2). Diabetics were classified into three categories: no clinically apparent retinopathy (NDR), non-proliferative diabetic retinopathy (NPDR), and proliferative diabetic retinopathy (PDR) [14].

All experiments were approved by our institutional ethical committee and conducted in compliance with the Declaration of Helsinki. Each participant provided a signed informed consent statement.

Demographic data: Age- and sex-matched samples were collected from 77 patients with T2DM (39 males and 38 females) and 27 healthy individuals (14 males and 13 females). The median ages of the patients and control subjects were 63.3 ± 6.7 years and 65.7 ± 7.6 years, respectively (p = 0.135). The diagnoses of the 77 diabetic patients were NDR (n = 25), NPDR (n = 26), and PDR (n = 26). The male to female ratios and mean ages (± SD) of NDR, NPDR, and PDR patients were 13:12 and 63.3 ± 7.0 years, 14:12 and 62.0 ± 6.2 years, and 12:14 and 64.7 ± 6.8 years, respectively.

Specimen collection: Twelve mL of whole blood was collected from each subject in a test tube with lithium heparin (Vacutainer; BD Biosciences, San Jose, CA) for quantification of protein and mRNAs, and venous blood was drawn to measure fasting plasma glucose (FPG) and glycated hemoglobin.

Isolation of PBMCs: Isolation of PBMCs from heparinized venous blood was performed using Ficoll-Hypaque density gradient centrifugation. PBMCs were incubated with lipopolysaccharide (100 ng/ml; Sigma-Aldrich Corp.,St Louis, MO) for 4 h, followed by incubation in RPMI 1640 medium with 1 mM ATP for another 15 min.

Collection of vitreous fluid: Using pars plana vitrectomy, 0.5 ml of undiluted vitreous fluid was obtained from each participant. Samples were stored at −80° C until analysis.

Quantitative real-time PCR: Total RNA was extracted from the PBMCs by the Trizol method and reverse-transcribed using the Qiagen QuantiFast SYBR Green PCR Kit on BioRad LightCycler CFX96 (Hercules, CA). The primers for human genes were designed according to the PrimerBank public database [15,16]. The following primers were used: *SIRT6* sense: 5′-GCTGGAGCCCAAGGAGGAATCT- 3′, antisense: 5′-AGCCTCACCTCTGGACAACACA −3′ [15]; *NMNAT2* sense: 5′- CCGCAATTGAAGGATGTTG-3′, antisense: 5′- CTCTGGCTCTTGGGATTCTG −3′; and β-actin sense: 5′-GGA CTT CGA GCA AGA GAT GG-3′, antisense: 5′-AGC ACT GTG TTG GCG TAC AG-3′. β-actin was included as a reference gene. Each assay was conducted in triplicate. The amplified products were resolved on agarose gel electrophoresis. Primer specificity was evaluated by melting curve analysis, and the $2^{-\Delta\Delta Ct}$ method was employed to measure relative mRNA expression levels.

Western blot analysis: Total protein was isolated from the PBMCs. Protein extract (60 µg) was resolved on 12% SDS–PAGE and then electroblotted to a PVDF membrane. The blot was detected with anti-SIRT6 or anti-*NMNAT2* antibodies (Abcam, Cambridge, UK). The target protein was visualized with the Pierce SuperSignal West Pico Substrate Kit. ImageJ software was employed to determine the protein band intensity relative to β-Actin.

ELISA: The levels of *IL-1β, IL-6*, and *TNF-α* in the vitreous fluid samples were determined with an ELISA kit (Sen-Xiong Company). Each sample, along with the standard, was measured three times. Background subtraction was applied to determine OD450, and a standard curve was made.

Immunohistochemistry: Surgical removal of fibrovascular membranes (FVMs) was performed on 26 PDR patients, and

epiretinal membranes (ERMs) were resected from 27 control subjects. There were no significant differences in age among the groups (Table 1). At the same time, retinas from mice and cadaver eyes served as positive controls (Appendix 1).

Surgically resected FVMs and idiopathic ERMs (3-µm thickness) were subjected to fixation with 4% paraformaldehyde, followed by paraffin embedding. Thereafter, the samples were deparaffinized and exposed to 3% H_2O_2 in methanol for 15 min to inhibit endogenous peroxidase activity. After being blocked in blocking solution for 10 min, the sections were incubated with polyclonal antibodies for *SIRT6* (1:150; Abcam, Cambridge, USA) or *NMNAT2* (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min, followed by incubation with HRP polymer (Thermo Scientific, Santa Cruz, CA) for 30 min at room temperature. Signal detection was performed using the AEC chromogen system (Thermo Scientific). Reactions were developed with diaminobenzidine (DAB), followed by hematoxylin counterstaining. Negative controls were isotype-matched. Images were captured by an Olympus BX60 microscope and analyzed with the use of Image Pro Plus 4.5 software (Media Cybertics). The H-score was used to evaluate the intensity of each signal.

Statistics: Statistical analyses were performed using SPSS version 19.0. Differences between the patients and control subjects were examined using multivariate ANCOVA (MANCOVA) or the nonparametric Kruskal–Wallis test based on assumptions of normality and homogeneity of variance. Mann–Whitney U tests or Student's t tests were performed to determine variations among all groups. The study parameters were compared using Spearman's correlation analysis. GraphPad Prism version 5 was used to draw graphs. A p value of less than 0.05 indicated significant difference.

RESULTS

Clinical characteristics: The clinical and laboratory parameters of the patients are summarized in Table 1. No significant differences in age and gender ($p = 0.249$ and $p = 0.951$, respectively) were observed among the different groups. T2DM patients had a significantly higher body mass index than the control subjects ($p < 0.001$). Compared with the NPDR and NDR groups, the mean extent of diabetes was markedly increased in the PDR group ($p < 0.001$). Normal HbA1c values ranged between 4.27% and 6.07%. Notably, the PDR group displayed a significantly higher level of HbA1c and fasting glucose than the NPDR and NDR groups ($p < 0.001$ for both).

Expression of SIRT6 and NMNAT2 in DR patients: We performed qRT-PCR assays to examine the expression levels of *SIRT6* and *NMNAT2* in both the patients and the control subjects. As depicted in Figure 1, the mRNA expression of *NMNAT2* and *SIRT6* was markedly decreased in PBMCs of NPDR and PDR cases as compared to those of NDR patients and control subjects ($p < 0.001$ for all comparisons).

We next investigated whether *SIRT6* and *NMNAT2* were downregulated at the protein level in DR patients. Western blot analysis revealed that the protein levels of *SIRT6* and *NMNAT2* in NPDR and PDR patients were significantly lower than those in NDR cases and control subjects ($p < 0.001$ for all comparisons; Figure 2). Collectively, these data indicate that the mRNA and protein expression of *SIRT6* and *NMNAT2* were significantly decreased in the PBMCs of patients with DR.

Concentrations of inflammatory cytokines in vitreous fluid: We further analyzed the level of *IL-1β, IL-6* and *TNF-α* to determine the inflammatory activity in T2DM patients. As shown in Figure 3, elevated production of cytokines was evident in PDR and NPDR cases as compared to NDR patients and control subjects.

Correlation analysis: Correlation analysis showed a positive correlation between protein and mRNA expressions of *SIRT6* and *NMNAT2* in DR patients ($r = -0.246$, $p < 0.01$).

As shown in Table 2, there was a linear regression between the expressions of *SIRT6* and *NMNAT2* and different variables. Moreover, we found that while the expression level of *SIRT* in PBMCs was significantly inversely correlated with vitreous concentrations of *IL-1β* and TNF-α, the expression levels of *SIRT6* and *NMNAT2* were all negatively correlated with FPG and HbA1c levels.

Expression of SIRT6 and NMNAT2 in patients with DR: We performed immunohistochemical staining to detect the expression of *SIRT6* and NMNAT2 in the FVMs of PDR patients. The stained area for *SIRT6* or *NMNAT2* was significantly reduced in PDR patients compared with the controls ($p < 0.001$ for both), as indicated by computer-assisted image analysis (Figure 4).

DISCUSSION

As a progressive, chronic microvascular complication of T2DM, DR can cause visual impairment and legal blindness [17]. Although inflammatory cytokines and nitric oxide have been found to be involved in DR pathogenesis, the exact mechanism remains to be addressed [18,19]. Herein, we comparatively examined the expression of *SIRT6* and *NMNAT2* at both protein and mRNA levels in DR patients and healthy controls. We found that the expression of the two factors was markedly downregulated in the patients (*p*

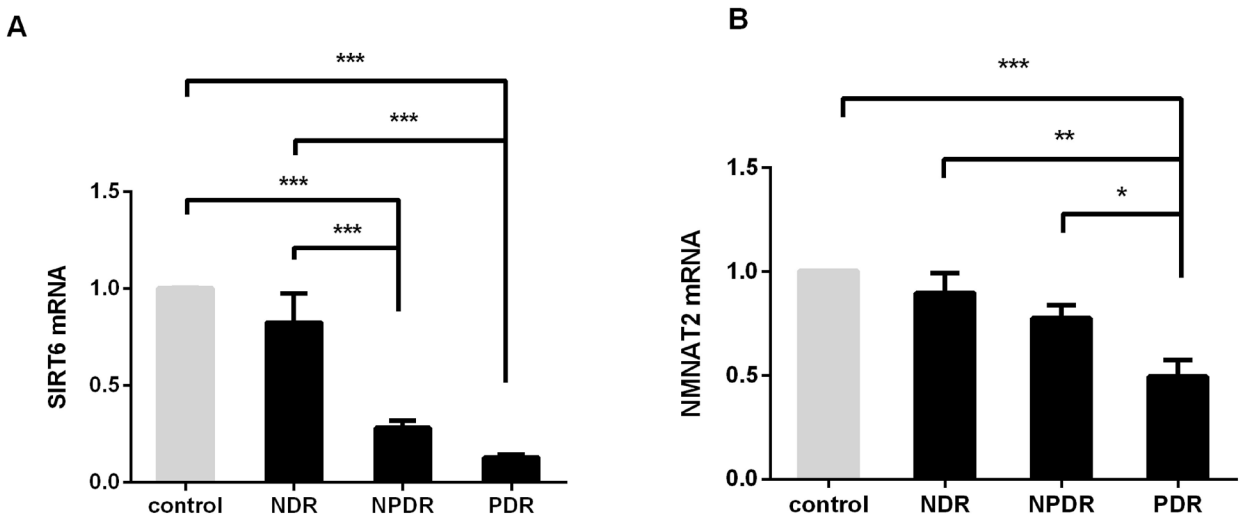

Figure 1. The mRNA levels of *SIRT6* and *NMNAT2* were downregulated in DR patients. The mRNA expression of *SIRT6* and *NMNAT2* in the PBMCs was quantified by real-time PCR and normalized to the level of β-actin. (PDR, $n = 26$; NPDR, $n = 26$; NDR, $n = 25$; controls, $n = 27$). Values are presented as fold-changes when compared with the controls. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

< 0.01). Moreover, immunofluorescent staining revealed a decreased expression of the two factors in PDR. Notably, we identified an association between the decreased expression of the two factors and the elevated level of inflammatory cytokines in DR ($p < 0.05$).

The *SIRT* family of histone deacetylases plays a role in controlling numerous cellular functions, including proliferation, differentiation, programmed cell death, metabolism, and aging [20,21]. *SIRT6*, a NAD+-dependent deacylase capable of regulating glucose metabolism, has recently been demonstrated to be critically involved in the physiopathological processes of T2DM [4,22]. High expression of *SIRT6* has also been found in the retina, while *SIRT6* retinal levels were significantly decreased in non-obese diabetic (NOD) mice compared to NOD normoglycemic littermates [23]. In line with this finding, decreased *SIRT6* levels in pancreatic islets from diabetic mice [24] and in the carotid plaques of asymptomatic diabetic patients [25] have also been observed. Moreover, *SIRT6*-overexpressing mice were found to be protected from developing high caloric diet-induced hyperglycemia and glucose intolerance [26], and *SIRT6* deficiency results in major defects in retinal transmission, while altering the expression of glucose homeostasis-related genes and glutamate receptors [23,27]. All these observations led us to propose that hyperglycemia may elicit downregulation of *SIRT6* and upregulation of inflammatory cytokines [23].

SIRT6 protein and mRNA expression levels have been found to be significantly reduced in colorectal cancer tissues; it was previously demonstrated that deletion of *SIRT6* may activate an energy metabolism program that promotes tumorigenesis [28]. However, *SIRT6*'s roles as a metabolic enzyme and a potential regulator of cancer cell metabolism remain to be investigated. It has been established that maintaining intracellular NAD levels is crucial for several biologic processes, such as energy metabolism and the activity of *SIRTs* [29-31]. It was also reported that *NMNAT2* mRNA was mainly expressed in high energy consumption organs, including the brain, heart, and skeletal muscle, while there was nearly no *NMNAT2* detected in kidney, lung, spleen, and testis [32]. Emerging evidence suggests that a decline in redox factor NAD+ is a hallmark of aging and neurodegenerative diseases [12,33]. *NMNAT2* is significantly decreased in glaucomatous retinal ganglion cells [28,34]. A decrease in the expression of *NMNAT2* and *SIRT6* was also observed in the spinal cord of amyotrophic lateral sclerosis patients [33]. Given the critical role of *NMNAT2* in cellular metabolism, its expression may be downregulated in endothelial cells in DR [32,35]. In this study, we found that *NMNAT2* expression was significantly reduced in DR patients. Other studies have revealed that decreased *NMNAT2* expression was markedly correlated with downregulation of *SIRT6* expression in DR. It has been reported that high glucose-induced endothelial damage is related to NAD depletion in cells [36]. A study on

cardiac hypertrophy showed that *SIRT6* participated in the anti-hypertrophic signals of *NMNAT2* overexpression, which suggested that upon activation of *SIRT6* via intracellular NAD, the protein level and enzymatic activity of *NMNAT2* were dramatically reduced [9]. Low *SIRT/NMNAT2* pathway expression in adipose tissue of BMI-discordant monozygotic twins was reported [12], which highlights a strong relationship of reduced *SIRT/NMNAT2* pathway expression with insulin resistance and inflammation. Immunohistochemical studies have provided data suggesting an association between decreased *NMNAT2* expression and *SIRT6* downregulation in PDR. Validation of the association of *SIRT6* with *NMNAT2* in DR may help to determine how DR's progression is inhibited by modulating the metabolism of retinal cells.

Elevated production of *IL-1β, IL-6* and *TNF-α* has been found to be correlated with decreased expression of *SIRT6* and *NMNAT2* in PDR and NPDR patients. In rat models, *SIRT6* functions as an immune regulatory factor responding to renal injury in diabetic nephropathy [37]. It has also been shown that low expression of *SIRT6* and high expression of NF-κB are linked to the inflammatory pathway in the abdominal subcutaneous fat of obese and pre-DM patients [38]. In central nervous system conditionally deleted *SIRT6* knockout mice, increased vascular endothelial growth factor

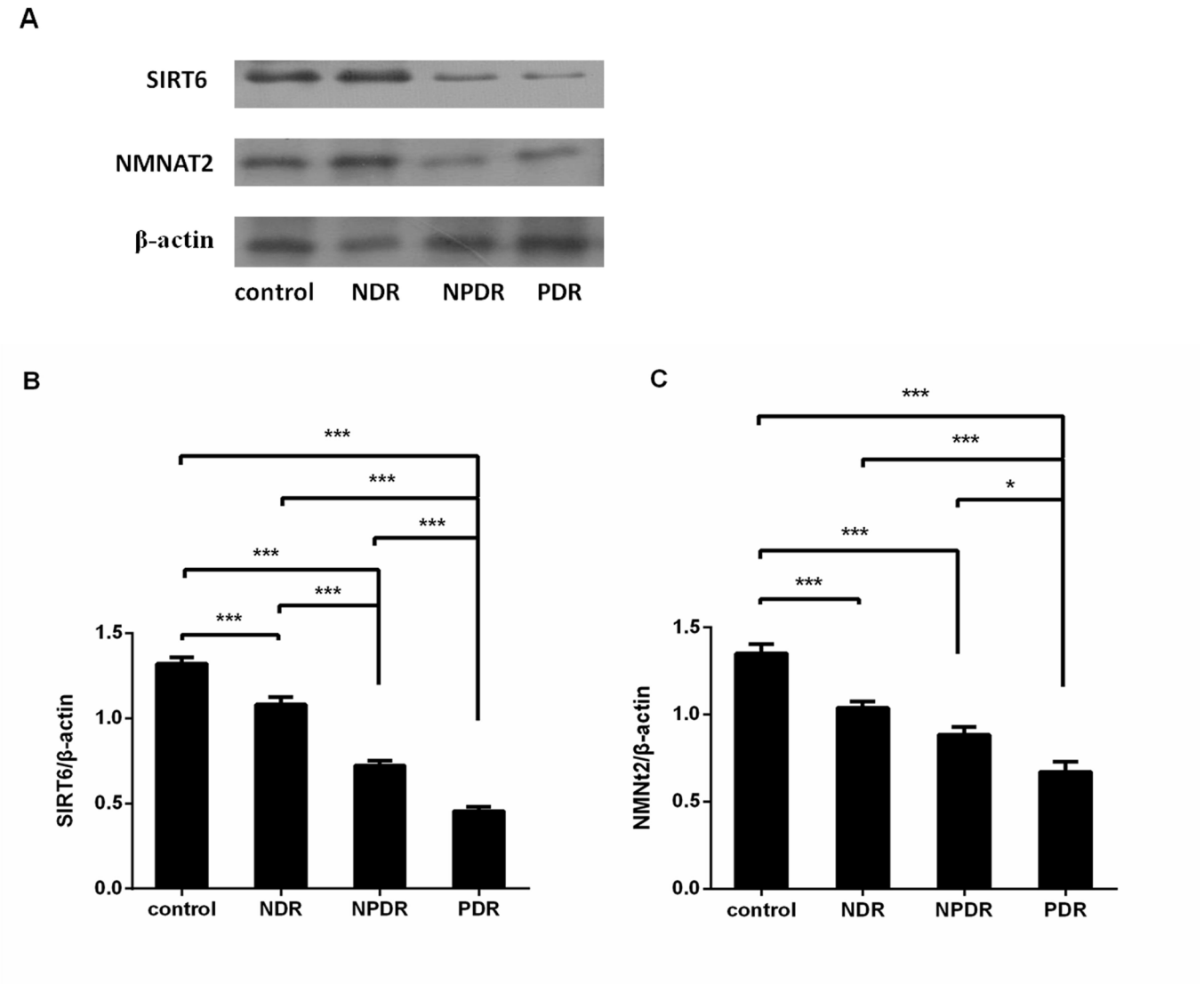

Figure 2. The protein expression of *SIRT6* and *NMNAT2* was decreased in DR patients (PDR, $n = 26$; NPDR, $n = 26$; NDR, $n = 25$; controls, $n = 27$). Western blot analysis (lane 1, the control; lane 2, NDR; lane 3, NPDR; lane 4, PDR) and quantification of *SIRT6* and *NMNAT2* expression in PBMCs. β-actin was included as a reference. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

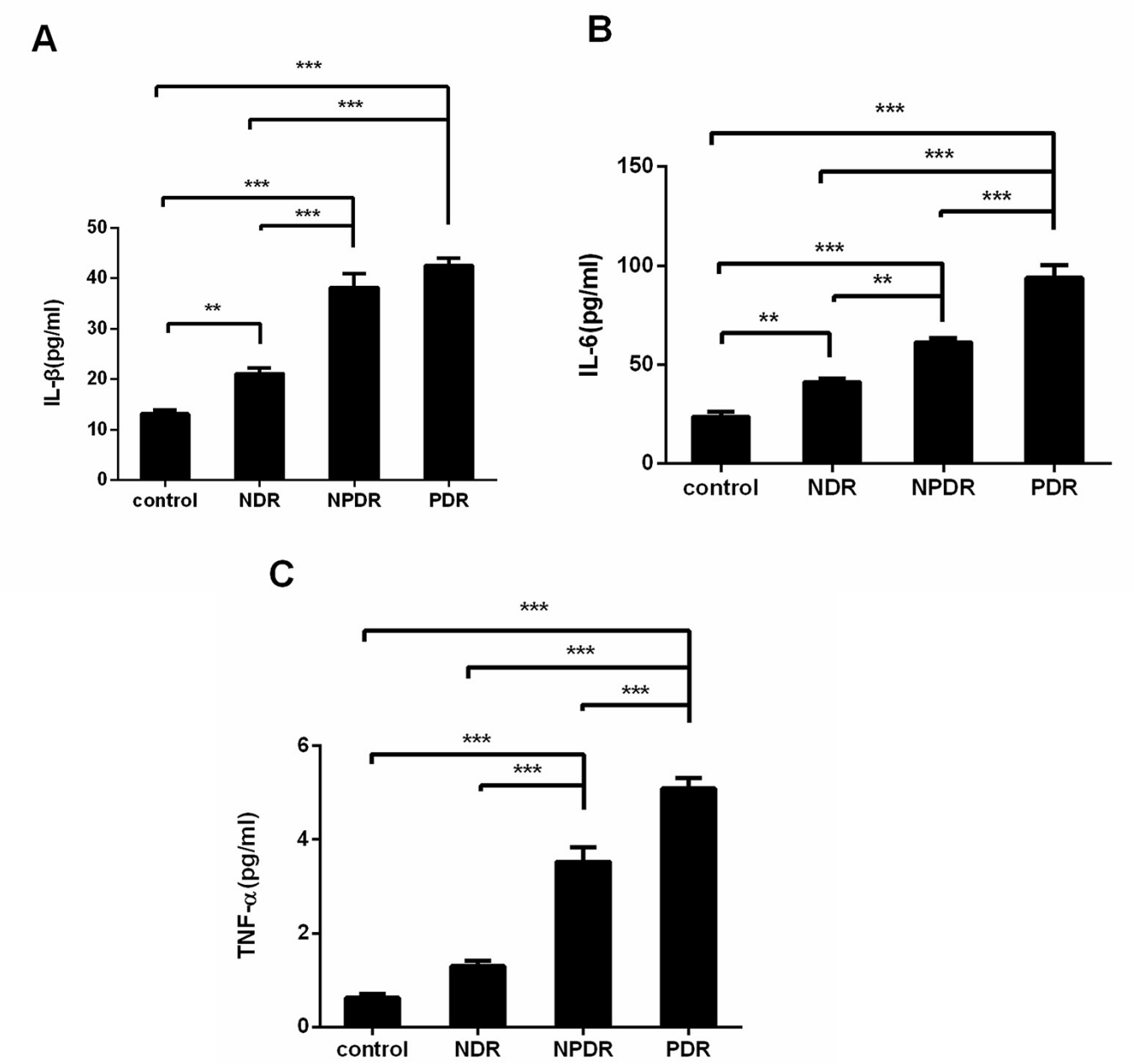

Figure 3. Concentrations of *IL-1β, IL-6,* and *TNF-α* in the vitreous fluid of T2DM patients and non-diabetic controls (PDR, $n = 26$; NPDR, $n = 26$; NDR, $n = 25$; controls, $n = 27$). The comparison was made by the Kruskal–Wallis test with Dunn's multiple comparison test. *$p < 0.001$.

levels, decreased brain-derived neurotrophic factor levels in retinas, and a significant reduction in the whole retinal thickness were observed [23]. Taken together, the findings in the present study demonstrate that decreased expression of *SIRT6* may aggravate the proinflammatory response in the progression of PDR [39].

The present study suggests that HbA1c levels are negatively correlated with the levels of *SIRT6* and *NMNAT2* in cases of DR. Given that the above correlation has also been observed in previous studies on animal models, we conclude that glycemic control is vital for the immune response of diabetic patients.

There are certain limitations in the current study. First, this study was a preliminary attempt that needs to be verified. Second, potential confoundment of the reported splice variant of *SIRT6* should be considered for future study, although whether this variant can be translated to protein and function needs to be identified experimentally [40]. Last, further

Table 2. Spearman's correlation between the expression levels of the studied genes in the PBMCs and clinical characteristics of the studied groups.

Studied gene	mRNA/ protein level	SIRT6 mRNA	SIRT6 protein	NMNAT2 mRNA	NMNAT2 protein	IL-1β Vitreous	IL-6 Vitreous	TNF-α Vitreous	FBG	HbA1c
SIRT6	mRNA	-	0.732***	0.377***	-	−0.742***	−0.666***	−0.749***	−0.801***	−0.792***
	protein	0.732***	-	-	0.593***	−0.694***	−0.736***	−0.829***	−0.767***	−0.770***
NMNAT2	mRNA	0.377***	-	-	0.334***	−0.511***	−0.507***	−0.512***	−0.487***	−0.523***
	protein	-	0.593***	0.334***	-	−0.607***	−0.567***	−0.649***	−0.645***	−0.632***

***p<0.001

studies with a larger sample of DR patients from more diverse populations are required.

In conclusion, we found downregulation of *SIRT6* and *NMNAT2* in patients with PDR. These findings indicate the role of *SIRT6* and *NMNAT2* in the pathogenesis of PDR. Moreover, downregulation of *SIRT6* and *NMNAT2* may lead to an increase in the expression of inflammatory cytokines. Future studies will be directed at investigating the potential of *NMNAT2* and *SIRT6* as diagnostic and prognostic indicators, as well as therapeutic targets for PDR.

APPENDIX 1. THE POSITIVE CONTROL FOR THE IMMUNOHISTOCHEMISTRY STAINING OF *SIRT6* AND *NMNAT2* IN RETINAS FROM MOUSE AND CADAVER EYES (×400).

To access the data, click or select the words "Appendix 1." (Top) Positive *SIRT6* and *NMNAT2* expression in cadaver retina with negative control; (Bottom) Positive *SIRT6* and *NMNAT2* expression in retina from mouse along with negative control.

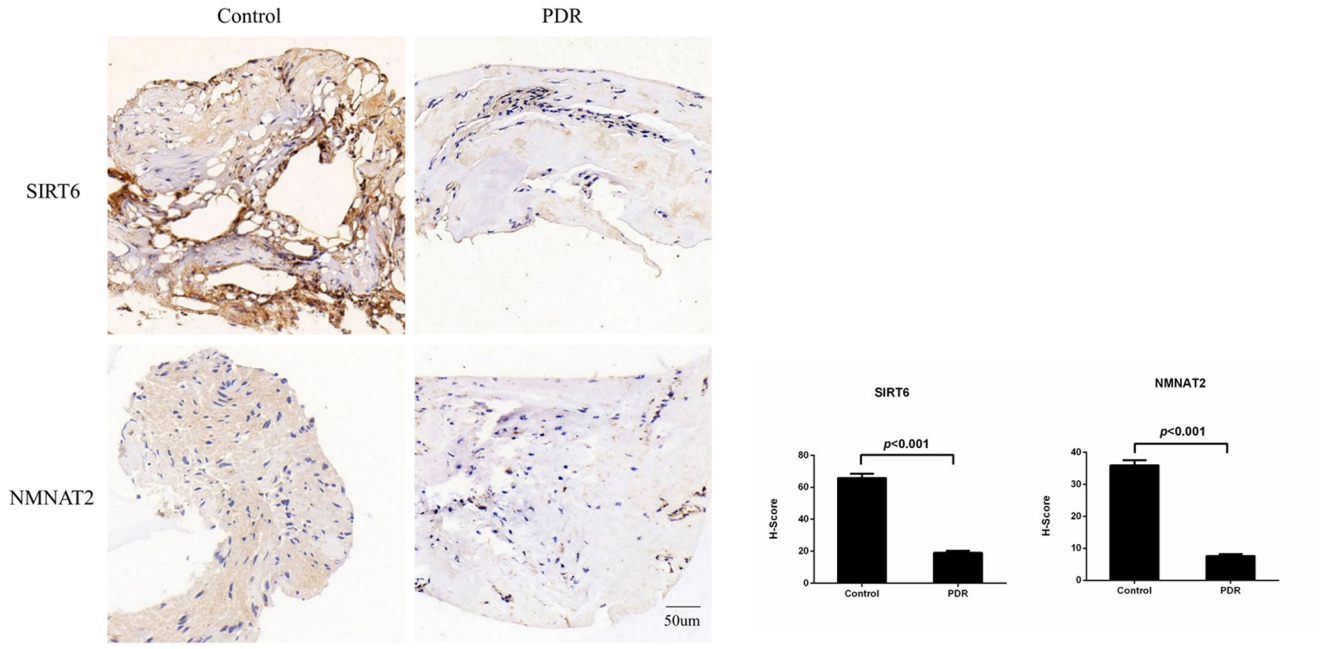

Figure 4. Immunohistochemistry staining reveals a significantly reduced expression of *SIRT6* and *NMNAT2* in FVMs of PDR patients (×200) compared with the epiretinal membrane subjects (control group). *$p < 0.001$.

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