EXTENDED REPORT

Vitreous polyamines spermidine, putrescine, and spermine in human proliferative disorders of the retina

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Background/aims: Many cytokines are involved in the pathogenesis of retinal proliferative diseases, but none has been shown to be related to a specific disorder. The aim of this study was to provide a selective marker of diabetes induced proliferative retinopathies.

Methods: 10 vitreous samples from 10 subjects affected by quiescent proliferative diabetic retinopathy (PDR), 20 vitreous samples from 20 subjects affected by active PDR, and 15 samples from 15 patients with proliferative vitreoretinopathy (PVR) were studied. Samples from 18 patients with a macular hole (n = 8) or pucker (n = 10) served as controls. Vitreous samples were obtained via pars plana vitrectomy. The polyamines spermidine, putrescine, and spermine, vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and transforming growth factor 1β (TGF-1β) were measured by high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA), and the correlation coefficients between the vitreous polyamine content and VEGF, IL-8, and TGF-1β levels were determined.

Results: Spermidine and putrescine were expressed in normal vitreous, but spermine was not detectable. In all the test groups spermidine was 3–4 times higher than in control vitreous and putrescine was similarly lower. The spermine content was up to 15 times higher only in vitreous from patients affected by PDR. Correlation coefficients showed that the spermidine and putrescine level variations correlated with the VEGF and IL-8 content in the active PDR and PVR groups, but not in those with quiescent PDR patients, while spermine was correlated to these cytokines in PDR, but not in PVR groups.

Conclusions: These data suggest a significant role for spermidine and putrescine as markers of proliferative diseases of the retina. The increase in spermine, restricted to diabetic states, may indicate that this polyamine is a unique and specific index of PDR.

I t has been well established that hypoxia induced alterations in the ocular vasculature are associated with retinal diseases, such as proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR), and retinopathy of prematurity (ROP).[1] In these conditions, vascular endothelial growth factor (VEGF) has been extensively studied as a potent stimulator of normal and abnormal vascular growth.[2–8] The level of VEGF is significantly higher in the vitreous of patients with proliferative retinopathies, especially those associated with type I and type II diabetes, than in normal subjects.[3]

Besides VEGF, other cytokines have been found to be implicated in the pathogenesis of proliferative vitreoretinal diseases. These include transforming growth factor (TGF-β_1),[1] hepatocyte growth factor (HGF),[4 7] interleukin 6 (IL-6), IL-8, tumour necrosis factor β (TNF-β), interleukin 1α (IL-1α), interferon β (IFN-β), platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor α_2 (TGF-α_2),[9–18] but none has been related to a specific retinal proliferative disorder. Chemokines,[19] tissue plasminogen activator, and plasminogen activator inhibitor[2] also do not differentiate proliferative retinopathies, despite their significant increase in all the proliferative alterations of the retina.

Therefore, there is no indicator among those studied which is able to discriminate between PVR and PDR.

The natural polyamines, spermidine, putrescine and spermine, widespread in all organisms, have been shown to have a basic role in the regulation of growth and differentiation of all cell types.[20] Their biosynthesis is known to increase with mitogenesis, and elevated polyamine concentrations have been found in hyperproliferative tissues.[21] Moreover, depletion of cellular polyamines has been associated with inhibition of growth and programmed cell death.[22]

It may be of particular interest to study the involvement of vitreous polyamines in patients affected by proliferative retinopathies, diabetes dependent and non-diabetes dependent, since these mediators exert a pathophysiological role either on proliferation or on the replication, metabolism, and insulin production of the pancreatic islet cells.[23–25]

The implications of polyamines in the regulation of proliferation and glycaemic homeostasis in physiological as well as diabetic conditions prompted us to evaluate their vitreous content in some proliferative retinal disorders, including PDR, with the aim of finding a pathogenesis related marker.

We also aimed to establish correlations between the vitreous concentrations of polyamines and the levels of VEGF, IL-8, and TGF-$\alpha1$ levels in the vitreous of patients affected by PDR and PVR to establish the pathogenetic significance of these amines in relation to the already well known positive involvement of VEGF and IL-8 and the negative involvement of TGF-$\alpha1$ in retinal proliferation.

MATERIALS AND METHODS
Study population
The study included 10 vitreous samples from 10 subjects affected by quiescent PDR (age range 45–71 years) (group I); 20 vitreous samples from 20 subjects affected by active PDR (age range 40–68 years) (group II); and 15 vitreous samples from 15 subjects affected by PVR (age range 42–46 years) (group III).

The time from the initial diagnosis of diabetes mellitus to a surgical procedure in PDR patients varied from 20–30 years, with a mean of 23 years. Retinopathy was graded in all eyes by clinical examination before surgery and by the surgeon at the

time of surgery. Neovascularisation was considered to be active when perfused, multibranching, preretinal capillaries existed and to be quiescent if only non-perfused gliotic vessels or fibrosis were present.[8] Almost all, and only, the diabetic patients of group I and II (26/30) had previously undergone a variable degree of peripheral laser photocoagulation at different time before the vitrectomy, ranging from few months to some years. Vitreous haemorrhage of various degree was present in 14 cases.

All patients in group III had an high grade of PVR needing vitrectomy.

Samples from patients with underlying inflammatory disease were excluded from this study.

Vitreous fluid samples from 18 eyes of 18 patients who had a macular hole (n=8) or pucker (n=10) served as controls.

Clinical examination

The ophthalmological examination included best corrected ETDRS visual acuity, applanation tonometry, and biomicroscopic evaluation of the anterior segment. Binocular indirect ophthalmoscopy, retinal biomicroscopy using high positive power precorneal lenses, and colour fundus stereophotographs (and fluorescein angiography, if necessary) were performed through dilated pupils.

Collection and storage of vitreous samples

Vitreous humour samples were obtained during pars plana vitrectomy. The samples were collected undiluted, by manual suction into a syringe through the air flushed aspiration line of the vitrectome, before the infusion line was opened.

Aliquots were transferred directly to cryotubes (Eppendorf, Hamburg, Germany) and stored at –80°C until analysis.

Protein assay

Vitreous protein levels were determined by a colorimetric assay (Bio-Rad, Milan, Italy). To 10 µl of undiluted vitreous humour 1 ml of dye reagent (diluted 1:5 with distilled water) was added. The absorbance within 60 minutes was read at 595 nm. Vitreous proteins were interpolated from a specific calibration curve prepared with known standard solutions. The detection limit was 1 µg/ml.

Cytokine measurement

Levels of VEGF, IL-8, and TGF-1β were determined by ELISA using commercial kits (Chemicon International, Inc, Temecula, CA, USA; Prodotti Gianni spa, Milan, Italy). For each data point, assay determinations were performed in duplicate.

VEGF assay

Immunoassay for the measurement of VEGF concentrations was performed in 96 well test plates coated with biotinylated rabbit anti-human VEGF polyclonal antibody. The microtitre plates were incubated with 100 µl of undiluted vitreous humour for 3 hours at room temperature, after which the plates were washed five times with wash buffer. In the following step, 50 µl of diluted streptavidin-alkaline phosphatase was added and the plates were incubated at room temperature for 45 minutes. Again, the plates were washed five times with wash buffer and 200 µl of prepared colour reagent solution was dispensed into each well. After 10 minutes the reaction was stopped with 0.5 M sulphuric acid. The absorbance was read at 490 nm. Vitreous levels of VEGF antigen were interpolated from specific calibration curves prepared with known standard solutions. The detection limit was 20 pg/ml (coefficient of variation, intra-assay 8.9%; interassay 11.1%). The results obtained have been converted to ng/mg protein.

IL-8 assay

For the detection of IL-8 antigen, microtitre well plates coated with monoclonal antibody (mouse) to human IL-8/NAP-1 were incubated with 50 µl of undiluted vitreous humour together with 50 µl of anti-IL-8/NAP-1 conjugate for 2 hours at room temperature. After this, antibodies and test samples were removed and the plate was washed three times with phosphate buffered saline solution containing 1% Tween 20. In the following step, 100 µl of substrate (tetramethyl-benzidine) solution was added and the plate was incubated for 15 minutes at room temperature. The reaction was stopped by the addition of 100 µl of 1M phosphoric acid and the microtitre plate was stored for 1 hour at 2–8° C in the dark before measurement. Absorbance was read at 450 nm. The detection limit for IL-8/NAP-1 was 11 pg/ml (coefficient of variation, intra-assay 3.8%; interassay 9.9%).

TGF-1α assay

Measurement of TGF-1α concentrations in vitreous humour was performed in 96 well microwell strips coated with antibody to human TGF-1α . Volumes of 100 µl of vitreous and 100 µl of assay diluent were added to the microwells and incubated at 2–8°C overnight. After this, 100 µl of monoclonal TGF-1α antibody (mouse) was added to each well, and the plate was incubated at room temperature for 2 hours. In the following step, 100 µl of biotin conjugate (anti-mouse IgG) was added and the plate was incubated at room temperature for 45 minutes, after which the microwell strips were washed three times with wash buffer. With the next step, 100 µl of the substrate (tetramethyl-benzidine) solution was dispensed into each well and incubated for 15 minutes at room temperature. The reaction was stopped by 50 µl of 0.5 M sulphuric acid. Absorbance was read at 450 nm. The detection limit for the determination of TGF-1α was 1.9 pg/ml (coefficient of variation, intra-assay 1%; interassay 7.5%).

Polyamine analysis by HPLC
Apparatus and chromatographic conditions
The HPLC system consisted of two pumps model LC-10 A*i* (Shimadzu) coupled to a high pressure mixer, and a Rheodyne injection valve fitted with a 20 µl loop. Separation was achieved on a Nucleosil ODS column (250 × 4.6 mm, internal diameter 5 µm).

The elution procedure was performed with two mobile phases, A (water) and B (methanol), at a flow rate of 0.8 ml/min. It first consisted of an isocratic elution with 80% solvent B for 2 minutes, then of a linear gradient elution built up to 85 % B within 15 minutes. A spectrofluorimeter (RF-10A*xl*, Shimadzu) at excitation wavelength of 360 nm and emission wavelength of 510 nm was used for detection.

Reagents
SP free base, SPD free base, and PUT dihydrochloride (Sigma-Aldrich, Milan, Italy) were dissolved in water (10 µg/ml). The *o*-phthaldialdehyde (OPA) reagent solution (Sigma-Aldrich, Milan, Italy), containing OPA (1 mg/ml), Brij 35, methanol, potassium hydroxide, boric acid, and 2-mercaptoethanol as reducing agent, was diluted in methanol (1:10). The organic solvents used (LC grade) were purchased from Sigma-Aldrich (Milan, Italy).

Processing of vitreous samples
The collected vitreous fluid samples were centrifuged at 3000 rpm for 30 minutes to remove cellular elements. The clear supernatant fluid was mixed with 5% perchloric acid (1:1) to remove proteins and centrifuged at 1500 rpm for 10 minutes. To 30 µl of acidic extract, 50 µl of borate buffer (0.01M, pH = 9.0) and 30 µl of the diluted OPA reagent solution were added. The derivation mixture was shaken for 4 minutes and 50 µl was injected into the HPLC system.

Statistical analysis
The results were analysed by analysis of variance (F). p Values less than 0.05 were considered significant. Non-parametric
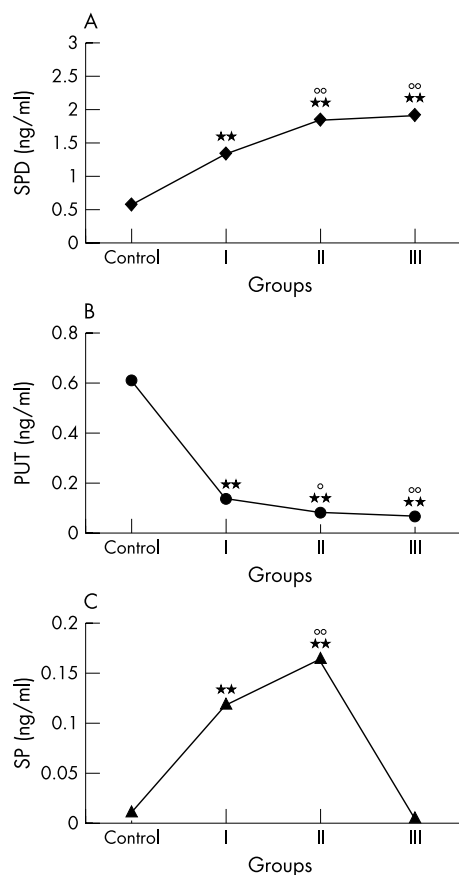
Figure 1 Spermidine (SPD) (A), putrescine (PUT) (B), and spermine (SP) (C) concentrations in the vitreous body from controls and patients affected by quiescent PDR (group I), active PDR (group II), and PVR (group III). Significant *p <0.05 and **p <0.01 by variance analysis compared with controls. Significant °p <0.05 and °°p <0.01 by variance analysis compared with group I.
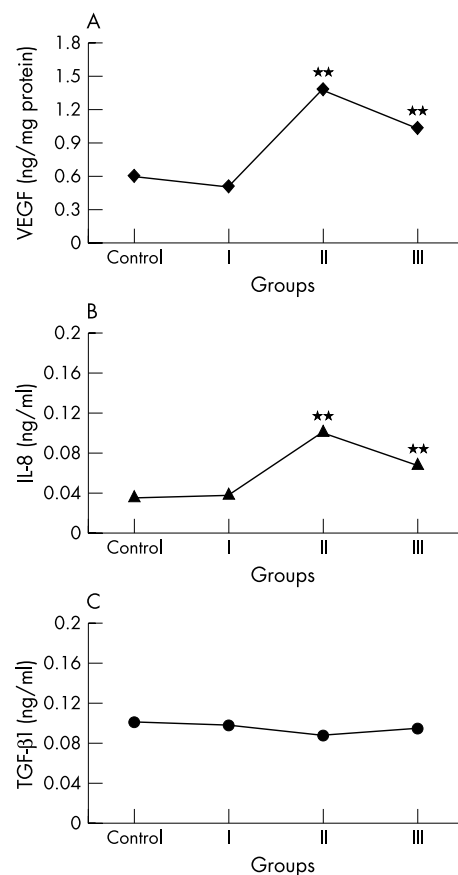
Figure 2 Vitreous concentrations of VEGF (A), IL-8 (B), and TGF-β1 (C) in controls and patients with proliferative retinopathy. Groups are designated as in Figure 1. Significant **p <0.01 by variance analysis compared with controls.

statistical analysis using Wilcoxon's test always gave similar results. Intra-assay and interassay variation coefficients were 5% and 10%, respectively, for all of the above methods.

Correlation between the intravitreous polyamine concentrations and the levels of VEGF, IL-8, and TGF-β1 were examined by Pearson correlation test. A level of p<0.05 was considered significant.

RESULTS

Polyamine levels in the vitreous body of the control subjects and patients affected by quiescent PDR (group I), active PDR (group II), and PVR (group III) are reported in Figure 1. Figure 1A shows that human vitreous contains appreciable amounts of spermidine, significantly more in all the test groups than in controls (p<0.01). Spermidine reached the highest values in patients suffering from active PDR and PVR, with a further significant difference (p<0.01) relative to quiescent PDR. Figure 1B shows that the vitreous putrescine concentration in basal conditions was slightly higher than that of spermidine and that it was significantly lower in all the proliferative diseases studied than in controls (p<0.01). The putrescine difference was more pronounced for active PDR (p<0.05) and PVR (p<0.01) than for quiescent PDR. Figure 1C shows that spermine was not detectable in the vitreous of control subjects. Its concentration was dramatically higher in patients with quiescent PDR and patients with active PDR (p<0.01), while no different in PVR relative to controls. Moreover, spermine levels in patients affected by active PDR were significantly higher than in the quiescent PDR group (p<0.01).

Figure 2 shows the variations of the mean values of VEGF (Fig 2A), IL-8 (Fig 2B), and TGF-β1 (Fig 2C) in groups I, II, and III, compared with controls. The VEGF concentration in the group of patients with quiescent PDR was no different from control, while the levels were markedly higher in patients affected by active PDR and PVR (p <0.01).The amount of IL-8 was significantly higher relative to controls in the active PDR and PVR groups (p <0.01), while TGF-β1 concentrations did not show significant variations from the controls in all the groups studied.

Table 1 reports Pearson correlation coefficients between vitreous polyamine content and VEGF, IL-8, and TGF-β1 levels in the group with quiescent PDR. No correlation could be

Table 1 Pearson correlation coefficients between vitreous polyamine content and VEGF, IL-8, and TGF β1 levels in group I

	Correlation coefficient for		
	VEGF	IL-8	TGF β1
Spermidine	r = −0.0175 p = 1	r = 0.2326 p = 1	r = −0.1576 p = 1
Putrescine	r = 0.0791 p = 1	r = −0.2563 p = 1	r = 0.1071 p = 1
Spermine	r = −0.0466 p = 1	r = 0.2716 p = 1	r = 5.4E-05 p = 1

Table 2 Pearson correlation coefficients between vitreous polyamine content and VEGF, IL-8, and TGF β1 levels in group II

	Correlation coefficient for		
	VEGF	IL-8	TGF β1
Spermidine	$r = 0.9574$ $p < 0.01$	$r = 0.9581$ $p < 0.01$	$r = -0.2356$ $p = 1$
Putrescine	$r = -0.9517$ $p < 0.01$	$r = -0.9637$ $p < 0.01$	$r = 0.1996$ $p = 1$
Spermine	$r = 0.9311$ $p < 0.01$	$r = 0.9599$ $p < 0.01$	$r = -0.1437$ $p = 1$

Table 3 Pearson correlation coefficients between vitreous polyamine content and VEGF, IL-8, and TGF β1 levels in group III

	Correlation coefficient for		
	VEGF	IL-8	TGF β1
Spermidine	$r = 0.9837$ $p < 0.01$	$r = 0.9481$ $p < 0.01$	$r = -0.4066$ $p = 1$
Putrescine	$r = -0.9899$ $p < 0.01$	$r = -0.9509$ $p < 0.01$	$r = 0.1313$ $p = 1$
Spermine	$r = -0.2012$ $p = 1$	$r = -0.1683$ $p = 1$	$r = 0.5891$ $p < 0.01$

observed, since neither the significantly higher levels of spermidine and spermine nor the reduced levels of putrescine were accompanied by differences in VEGF, IL-8. or TGF-β1 (p = 1).

Table 2 shows the good positive correlation between the levels of spermidine and spermine and VEGF and IL-8 in the vitreous of patients affected by active PDR (p<0.01). No correlation with the unchanged levels of TGF-β1 (p = 1) was found. A very significant inverse correlation was observed between the significantly reduced levels of putrescine and the increased concentrations of VEGF and IL-8 (p<0.01), while no correlation between putrescine decreases and TGF-β1 unvaried values occurred (p = 1).

Table 3 shows a high positive correlation between the higher spermidine levels and VEGF and IL-8 elevation (p<0.01) and no correlation with the unchanged concentration of TGF-β1(p = 1) in the PVR group. A very significant inverse correlation between putrescine and VEGF and IL-8 was observed (p<0.01), while no correlation was found with the unmodified levels of TGF-β1 (p = 1). The unmodified spermine levels did not correlate with the higher concentrations of VEGF and IL-8 (p = 1), while they were positively correlated with TGF-β1, similar to control values (p<0.01).

DISCUSSION

Results reported here are the first demonstration that the polyamines spermidine, spermine, and putrescine may have a physiological as well as pathophysiological role in the homeostasis of human vitreous. Measurable amounts of spermidine and putrescine were present in basal vitreous conditions and showed very significant variations in patients affected by the proliferative retinopathies studied by us. On the contrary, the spermine content was nearly zero in healthy vitreous and increased very significantly only in proliferative diabetic retinopathy. Spermidine concentrations were elevated

about threefold in quiescent PDR and about fourfold in active PDR and PVR, compared with controls, indicating that all the proliferative processes of retina, irrespective of aetiopathogenesis and disease stage, are mediated by this polyamine. Putrescine levels were very significantly lower in quiescent PDR relative to controls and in active PDR and PVR relative to quiescent PDR. Since putrescine is the physiological precursor of spermidine and spermine and is the decarboxylated product of L-ornithine by ornithine decarboxylase,[26] whose gene is activated in all the rapidly growing and dividing cells,[27 28] it is conceivable that its decrease and the parallel spermidine increase observed here in all the retinal proliferative disorders studied were caused by an excess of consumption of putrescine to produce spermidine. It is also well known that putrescine enhances the formation of decarboxylated S-adenosylmethionine (SAM) by stimulating S-adenosylmethionine decarboxylase (SAMDC) activity and thereby accelerating further conversion of putrescine into spermidine and spermine by the aminopropyltransferases.[29]

Data obtained by us are in agreement with the well established role of whole polyamines in proliferation and differentiation,[18] but they underline also a different behaviour of the three polyamines in relation to the proliferative retinal pathology. The higher vitreous spermidine levels and the lower putrescine concentrations in all the proliferative vitreoretinal disorders investigated indicate a significant but non-specific role of these two polyamines in proliferative processes of the retina, while the higher spermine level only in PDR, either quiescent or active, indicates a precise involvement of this polyamine in diabetes. Recently, it has been reported that polyamines as a whole cause a time and concentration dependent inhibitory effect on protein phosphatase activities of insulin secreting cells, which may contribute to the increase in phosphorylation state occurring after secretory stimulation.[30] But different roles for each polyamine in the metabolism and insulin production of pancreatic islet cells have also been described.

In diabetic rats a decrease of pancreas putrecine levels was observed, which were restored with L-arginine, indicating that this polyamine may be utilised in regenerating processes or for recovering the endocrine pancreatic function.[31] In vitro researches by Welsh and Sjoholm[32] showed differential effects exerted by the three polyamines on islet insulin release and on (pro)insulin biosynthesis. Depletion in the putrescine and spermidine content of islets did not affect insulin release in response to glucose, while the concomitant reduced levels of spermine decreased insulin m-RNA content. Furthermore, only spermine was able to stimulate (pro)insulin biosynthesis, since spermine inhibition reduced its percentage selectively.

These findings, together with the high islet spermine to spermidine ratio observed by Hougaard et al[33] in pancreatic cells, strongly suggest that spermine is the most important of these polyamines in maintenance of normal insulin biosynthesis and secretion. A selective and exclusive involvement of spermine in modulating insulin binding[34] and in proliferation and insulin production by normal and tumoral pancreatic α cells is also well established.[21 22 35] Moreover, spermine was shown to be a mediator of insulin pyruvate dehydrogenase phosphate phosphatase activation.[36] Such a role for spermine in the endocrine regulation of glucose metabolism accounts for the exclusive spermine increase in vitreous from PDR affected patients observed by us and for its relevance as a unique specific marker of diabetes induced retinal proliferation.

The selective increase of spermidine levels reported by Seghieri et al[37 38] in erythrocytes from diabetic patients affected by retinopathy may be explained by a diversified tissue distribution of polyamines, which accounts for the diversified content of polyamines in the periphery in respect to the site of their biosynthesis.

Our data regarding the two angiogenic factors, VEGF and IL-8, implicated in the development of intraocular

neovascularisation[2–8 10 11 14] and TGF-β1, which is known to have an angiostatic property in the eye,[1 18] are in agreement with literature. Their concentrations showed a diversified correlation with the polyamine content in the various disorders considered, indicating that in the balance between angiogenic factors, recently studied by Funatsu *et al* in diabetic retinopathy,[39] the role of polyamines, especially spermine, has to be taken in account. While VEGF and IL-8 increased in active PDR and PVR, irrespectively of aetiopathogenesis, polyamines displayed a more significant involvement, not only in retinal proliferative disorders, being able to discriminate conditions with different aetiologies. Therefore, no correlation was observed between the higher spermidine levels and the lower putrescine levels, on the one hand, and the unmodified levels of VEGF and IL-8 observed, on the other, in the quiescent PDR group. On the contrary, a positive correlation between higher spermidine and the augmented VEGF and IL-8 levels and a negative correlation between lower putrescine and VEGF and IL-8 concentrations were found in all the other groups with active vascular proliferation. TGF-β1 did not correlate with the modified levels of spermidine, spermines, and putrescine in quiescent or active PDR and with higher spermidine and lower putrescine observed in the other disorders. Our data account for a significant role of spermidine and putrescine as markers of all the proliferative diseases of retina, even in conditions of poor proliferation activity which are not characterised by VEGF and IL-8 modifications. The spermine increment, restricted to diabetic states, indicates that this polyamine is a unique and specific index of PDR, able to distinguish proliferative diabetic retinopathies, of either quiescent or active status, from other non-diabetic vitreoretinal disorders.

Further studies on mechanisms involving polyamine in ocular pathologies are required to better understand how this knowledge should be useful for targeting therapeutic molecules or for using polyamine itself to block proliferative processes.

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