Systemic Erythropoietin Concentration and its Correlation with Stage of Diabetic Retinopathy

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Abstract

Summary: Background: Erythropoietin (Epo) is one of systemic angiogenic factors, and its role in ocular angiogenesis and in diabetic retinopathy (DR) is not yet fully understood. Latest research data reveal possible correlation of higher EPO concentrations of erythropoietin in blood and in the eye, with more severe of stages of DR. The main aim of this work was to examine the possible influence of serum concentrations of erythropoietin on the development and stages of diabetic retinopathy in patients with diabetes mellitus type 2. Methods: The research involved 90 patients examined at University Eye Clinic in Clinical Center of Vojvodina in Novi Sad, Serbia. First group comprised of 60 patients with diabetes mellitus lasting 10 years or more, with diabetic retinopathy. Second, control group, consisted of 30 healthy individuals. In the first group of 60 diabetic patients, 30 of them had non-proliferative diabetic retinopathy (NPDR), and 30 had proliferative diabetic retinopathy (PDR). Laboratory EPO serum levels were determined, and they were correlated to the stage of DR. Concentration of EPO was assessed by ELISA method at the end of the study. Results: The highest average concentration of EPO in serum (9.95 mIU/ml) was determined in group of diabetics with PDR. The lowest average concentration of EPO in serum (6.90 mIU/ml) was found in control group. The average concentration of Epo in serum in group of diabetics with NPDR was 7.00 mIU/ml. EPO concentration in serum was elevated in group of PDR, and it was directly proportional to the level of clinical stadium of PDR, being significantly higher in moderate and severe subgroup of PDR comparing to control healthy subjects, NPDR and mild PDR (h=9.858, p=0.007). Conclusions: Significantly elevated serum concentration of EPO in advanced stages of DR, and positive correlation between EPO serum concentration and clinical stadium of PDR, suggest that erythropoietin presents one of the important growth factors from blood, which plays role in retinal ischemia and angiogenesis in diabetic retinopathy, especially in the proliferative stage of this disease.

Keywords: diabetic retinopathy; erythropoietin; glycated hemoglobin; non-proliferative diabetic retinopathy; proliferative diabetic retinopathy.

Introduction

Diabetes mellitus (DM) is a multi organic disease with a high incidence in the population, whose main cause is a carbohydrate metabolism disorder. One of the most common and at the same time one of the most difficult chronic complications of DM is diabetic retinopathy (DR). Diabetic retinopathy leads to changes in the small blood vessels of the eye, and DR is considered nowadays as one of the main causes of impaired visual function and blindness, with significant socioeconomic consequences due to the big, mainly working population which it affects (1).

Changed quality of blood vessels in DM and DR, leads to increased liquid permeability which manifests as bleeding, edema and exudates in the eye. Another key point in DM and DR pathogenesis is ischemia in the tissues, which through production of vasoproliferative, angiogenic factors leads to neovascularization- growth of pathological blood vessels in the eye. These vessels have poor wall quality, and they will furthermore lead to circulus vitious of new ischemia attacs and bleeding. Increased vascular permeability and pathologic neovascularisation are considered as two major vascular pathogenic

pathways for the development of diabetic retinopathy (2).
Development of DR has been studied for decades. It has been known that local angiogenic factors in the eye are dominant, but many older as well as many recent studies indicate the importance of systemic angiogenic growth factors, too (3,4,5). One of angiogenic factors is erythropoietin, which is excreted in organism, as a response to tissue hypoxia, with a role to increase erythropoiesis.

Growth factor is the substance that stimulates cell growth, proliferation and differentiation. Most frequently these factors work as cytokines and/or hormones, i.e. as signal transmitters between cells, and it is important to distinguish them from cytokines. Cytokines could or could not affect the proliferation, while the growth factors positively affect the cell division (6). The most important representatives of growth factors are: epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), insulin-like growth factor (IGF), erythropoietin (EPO), thrombopoietin (TPO), etc. (6,7).

Erythropoietin is a glycoprotein by its structure, and a hormone with the circulatory growth factor by its function (8). It is produced mainly in the kidneys, and only in small part in the liver (10%); main stimulus for its releasing is tissue hypoxia (9). It exhibits it's influences by binding to transmembrane erythropoietin receptors (EPOR), which primarily are found on the hematopoietic cells, but also can be found on the endothelial, myocardial, neural cells, and as well on the cells of liver, uterus and retina (10). Circulating erythropoietin primarily affects hematopoietic cells, and afterwards by stimulating angiogenesis, it is even considered as tumor-cells stimulator (11,12). There are theories about erythropoietic induction of neovascularization caused by inflammation or ischemia. This occurs by mobilizing the endothelial progenitor cells from the bone marrow, and thus increasing the number of cells in the circulation. From existing capillaries and postcapillary venules, new blood vessels are being formed by degradation of basal membrane of the blood vessel, migration of the endothelial cells and their mitosis, by forming bourgeons, lumens and vascular loops**.** All these steps represent angiogenesis that has to be distinguished from vasculogenesis (13). Vasculogenesis occurs by the differentiations of endothelial cells from angioblasts Angiogenesis can be physiological and pathological. Physiological angiogenesis presents the balance between proangiogenic and antiangiogenic factors in organism, and occurs in the reproductive cycle, pregnancy and wound healing (14, 15). While pathological occurs in neoplastic diseases leading to acceleration of disease progression, or it is the pathological mediator like in diabetic retinopathy (16, 17, 18).

Numerous new studies indicate the main role of angiogenic growth factors on the development of proliferative diabetic retinopathy, and VEGF is considered to be at the first place (19), followed by erythropoietin, IGF-1, PDGF, etc. Intraocular synthesis of proangiogenic factors is in counterbalance with production of antiangiogenic ones. It is considered that erythropoietin can have direct role in pathophysiology of diabetic retinopathy, however there are still contradictory opinions about whether the role is aggravating or protective. It has been proved that erythropoietin is significantly correlated with origin of proliferative diabetic retinopathy (20). In DM, retinal cells are trying to compensate hypoglycemia and hypoxia by manifestation of increased number of erythropoietin receptors on them. It

is considered by some authors this might be how they survive in unfavorable conditions like in DM, inducing increased binding of erythropoietin molecules (20, 21). In experimental rats with DM, intravitreal injection of Epo caused increased number of erythropoietin receptors (EpoR) in neurosensory retina, with protective effect against retinal neovascularization and degenerative changes of photoreceptors (22, 23).

In certain researches is found that the same Epo injection slows down retinal cells death and promotes the function of hematoretinal barrier, therefore Epo is being considered as one of new therapeutical options in the treatment of early DR and diabetic macular edema (24). However, there are different theories between authors about favorable influence of Epo on progression of non-proliferative and proliferative DR, some data show improvement of DR by blocking of production and effects of Epo in the eye (25).

Normal or low concentrations of erythropoietin are found in conditions of primary polycythemia, some erythropoietin-independent anemias, but also in kidney-derived anemia (26). The importance of reduced concentration of erythropoietin is clinically confirmed in early diabetic nephropathy, as it resulted in anemia which worsened diabetic retinopathy (27). Also, in curing anemia of renal origin, Epo given intravenously had positive effects on macular edema, had improved visual acuity in patients with DR, and also led to reduction of exudative maculopathy and proliferative changes of the disease (28, 29).

Material And Methods

Study group

This cross-sectional study included 90 examinees, over 50 years of age, 60 of them with verified diabetes mellitus (DM) and consequent ocular changes, and 30 of them as control subjects. Patient were selected from the population of Vojvodina - Northern Serbia region, and all of the participants undertook detailed eye examination at University Eye Clinic in Novi Sad, Clinical center of Vojvodina, in the period from 2009 to 2011. The study was conducted according to the principles of the Helsinki Declaration and approved by the signed consent of Ethics Committee of The Clinical Center of Vojvodina. Informed patient consent for participating in the study was obtained from all participants prior to inclusion in the study.

The first group consisted of 60 patients suffering from DM type 2 who were treated for 10 years or more with oral, insulin or combined therapy, and who were referred to the University eye clinic for complete eye examination, and evaluation of possible diabetic ocular fundus changes The second group of 30 patients served as control group of healthy subjects who have not been diagnosed with any systemic disease or ophthalmic disease, except for the senile cataract. The study did not include patients with an asymmetric finding on the eyes in terms of diabetic retinopathy, as well as patients with unregulated arterial hypertension, anemia, diabetic patients with severe kidney damage, and patients on recombinant erythropoietin therapy.

The following data were recorded: name, surname, age, gender, and duration of diabetes mellitus. The height of the intraocular pressure was measured by the applanation tonometer, where the reference

values ranged from 10 to 21 mmHg. Best corrected visual acuity was determined by the Snellen chart, and expressed in decimal values (Table 1).

In the group of patients with diabetes, all patients had DM for more than 10 years. The presence of diabetes retinopathy and the progression of changes were determined. Patients with DR were according to ocular fundus changes further classified according to the existing international classification into two main groups. As a non-proliferative or proliferative DR group, and further into one of three subgroups, depending on the progression of changes - mild, moderate and severe (Table 2.) (30).

Laboratory analysis was performed at the Center for Laboratory Medicine of the Clinical Center of Vojvodina. Collected peripheral blood samples were labeled according to the standard protocols, transported and stored until the time of the laboratory analysis.

Special blood samples (5ml) were separated for the ELISA human erythropoietin immunoassay (Enzyme-Linked Immunosorbent Assay). They were frozen and stored at a temperature of -20 Celsius degrees within the hour of sampling and centrifugation, until the analysis took place. Epo was measured by the commercially available human Epo ELISA commercial kit by the manufacturer R&D Systems Quantikine IVD for the quantitative determination of human serum or plasma erythropoietin. According to the manufacturer, medicines should not affect the accuracy of the results. The sensitivity of this test is very high, less than 0.6 mIU/ml. In addition, from blood samples of examinees, there were implemented laboratory measurements of concentration of the glycated hemoglobin (HgA1c) in serum.

Statistical analysis

All the data were statistically analyzed using statistical software Statistical Package for Social Sciences - SPSS 17 and were presented graphically and in tabular presentation. Descriptive statistics, including median, arithmetic mean, and standard deviation (SD) were used to describe the studied parameters. Differences in distributions of individual parameters between study groups were analyzed using the parametric Student's t-test, or the nonparametric Mann-Whitney test in case a distribution showed a significant deviation, while Chi-square test was used for categorical data. Value of $p<0.05$ was considered statistically significant. Multiple regression analysis was used to form a model for predicting disease indicators.

Results

Table 1 shows basic characteristics of the examinees, and ophthalmic clinical and laboratory data of the parameters that were analyzed in the study. Out of total number of examinees, there were 48 women and 41 men, the average age was 62.65 years (in the range from 50 to 81 years).

In all patients in the non-proliferative and proliferative DR group, existence of diabetes mellitus type 2 was proven, as well as the presence and type of diabetic retinopathy, according to the International Clinical Diabetic Retinopathy Disease Severity Scale – ICDRDSS (Table 2).

The duration of diabetes mellitus in the NPDR group was 17.1 years (16±7.36 years), and in the PDR group it was 18.13 (16± 7.76 years). There was no statistically significant difference in the duration of diabetes mellitus between the observed groups (p=0.589).

The maximum duration of DM in the NPDR group was found in subgroups with an advanced form of illness18±2.83 years, followed by subgroups of moderate and mild form of NPDR. In the PDR group, the longest duration of DM was recorded in a subgroup of patients with a mild form of disease of 20.5±6.63, followed by subgroups of moderate and severe PDR.

A statistically significant difference in the duration of DM between six analyzed subgroups of patients in relation to the clinical stage of DR, was not determined, p=0.707.

The value of glycated hemoglobin (HgA1c) was measured in all patients involved in the study. The average concentration of glycated hemoglobin in the blood of all patients is 7.15%±1.68%. A significant difference in the average level of glycated hemoglobin between observed main patient groups was determined, p=0.001. The highest average HgA1c value was observed in the group of patients with proliferative diabetic retinopathy (8.14%).

In the group of subjects with NPDR concentration, HgA1c was 7.96%, and in the control group 5.43%. A statistically significant difference was found between the average value of HgA1c in the control group and NPDR, p=0.001, and between the control and PDR, p=0.001. While a statistically significant difference was not found between the average values of HgA1c in the blood of patients with NPDR and PDR, p=0.599.

By examination of the fundus of the eye by direct or indirect ophthalmoscopy and biomicroscopy, the control group of the subjects excluded the existence of pathological changes in the retina, except changes in arterial hypertension, which were found in 67% of persons. The study did not include patients who were previously treated with laser photocoagulation, or who had any other eye treatment.

A statistically significant difference in mean visual acuity (BCVA) values was found between observed groups for right and left eye, p = 0.001, and for BCVA on average for both eyes together p = 0.001. The values of the best corrected visual acuity BCVA were significantly lower in the PDR group (mean 0.44, median 0.45) compared to values in patients with NPDR (mean 0.78, median 0.90) and patients from the control group (mean 0.97, median 1.0).

The mean intraocular pressure (IOP) value, measured by applanation tonometer, in this study in the control group of the subjects was 14.62 mmHg, in the group of NPDR 14.84 mmHg, and in group PDR 13.92 mmHg.

A statistically significant difference was not found in the average IOP values between the observed groups for the right p=0.347 and left eye p=0.467, and for the IOP average for both eyes together p=0.383.

The mean concentration of erythropoietin (Epo) in our analyzed blood sample was 8.48 mIU/ml. The highest average Epo serum concentration was found in the PDR group of patients, 9.95mIU/ml, then 7.0 mIU/ml in the NPDR group. The lowest Epo concentration was found in the control group of patients, 6.9mIU/ml (Graph 1). The Kruskal-Wallis test did not show a statistically significant difference in the Epo concentration between the observed groups, p=0.123. The Mann-Whitney test did not show a statistically significant difference between the patients of the main observed groups - between the control group and the NPDR (p=0.805), the control group and the PDR (p=0.087), as well as among the subjects in NPDR and PDR (p=0.071).

The mean Epo concentration in the NPDR group was 7.0mIU/ml, the highest concentration was recorded in the moderate NPDR group, while the lowest was found in the subgroup of patients with advanced NPDR. The Kruskal-Wallis analysis did not show significant differences in the erythropoietin level in the observed patient subgroups, p=0.781.

Further, with the Mann-Whitney test, a statistically significant difference was not found in the mean value of erythropoietin by comparison with the observed NPDR: mild and moderate p=0.519, mild and advanced p=0.933, moderate and advanced p=0.686.

The average Epo concentration in the PDR patients group was 9.95 mUI/l. Using the Kruskal-Wallis test, a statistically significant difference in Epo values in PDR patients was found in relation to the stage of clinical picture, p=0.007. By using the Mann-Whitney test, statistically significantly higher concentrations of Epo in the blood of patients in subgroups were moderate and advanced in PDR, relative to the mild, p=0.001. Whereas, there is no statistically significant difference in the serum Epo concentration between the two most severe forms of PDR, p=1,000.

Statistically significantly higher concentrations of Epo in the blood were found in the subgroup of advanced PDR compared to: control group (p=0.004), but also each subgroup of NPDR, compared to mild NPDR (p=0.035), moderate NPDR (p=0.039), and advanced NPDR (p=0.022).

Discussion

A common complication of diabetes mellitus is diabetic retinopathy which, in addition to cataract, glaucoma and senile macular degeneration, is one of the leading causes of visual impairment and blindness in the world (31, 32). Although investigated in numerous studies for decades, pathophysiological mechanism of the development of diabetic retinopathy has not been completely clarified. The eye, although a small organ, is by its metabolic properties most similar to the brain, and from the general circulation it is separated by blood-ocular barrier. In the vitreous body, an increased concentration of vasoproliferative, angiogenic substances was found in patients with diabetes mellitus. However, it is not entirely clear whether this is a consequence of local production due to the presence of eye ischemia and/or the systemic impact of these factors on the eye and its metabolic processes. In explanation of the onset of diabetic retinopathy nowadays, increasing attention is also attributed to

systemic angiogenic factors, and their possible ability to promote and increase diabetic vascular changes and occurring of neovascularization of the retina (33, 34).

Apart from vascular endothelial factor, as a main factor, erythropoietin is one of the most prominent other representatives of systemic angiogenic factors, and its role in the occurrence of neovascularization in the eye has not yet been fully determined and understood. It plays a role as a growth factor in normal processes of erythropoiesis, by stimulating erythrocytes, their proliferation and differentiation, and the prevention of the apoptotic death of erythroid precursors with erythropoietin receptors (9,13). However, in numerous studies, the presence of erythropoietin receptors has been confirmed in various tissues such as kidney, liver, uterus, retina (10, 35). In some studies, in diabetic patients was found significantly higher erythropoietin concentration in the vitreous body, along with VEGF receptors, which is explained by local production of erythropoietin in tissue hypoxia in the eye, in conditions of elevated blood sugar levels, in the presence of other cytokines in DR as well (19, 36).

In various studies has been shown that erythropoietin affects endothelial cells the same as vascular endothelial growth factor (VEGF), and for instance, in renal anemia diabetic patients treated with human recombinant erythropoietin (rhEpo), rhEpo exibits the same effect promoting increased intraocular angiogenesis and consequently impairment of DR (37, 38). A statistically significant worsening of DR in the group of patients using rhEpo compared to the non-receiving group was also noted, and a direct proportionality of the serum erythropoietin concentration and the deterioration of diabetic eye disease was determined (39, 40). Certain number of authors claim that the recombinant therapeutic Epo application allows better delivery of oxygen to ocular tissues, and reduction of diabetic changes after its local or systemic administration (41, 42).

The role of Epo is still interesting for many researches nowadays. It is still unclear whether erythropoietin in DR has a protective or aggravating role, comparing results of various studies. Certainly, an increase in the number of erythropoietin receptors on retina cells in DR has been confirmed, which is considered as a compensatory response to tissue hypoxia and hyperglycemia during DM. In these conditions, increased production and binding of Epo to erythropoietin receptors is the mechanism of survival of retinal nerve cells. It has been shown that erythropoietin inserted exogenously contributes to the preservation of the external blood-retinal barrier, acting on processes at the level of the retinal pigment epithelium. In addition, intravitreal injections of rhEpo have led to the inhibition of VEGF and stabilization of the function of the blood-retinal barrier, and also to a short-term positive effect on the chronic macular edema that has until then been practically refractory to any potential therapy (43).

During our study, we found that serum erythropoietin concentrations were increased directly proportional to the severity of the clinical stage of proliferative diabetic retinopathy. The highest average value of erythropoietin in serum was found in the group of subjects with the most severe forms of proliferative diabetic retinopathy (9.95 mIU/ml). The lowest average concentration of EPO in serum (6.90 mIU/ml) was found in control group. The average concentration of Epo in serum in group of diabetics with NPDR was 7.00 mIU/ml. There was no statistically significant difference in average Epo concentration within

main groups of healthy control subjects, and groups of patients with NPDR and PDR. EPO concentration in serum was markedly elevated in group of PDR, and it was directly proportional to the level of clinical stadium of PDR, being significantly higher in moderate and severe subgroup of PDR comparing to controls, NPDR and mild PDR (h=9.858, p=0.007).

These data correspond to recent publications, where most elevated serum Epo concentration was determined along with more advanced stages of diabetic retinopathy, like in work of Semeraro, Mustafa and Reida (44, 45, 46). Moustafa et al compared vitreous and serum levels of Epo, as well as their correspondence with certain Epo gene, and their results are in accordance with our study report, where bigger concentrations of Epo in the eye and in serum were correlating with PDR forms of DR.

Limiting factor of our study is the relatively small sample of 90 participants, and the lack of information of simultaneous intravitreal erythropoietin concentrations, correlated to clinical stage of DR.

Further, more comprehensive studies should include also intravitreal along with same time serum Epo concentration measurements, in order to obtain more precise role of Epo in different stages of ischemic diseases, including diabetes mellitus, in order to find new possibilities for better quality treatment and a better quality of life for patients with diabetic retinopathy.

Conclusion

In our study was determined a significantly higher concentration of erythropoietin in serum in severe forms of diabetic retinopathy, therefore we can conclude that erythropoietin also presents one of the important growth factors that, together with other angiogenic factors, as vascular endothelial factor, participates in ischemic and angiogenic processes in diabetic retinopathy, especially at its proliferative stage.

Further studies are needed, involving larger number of subjects in longer follow up period, to determine more precisely the effect of intravitreal and systemic erythropoietin and its concentration in diabetic retinopathy, in order to find new possibilities for better treatment and better quality of life of patients with diabetic retinopathy.

List Of Abbreviations

Epo- erythropoietin

DR- diabetic retinopathy

DM- diabetes mellitus

VEGF- vascular endothelial growth factor

NPDR- nonproliferative diabetic retinopathy

PDR- proliferative diabetic retinopathy

HgA1c- glycated hemoglobin

BCVA- best corrected visual acuity

IOP- intraocular pressure

RE- right eye

LE- left eye

ELISA- Enzyme-Linked Immunosorbent Assay

rhEPO- human recombinant erythropoietin

Declarations

Ethics approval and consent to participate

Ethics approval was obtained in the written and officially signed paper form by the authorities of Ethical Committee of Clinical Center of Vojvodina, Novi Sad, Serbia, prior to starting of collecting data and patients for the study. Original document from archive is available by corresponding author on request. All patients signed informed consent to participate.

Consent for publication

All authors have read through the paper, and agreed upon consent for publication of this paper.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author, and available in supplement, as excel sheet file.

Competing interests

All authors confirm there is no competing interests, and no financial disclosure.

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Authors' contributions

SD conceived the idea for the study. SD, SJ, AM, and NB contributed to the design of the research. All authors were involved in data collection. DG, SB and AM analyzed the data. SD coordinated funding for the project. All authors edited and approved the final version of the manuscript.

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Tables

Table 1. Characteristics of patients in main groups in relation to analyzed parameters.

Subjects		Diagnosed NPDR	Diagnosed PDR	Control group
Total number	90 (100%)	30 (33.3)	30 (33.3)	30 (33.3)
Male (%)	46.7	51.7	46.7	43.3
Female (%)	53.3	48.3	53.3	56.7
Age (mean ± SD, years)	62.0±7.75	61.0±7.13	64.67±8.18	61.0±7.72
Duration of DM	-	16.0±7.36	16.0±7.67	-
Mild	-	13.50±7.93	20.5±6.63	-
Moderate	-	16.0±7.55	18.5±6.29	-
Severe	-	18.0±2.83	13.5±9.57	-
HgA1c (%)		7.87±1.15	8.03±1.46	5.27±0.64
BCVA (RE)		0.9±0.26	0.45±0.36	1.0±0.05
BCVA (LE)		0.8±0.26	0.35±0.39	1.0±0.18
BCVA (average for both eyes)		0.9±0.23	0.45±0.32	1.0±0.1
IOP (RE)		16.0±2.64	13.0±3.27	14.0±2.8
IOP (LE)		16.0±2.37	14.0±2.52	14.5±2.95
IOP (mmhg) (average for both eyes)		16.0±2.44	13.0±2.73	14.25±2.83
EPO (mIU/ml)		6.2±3.92	8.0±7.21	6.63±3.33
Mild	-	5.8±4.54	4.6±2.05	-

Moderate	-	6.5±3.59	10.6±10.88	-
Severe	-	6.1±0.57	9.6±3.8	-

Legend: *DR- diabetic retinopathy, NPDR - non-proliferative diabetic retinopathy, PDR – proliferative diabetic retinopathy, DM – diabetes mellitus, HgA1c - glycolized hemoglobin, BCVA – best corrected visual acuity, RE – right eye, LE – left eye, IOP – intraocular pressure, EPO – erythropoietin.

Table 2. International Clinical Diabetic Retinopathy Disease Severity Scale, American Academy of Ophtalmology.

Proposed Disease Severity Level		Finding Observable upon Dilated Ophtalmoscopy
DM, without DR		No abnormalities
Non-proliferative DR	Mild	Microaneurysms only
	Moderate	Microaneurysms, exudate, venous beading, IRMA
	Severe	More than 20 intraretinal hemorrhages in each of 4 quadrants, definite venous beading in 2+ quadrants, prominent IRMA and 1+ quadrant; and no signs of proliferative retinopathy (*4:2:1 rule)
Proliferative DR	Mild	Neovascularization Vitreous/preretinal hemorrhage
	Moderate	
	Severe - High-risk - Advanced	

Table 2. International Clinical Diabetic Retinopathy Disease Severity Scale, American Academy of Ophtalmology.

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