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Original Article

CORRELATION BETWEEN SERUM MELATONIN LEVEL AND OTHER INDICATORS WITH STAGES OF DIABETIC RETINOPATHY

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Abstract

Purpose: *Diabetic retinopathy is considered the ghost cause of vision deterioration; it is attributed to elevated oxidative stress. Antioxidants, like melatonin, were found to be affected in diabetic retinopathy. In the present paper, we aim to explore melatonin levels in various stages of diabetic retinopathy.* ***Method:*** *Patients with diabetic retinopathy were recruited and divided into 3 groups (non-proliferative diabetic retinopathy (NPDR), proliferative diabetic retinopathy (PDR), and advanced diabetic eye disease). One control group was recruited (diabetics without diabetic retinopathy). Each participant was subjected to full ophthalmological examination, internal medicine evaluation, fluorescein angiography F/A, as well as optical coherence tomography (OCT) to classify the stage of diabetic retinopathy. The laboratory evaluation of melatonin, bilirubin, aspartate transferase AST, alanine transferase ALT, fasting plasma glucose FBG, hemoglobin A1c HA1c, and lipid profile were done. Data were collected and analyzed.* ***Results:*** *Each group includes 60 participants. We found no different sex distribution in the four groups as (P value =0.77), age in the four groups was as follows 58.35±8.56, 55.77±14.43, 59.3±10.73, 60.98± 7.60 years with (P =0.06). For diabetes-associated variables like duration, fasting glucose levels, HA1c, and medications (insulin or oral hypoglycemic), we found this result; increased duration of diabetes with raised levels of FBG, HA1C, and insulin treatment were linked with severe diabetic retinopathy affection (P value <0.0001). When assessing the plasma melatonin levels, the mean melatonin levels were decreased significantly with increased severity of diabetic retinopathy (86.04 ± 2.71, 73.05 ± 6.91, 25.82 ± 2.89, 24.28 ± 2.37 pg/ml) in the studied groups NDR, NPDR, PDR and advanced diabetic retinopathy with (P-value < 0.0001). Decreased melatonin could be a risk factor for diabetic retinopathy. As regards total bilirubin, its mean values decreased significantly with increasing severity of DR (P-value < 0.0001).* ***Conclusion:*** *There is a relation between the levels of serum melatonin and the severity of diabetic retinopathy.*

Keywords: *NPDR, PDR, Melatonin, HA1c, Bilirubin.*

1. Introduction

Diabetic retinopathy is considered the ghost cause of vision deterioration worldwide [1]. It is estimated that millions of diabetics are blind due to diabetic retinopathy [2]. The elevated glucose levels in diabetics with increased oxidative stress lead to

microangiopathy of retinal vasculature with endothelial cell loss, basement membrane thickening, and neovascularization [3]. Many classifications are applied to diabetic retinopathy. Retinal imaging by F/A and OCT are essential for the detection of the degree of retinal affection. The degree of affection ranges from mild NPDR affection to high-risk PDR and advanced diabetic eye disease with irreversible damage and irreversible vision loss [4]. Many studies postulated that oxidative stress resulting from high glucose levels and other oxidative factors have a major impact on the pathological changes of diabetic retinopathy. One of these antioxidants is melatonin [5]. Melatonin, which is N-acetyl-5-methoxytryptamine, can be synthesized from tryptophan (an essential amino acid) and produced from (the brain) pineal gland, (eye) retina and lacrimal gland, digestive system, and other organs [6]. Melatonin has very important functions in regulating the circadian rhythm, cardiovascular function, regulating the immune system, vague retinal functions, and pancreatic function. It is widely known for its antioxidant functions and neuroprotective effects [7]. Bilirubin was found to have some antioxidant properties. Its decrease can worsen oxidative stress, leading to the worsening of diabetic retinopathy. Many studies correlated the decrease in total bilirubin with the increased severity of diabetic retinopathy [8]. Many studies showed that there is a strong relation between diabetic retinopathy and serum melatonin levels [9]. This paper aims to find the correlation between blood melatonin and the different stages of diabetic retinopathy.

2. Patients and Methods

This study was done in the Ophthalmology Department, Biochemistry Department, Internal Medicine Department, and Clinical Pathology Department at Sohag University, from April 2021 to April 2022.

2.1. *Ethical consideration*

This study was validated by Sohag Faculty of Medicine Ethical Board with IBR number: Soh- med- 21 -02 – 34. The study was done under the Helsinki declaration principles. An informed written comprehensive consent was taken from each patient.

2.2. *Study population*

In this case-control study, patients with diabetic retinopathy were recruited and divided into 3 groups (NPDR, PDR, and advanced diabetic eye disease). One control group was recruited (diabetics without diabetic retinopathy). The patients were recruited randomly from our retina clinic, and the control group patients were recruited from our general ophthalmology outpatient clinic. *Inclusion criteria*: Patients with diabetic retinopathy. *Exclusion criteria:* Media opacity interfering with F/A imaging, hepatic patients, those under 18 years old, and patients with sleep disturbance or psychological problem which affects the level of melatonin, as well as patients with Melatonin supplementation in the last month. Age, sex, duration of diabetes, oral hypoglycemic therapy, insulin, smoking, hypertension history, and body mass index were collected from each participant. Eligible participants underwent detailed eye examination, best corrected visual acuity measurement by the Snellen chart (auto chart projector CP 670; Nidek Co., Ltd, Japan), measurement of intraocular pressure by the Goldmann applanation tonometer (AT900, Haag-Streit, Koeniz, Switzerland); undilated and dilated slit-lamp biomicroscopy examination (Photo-Slit Lamp BX 900; Haag-Streit, Koeniz, Switzerland) to evaluate the anterior and posterior segments. Diabetic patients had fundus fluorescein angiography (FFA) with Spectralis™ and spectral domain optical coherence tomography (SD-OCT) of the macula (Heidelberg Engineering,

Heidelberg, Germany) according to the preferred guidelines. Diabetics were evaluated according to the American Diabetes Association [11].

2.3. *Laboratory investigations*

Eight ml of fasting venous blood was taken from each participant at 8:00 Am. under aseptic conditions. The following investigations were performed: ***)** Biochemical assays for fasting blood glucose, total bilirubin, alanine transferase (ALT), aspartate transferase (AST), high-density lipoprotein cholesterol (HDL- cholesterol), Triglycerides (TG), total cholesterol, as cholesterol (LDL-cholesterol) were evaluated using the Cobas c311 Chemistry Analyzer System (Roche Diagnostics GmbH, Indianapolis, IN, USA). ***)** HbA1C (%) was determined by a D-10 HPLC autoanalyzer (Bio-Rad Laboratories, Hercules, California, the United States of America).

2.4. *Detection of plasma melatonin*

The evening blood sample was collected to avoid fluctuations [12]. EDTA tubes must be centrifuged for 15 min at 1000×g at 2 - 8℃ around 30 min of collection. The plasma supernatants were collected, distributed into aliquots, and packed at -80°C till use. Measuring the plasma concentration of human melatonin was conducted by the enzyme-linked immunosorbent assay (ELISA) Kit (Elabscience Biotechnology Co., Ltd, 14780 Memorial Drive, Suite 216, Houston, Texas 77079, USA, CATALOG #: E-EL-H2016), following the guidelines. Ideal standard curves were done by repeated dilution of the ideal standards by the kit. Fifty µl of calibrators and patient samples were added to the wells, and 50 µl of detection antibody solution was added to every well. The plate was put in the incubator for 45 minutes at a temperature of 37°C. Following complete good washing, 100 µl of Horseradish Peroxidase (HRP) working solution was put in every well and put in the incubator for 30 minutes at a temperature of 37 °C. After good washing, 90 µl of substrate solution was added. The well was put in the incubator for 15 min at a temperature of 37 °C with good protection from light. The reaction was ended using 50 µl of sulphuric acid. Color change from blue to yellow was evaluated at 450 nm by a Thermo Fisher Scientific Multiskan EX Microplate Reader (Thermo Fisher Scientific Oy, FI-01621 Vantaa, Finland). The samples' melatonin concentration (pg/mL) was detected by a comparison of the O.D. of the samples to the standard curve.

2.5. *Statistical analysis*

Data were analyzed using STATA v. 14.2 (STATA version 14.2 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.). Quantitative data were demonstrated as range, median, standard deviation, and mean. ANOVA with Bonferroni post-hoc test was used for comparing the means of four groups. Qualitative data were demonstrated as numbers and percentages and compared by the Chi-square test. The receiver operating characteristic (ROC) curve was indicated to evaluate the efficacy of related variables. The Pearson coefficient was indicated to find how different variables correlated. $P < 0.05$ was the significant value. Graphs were produced by the STATA program.

3. Results

Using the Shapiro–Wilk test, the collected data were distributed normally. In this study, four included groups were diabetics with no diabetic retinopathy NDR, diabetics with NPDR, diabetics with PDR, and diabetics with advanced diabetic retinopathy. Each group included 60 participants. We found no different sex distribution in the four groups as (*P value* =0.77). The age in the four groups

was as follows 58.35±8.56, 55.77±14.43, 59.3±10.73, 60.98±7.60 years with (*P value* =0.06). For diabetes-related variables, like duration, fasting blood glucose levels, HA1c, and medications (insulin or oral hypoglycemic), the NPDR group had a significantly lower duration of DM than the other three groups. Regarding other laboratory findings, including Triglycerides, LDL, cholesterol, and HDL, their mean values significantly decreased with increased severity of DR (P-value < 0.0001). As regards total bilirubin, its mean values significantly decreased with increased severity of DR (P-value < 0.0001). ALT's mean values decreased none significantly with increased severity of DR with a p-value of 0.18. As regard AST, its mean values significantly decreased with increased severity of DR with a p value of 0.01. As regard melatonin, it was found that the decrease in melatonin values was associated with more severe diabetic retinopathy (*P* value <0.0001). Regarding cataract, glaucoma, and AMD, the P value was (0.02, 0.25, 0.96), respectively, with low significant values. The macular edema risk was increased with the severity of diabetic retinopathy (P value<0.0001). The characters, as well as the laboratory and clinical data of the study groups, can be found in tab. (1).

Table 1: showing the characters, as well as laboratory and clinical data of the study groups

Variable	NDR N=60	NPDR N=60	PDR N=60	Advanced DR N=60	P value
Age in years • Mean ±SD • Median (range)	 58.35±8.56 56 (42:78)	 55.77±14.43 57.5 (25:85)	 59.3±10.73 59.5 (35:82)	 60.98±7.60 60 (45:82)	0.06
Gender • Females • Males	 29 (48.33%) 31 (51.67%)	 29 (48.33%) 31 (51.67%)	 34 (56.67%) 26 (43.33%)	 31 (51.67%) 29 (48.33%)	0.77
Duration of DM • Mean ±SD • Median (range)	 2.17±1.18 2 (1:5)	 12.73±3.48 13.5 (5:20)	 18.23±3.36 18 (12:25)	 15.95±3.78 16 (8:25)	<0.0001
P value1<0.0001, P value2<0.0001, P value3<0.0001, P value4<0.0001, P value5<0.0001, P value6=0.001					
FBG (mg/dl) • Mean ±SD • Median (range)	 113.43±24.14 109.5 (78:220)	 176.1±25.05 177.5 (139:250)	 205.37±19.45 201 (147:254)	 231.5±20.74 237.5 (190:262)	<0.0001
P value1<0.0001, P value2<0.0001, P value3<0.0001, P value4<0.0001, P value5<0.0001, P value6<0.0001					
HA1c (%) • Mean ±SD • Median (range)	 6.43±0.83 6.35 (4.9:8)	 7.91±0.74 7.85 (6.7:9.7)	 10.81±0.45 10.8 (9.9:12.5)	 10.78±0.99 10.9 (7:12.5)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4<0.0001, P value 5<0.0001, P value 6=1.00					
ALT (U/I) • Mean ±SD • Median (range)	 24.93±2.34 25 (20:30)	 24.85±3.61 26 (18:30)	 25.72±1.67 26 (23:28)	 24.9±1.92 25 (21:28)	0.18
AST (U/I) • Mean ±SD • Median (range)	 22.31±4.20 23 (2:28)	 24.38±3.07 26 (18:28)	 23.43±3.52 23 (16:28)	 22.83±3.05 23 (16:28)	0.01
P value 1=0.008, P value 2=0.49, P value 3=1.00, P value 4=0.83, P value 5=0.10, P value 6=1.00					
T bilirubin (mmol/L) • Mean ±SD • Median (range)	 19.09±0.51 19.2 (18:19.8)	 15.93±1.23 16.1 (13.2:18)	 9.00±0.45 8.95 (8:10)	 8.75±0.45 8.8 (7.9:9.8)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4<0.0001, P value 5<0.0001, P value 6=0.39					
Cholesterol (mg/dl) • Mean ±SD • Median (range)	 183.32±35.30 184.5 (100:250)	 225.43±16.90 220 (200:255)	 244.42±16.25 243 (214:265)	 250.73±15.05 255.5 (212:275)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4<0.0001, P value 5<0.0001, P value 6=0.69					

Triglycerides (mg/dl) • Mean ±SD • Median (range)	173.07±31.16 183.5 (115:230)	164.7±22.39 163.5 (102:198)	181.4±14.51 188 (140:199)	184.42±15.14 188 (142:220)	<0.0001
P value 1=0.22, P value 2=0.23, P value 3=0.03, P value 4<0.0001, P value 5<0.0001, P value 6=1.00					
HDL (mg/dl) • Mean ±SD • Median (range)	51.13±6.30 52 (32:59)	41.93±7.22 39.5 (24:58)	37±3.09 37 (29:42)	36.18±2.84 36 (28:41)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4<0.0001 P value 5<0.0001, P value 6=1.00					
LDL (mg/dl) • Mean ±SD • Median (range)	131.37±20.82 130 (100:180)	128.87±14.04 124.5 (103:169)	138.92±6.55 140 (120:156)	139.45±8.60 140 (120:163)	<0.0001
P value 1=1.00, P value 2=0.02, P value 3=0.008, P value 4<0.0001, P value 5<0.0001, P value 6=1.00					
Melatonin (pg/ml) • Mean ±SD • Median (range)	86.04±2.71 86.55 (78.8:90.1)	73.05±6.91 73 (59.74:84.45)	25.82±2.89 25.89 (19.05:31.41)	24.28±2.37 24.33 (18:30)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4<0.0001, P value 5<0.0001, P value 6=0.26					
Cataract • Negative • Positive	48 (80.00%) 12 (20.00%)	36 (60.00%) 24 (40.00%)	50 (83.33%) 10 (16.67%)	43 (71.67%) 17 (28.33%)	0.02
P value 1=0.02, P value 2=0.64, P value 3=0.29, P value 4=0.005, P value 5=0.18, P value 6=0.13					
Glaucoma • Negative • Positive	54 (90.00%) 6 (10.00%)	48 (80.00%) 12 (20.00%)	54 (90.00%) 6 (10.00%)	49 (81.67%) 11 (18.33%)	0.25
AMD • Negative • Positive	53 (88.33%) 7 (11.67%)	52 (86.67%) 8 (13.33%)	52 (86.67%) 8 (13.33%)	51 (95.00%) 9 (15.00%)	0.96
Macular oedema • Negative • Positive	60 (100%) 0	42 (70.00%) 18 (30.00%)	41 (68.33%) 19 (31.67%)	42 (70.00%) 18 (30.00%)	<0.0001
P value 1<0.0001, P value 2<0.0001, P value 3<0.0001, P value 4=0.84, P value 5=1.00, P value 6=0.84					
Hypertension • Negative • Positive	51 (85.00%) 9 (15.00%)	40 (66.67%) 20 (33.33%)	33 (55.00%) 27 (45.00%)	35 (58.33%) 25 (41.67%)	0.002
P value 1=0.02, P value 2<0.0001, P value 3=0.001, P value 4=0.19, P value 5=0.35, P value 6=0.71					
Medication • Insulin • Oral hypoglycemia	13 (21.67%) 47 (78.33%)	24 (40.00%) 36 (60.00%)	40 (66.67%) 20 (33.33%)	52 (86.67%) 8 (13.33%)	<0.0001
P value 1=0.03, P value 2<0.0001, P value 3<0.0001, P value 4=0.003, P value 5<0.0001, P value 6=0.01					

P-value *compared the four groups.* ***P1*** *compared* ***NDR*** *and* ***NPDR***, ***P2*** *compared* ***NDR*** *and* ***PDR***, ***P3*** *compared* ***NDR*** *and advanced* ***DR***, ***P4*** *compared NPDR and PDR, P5 compared NPDR and* ***advanced DR***, *and* ***P6*** *compared* ***PDR*** *and* ***advanced DR***. ***ANOVA*** *with Bonfrrroni post hoc was used in p to compare the means of four groups, and the Chi-square test was utilized for p1, p2, p3, p4, p5, and p6.*

3.1. *Plasma melatonin concentration and severity of diabetic retinopathy*

When assessing the plasma melatonin levels, the mean melatonin levels were decreased significantly with increased severity of diabetic retinopathy (86.04 ± 2.71, 73.05 ± 6.91, 25.82 ± 2.89, 24.28 ± 2.37 pg/ml) in the studied groups NDR, NPDR, PDR, and advanced diabetic retinopathy with (P-value < 0.0001), fig. (1) & and tab. (1) show this relation.

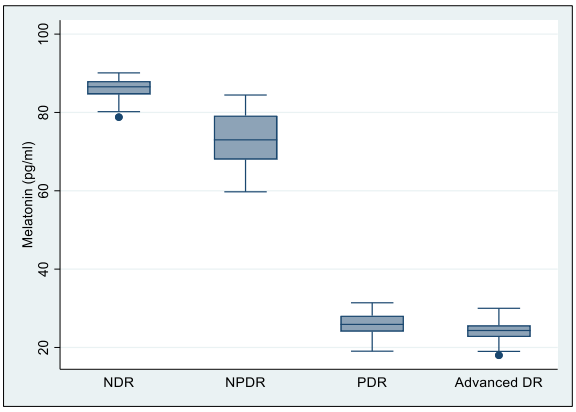

Figure 1: showing the plasma melatonin concentration in the study group

3.2. *The correlation between plasma melatonin concentration and clinical and laboratory results*

As shown tab. (2), we found a strong negative correlation between serum melatonin levels and the duration of diabetes mellitus, FBG, HA1c, HDL, LDL, triglycerides, and cholesterol, with (p-value <0.0001). Regarding melatonin and total bilirubin, we found a strong positive correlation.

Table 2: Correlation between plasma melatonin concentration and clinical and laboratory findings in three groups with DR

Variable	Plasma melatonin concentration	
	Correlation coefficient (r)	*P-value*
Age in years	-0.12	0.07
Duration of DM	-0.78	<0.0001
FBG (mg/dl)	-0.80	<0.0001
HA1c (%)	-0.91	<0.0001
ALT (U/I)	-0.06	0.35
AST (U/I)	-0.03	0.70
Total bilirubin (mmol/L)	0.99	<0.0001
Cholesterol (mg/dl)	-0.70	<0.0001
Triglycerides (mg/dl)	-0.29	<0.0001
HDL (mg/dl)	0.69	<0.0001
LDL (mg/dl)	-0.32	<0.0001

3.3. *The relation between melatonin concentration and the probability of diabetic retinopathy*

In the ROC curve figs. (2 & 3), the probability of diabetic retinopathy is increased with the decrease in plasma melatonin levels.

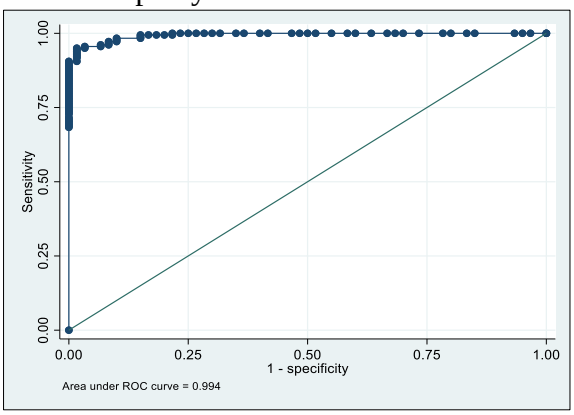

Figure 2: ROC curve study to evaluate the performance of plasma melatonin concentration in predicting DR. The AUC is 0.994 (95% CI 0.974:1.00), p value<0.0001, cutoff point≤80.15 (senstivity= 95.0%, specicity=98.3%, PPV=99.4%, and NPP=77.6).

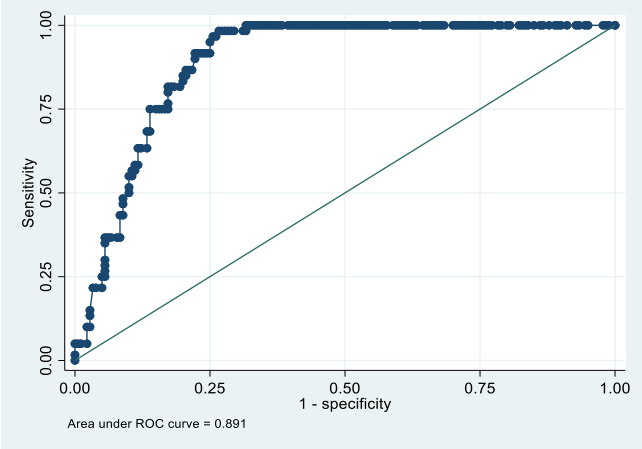

Figure 3: ROC curve study to evaluate the performance of plasma melatonin concentration in predicting Advanced DR. The AUC is 0.891 (95% CI 0.844:0.927), p<0.0001, cutoff point≤28.36 (senstivity= 98.3%, specicity=73.3%, PPV=55.1%, and NPP=99.2).

3.4. *Total bilirubin concentrations in the studied population*

Regarding total bilirubin concentrations figure (4) shows the decrease in total bilirubin with the increase in the severity of diabetic retinopathy.

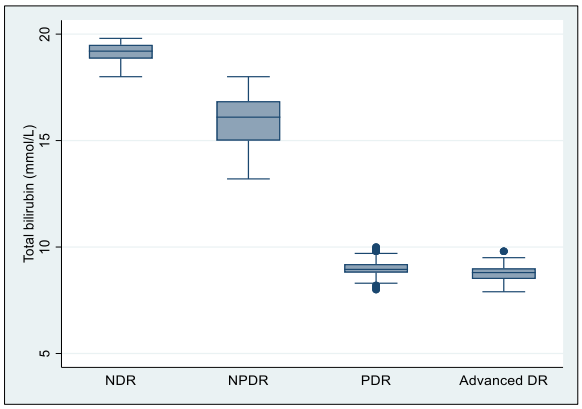

Figure 4: The plasma bilirubin concentration in the study group

3.5. *Correlation between melatonin and bilirubin*

In our study tab. (3) shows the strong and positive correlation between serum melatonin levels and total bilirubin levels.

Table 3: Correlation between total serum bilirubin and melatonin levels

	Correlation coefficient r	P value
All patients	0.99	P <0.0001
NDR group	0.2	P=0.89
NPDR group	0.77	P <0.0001
PDR	0.11	P=0.42
Advanced DR	0.04	P=0.74

4. Discussion

Melatonin is produced from the pineal gland and, to some extent, from the retina [13]. It has antioxidant functions and a major role in neurovascular and cardiovascular diseases [14,15]. It has antioxidant properties, so its decrease is linked to more severe diabetic retinopathy; the decrease in melatonin leads to increased oxidative stress with more damage to capillary endothelium. We can find a relation between its levels in the blood and diabetic retinopathy stages [16].

This relation was demonstrated in many studies. Abdel-Kawi SH et al. found that it improves the efficacy of stem cells treatment [17]. Romeo A studied its effects in diabetic retinopathy in vivo model using Nano medicine [18]. In this study, there was a significant decrease in the levels of melatonin in the advanced diabetic retinopathy group along with the levels of bilirubin; the more decrease of melatonin, the severer retinal affection. Melatonin and bilirubin significantly affect the pathogenesis of diabetic retinopathy. Ding Y et al. found that the diabetic retinopathy progression is associated with the levels of total bilirubin [19]. The ROC curve gives high specificity and sensitivity values in detecting diabetic retinopathy; this means that melatonin is a key risk factor in the pathogenesis of retinopathy. Thus, trials giving melatonin as an antioxidant for the treatment of diabetic retinopathy could be implemented [20,21]. Wan WC et al. [22]. found that total bilirubin also has antioxidant properties, so its levels decrease with the advanced diabetic retinopathy stages [23-25]. The bilirubin results from the catabolism of Heme in the liver. Many studies related its decrease to the increased severity of diabetic retinopathy. It has a strong +ve correlation with melatonin in all stages of diabetic retinopathy. This strong +ve correlation enforces the role of both of them in the pathogenesis of diabetic retinopathy [22]. This study enforces this +ve correlation with more decrease in bilirubin levels with advanced diabetic eye disease. When evaluating the other parameters, such as the duration of diabetes, medications, FBG, HA1c, and lipid profile, we found a negative correlation with diabetic retinopathy. These are essential factors for predicting the severity of retinal affection; this was discussed in different studies [26,27]. The increase of FBG levels, HA1c, duration of diabetes, and blood lipids leads to increased severity of diabetic retinopathy. This necessitates the control of these factors to improve the treatment of diabetic retinopathy. When evaluating the correlation between melatonin and bilirubin, we found a +ve correlation; this means that they decrease with each other with the progression of diabetic retinopathy. The overall estimation of these parameters (melatonin, bilirubin, FBG, HA1C, and lipid profile) is essential in diabetic retinopathy cases as each parameter of them has a relation with the severity of diabetic retinopathy.

5. Conclusion

There is a strong relation between diabetic retinopathy risk factors and the stage of retinal affection. The increase in these indicators, the more affection. The good control of these factors could lead to better prognosis. Melatonin and other antioxidants such as Bilirubin have an important role in pathogenesis and could be implemented in treatment.

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