Biomedical & Pharmacology Journal, June 2024. Vol. **17**(2), p. 1317-1327

Serum Total Bilirubin and Oxidative Stress Status in Diabetic Retinopathy – A Hospital-Based Observational Study

K. Prabhavathi[1], Mamatha Kunder[2]*, K.N. Shashidhar[1], K. Kanthamani[3] and A. Raveesha[4]

[1]Department of Biochemistry, Sri Devaraj Urs Medical College attached to Sri Devaraj Urs Academy of Higher Education and Research University, Tamaka, Kolar, Karnataka, India.
[2]Department of Biochemistry, Yenepoya School of Allied Health Sciences, Yenepoya, Mangalore Karnataka, India.
[3]Ophthalmology, Bangalore, Karnataka, India.
[4]Department of Medicine, Sri Devaraj Urs Medical College attached to Sri Devaraj Urs Academy of Higher Education and Research University, Tamaka, Kolar, Karnataka, India.
*Corresponding Author E-mail: mamathayogesh79@gmail.com

https://dx.doi.org/10.13005/bpj/2945

(Received: 02 April 2020; accepted: 21 December 2023)

Diabetic retinopathy (DR) is one of the common microvascular complications of Type 2 Diabetes Mellitus (T2DM). As an antioxidant, the serum total bilirubin is associated with vaso-occlusive disorders. Oxidative stress parameters such as Erythrocyte Glutathione (GSH) as an antioxidant and Malondialdehyde (MDA) as an oxidant are critical in the pathogenesis of diabetic complications. This study aimed to explore the possibilities of the endogenous protective role of serum total bilirubin on the retinal vasculature in DR patients by estimating and correlating the levels of serum total bilirubin, GSH, and MDA in DR cases. In this hospital-based case-control study, 288 participants were selected from R.L. Jalappa Hospital and Research Centre, Kolar, divided into three groups with 96 subjects per group. Group I: Controls, Group II: T2DM, and Group III: DR subjects. The fasting blood sugar, glycated hemoglobin, liver function test, and lipid profiles were estimated by standard methods. Oxidative stress parameters viz, GSH and MDA were assayed by chromogen 5,5'- di thiobis 2-nitrobenzoic acid (DTNB) and thiobarbituric acid reactive substances (TBARS) methods, respectively. The prevalence of DR was significantly lower among subjects with the highest bilirubin quartile than those with the lowest. There was a significant mean difference with $p<0.001$ between the groups for total bilirubin, FBS, HbA1c, GGT, TC, TG, LDL, GSH, and MDA. A Negative correlation of serum total bilirubin with FBS ($r = - 0.375$), HbA1c ($r = -0.351$), and MDA($r=-0.323$), and a positive correlation with GSH ($r = 0.335$) was observed in DR group with a significant p-value. T2DM subjects with higher levels of bilirubin within biological reference intervals were less likely to develop retinopathy. The severity of DR was inversely proportional to the total bilirubin levels. Therefore, serum total bilirubin levels could be a biomarker to predict the risk of developing retinopathy in people with T2DM.

Keywords: Diabetic Retinopathy; Erythrocyte Glutathione; Malondialdehyde; Oxidative stress; Total Bilirubin.

Type 2 Diabetes mellitus (T2DM) is characterized by hyperglycemia due to defects in insulin secretion, action, or both. Global data of diabetes prevalence in 2021 in the age group of 20–79 years is estimated at around 10.5% (536.6 million people), with an expected rise to

12.2% (783.2 million) by 2045. The Prevalence of Diabetes in either of the genders was highest in the age group of 75–79 years[1]. Data from 2021 has predicted that there may be a preponderance in middle-income countries of 21.1% compared to high and low-income countries of 11.9% and 12.2%, respectively, by 2045[1]. According to 2019 estimates, the number of diabetics in India could double approximately from 77 million to 134 million by 2045[2].

Among the major microvascular complications of diabetes, Diabetic retinopathy (DR) is the cause of vision loss among adults who are in the earning age group. A combined survey of the R. P. Center for Ophthalmic Sciences, National Diabetic Retinopathy Rapid Assessment of Avoidable Blindness (RAAB) Survey, and the Ministry of Health and Family Welfare, Government of India between 2015- 2019 has predicted the prevalence of DR to be 16.9%[3].

One of the crucial factors in the development of DR is Oxidative Stress (OS). Chronicity of hyperglycemia plays a pivotal role in the formation of reactive oxygen species (ROS), activation of the polyol, protein kinase C (PKC), and overactivity of the hexosamine pathways. Oxidative stress results in inflammation, mitochondrial dysfunction, pyroptosis, apoptosis, or autophagy. The consequential effect of oxidative stress in conjunction with neurodegeneration leads to neural, vascular, and retinal tissue damage. DR is a consequence of the synthesis of Advanced Glycation End products (AGEs) and expression of Receptors for Advanced Glycation End products (RAGEs), which generate free radicals with sequential oxidative tissue damage and glutathione (GSH) depletion[4]. Malondialdehyde (MDA), a marker of lipid peroxidation, affects the cell membrane phospholipids and correlates well with higher oxidative stress[5].

The United States National Health and Nutrition Examination Survey (NHANES) data of 1999-2006 on sixteen thousand subjects documented the upper bilirubin range in the Biological reference interval. This elevation observed is beneficial to the subjects, with a 26% reduction in the risk of developing T2DM[6]. Once considered a biological waste product of heme catabolism, bilirubin has been recognized as a potential endogenous antioxidant under physiological conditions. Bilirubin is been documented to have anti-inflammatory activity on the vasculature[7].

A study has reported that T2DM patients with higher serum bilirubin, however, within the biological reference interval, will have a lower risk of developing retinopathy[8]. These factors made us study the role of oxidative stress in the pathogenesis of DR and its association with the antioxidant effect of bilirubin.

MATERIALS AND METHODS

Study Design

A Hospital-based case-control study was conducted from 2015 to 2018 at R L Jalappa Hospital and Research Centre, a tertiary care rural referral hospital attached to Sri Devaraj Urs Medical College, affiliated to Sri Devaraj Urs Academy of Higher Education and Research, Kolar.

The central ethical committee of SDUAHER, Kolar, approved the study, Ref. No.: SDUAHER / Res. Project / 89 /2013-14. Written informed consent obtained from all study subjects. All the parameters analyzed at the Central Diagnostic Laboratory Services' biochemistry section at RLJH and RC.

A total of two hundred eighty-eight subjects of either gender, in the age group of 30-70 years were enrolled. Subjects, divided into three groups. Group I: 96 clinically proven healthy individuals, Group II: 96 clinically proven T2DM subjects without retinopathy (T2DM), and Group III: 96 clinically proven cases of T2DM with retinopathy (DR) of all stages. Group III categorization based on fundoscopy changes. Factors that affect or alter the cases or controls were excluded from the study.

Sample collection

After the individuals had fasted for eight hours the previous night, 5 mL of blood was extracted from the median cubital vein with complete aseptic precautions in the supine position. Precautions were taken to prevent sample hemolysis. The standard sample collection protocol prevented the factors affecting the parameters, such as bilirubin. The samples centrifuged for 10 minutes at 3000 rpm. The supernatant was separated, and carried out the analysis.

Methods

Plasma glucose estimated by glucose oxidase peroxidase method[9], serum urea by urease method,[10] serum creatinine by enzymatic creatinine-amidohydrolase method[10], total cholesterol(TC) by cholesterol oxidase peroxidase method[11], triglycerides(TG) by enzymatic colorimetric test GPO-PAP[12],high-density lipoproteins cholesterol(HDL-C) by phosphotungstic acid enzymatic method[13],total Bilirubin and direct bilirubin by azobilirubin and duly wavelength spectrophotometric method[14] and other liver function test by standard methods[15] using Vitros 5.1 FS, Ortho Clinical Diagnostic dry chemistry analyzer instrumentation, based on reflectance photometry. Low-density lipoprotein cholesterol (LDL-C) was calculated using Friedewald's equation, considering its limitations. HbA1c was analyzed by the HPLC method using a Bio-Rad D10 analyzer (Biorad, Hemel Hempstead, UK) as a Laboratory reference method. Erythrocyte-reduced glutathione was assayed by spectrophotometer using chromogen 5,5'- di thiobis 2-nitrobenzoic acid (DTNB)[16] and MDA assayed by thiobarbituric acid reactive substances (TBARS) method[17].

Statistical analysis

Data was analyzed using the licensed version of Statistical Product and Service Solutions (SPSS) software version 22 for statistical significance. Results were expressed as mean ± standard deviation. ANOVA test was used to determine significance, and posthoc Bonferroni was used to validate results. The p-value of <0.05 was considered statistically significant. Pearson's correlation was performed for the association of serum total bilirubin and oxidative stress markers in DR subjects.

RESULTS AND DISCUSSION

In this study, 288 subjects enrolled, and there was male preponderance in all three groups (62.5%, 64.6%, 57.3%) compared to female subjects (37.5%, 35.4%, 42.7%).

The mean age of the subjects and duration of diabetes are shown in Table 1. Duration of diabetes was significantly higher in DR patients compared to T2DM patients (p<0.001).

Out of 96 DR subjects considered for the study, 36 had mild Non-Proliferative

Table 1. Mean age and Duration of diabetes of study groups

Groups	Group I No.=96	Group II No.=96	Group III No.=96	p- value
Mean age (years)	52.31 ± 12.09	56.36 ± 8.65	57.12 ± 7.33	>0.05
Duration of DM (years)	-	5.31 ± 0.97	12.79 ± 3.92	< 0.001*

Values are expressed as Mean ± SD. *p value < 0.001 is highly significant.
Group I (Controls), Group II (T2DM) and Group III (DR).

Table 2. Prevalence of DR by quartiles of serum concentration of total bilirubin

Cases and Controls	Quartiles based on serum total bilirubin(mg/dL)							
	I quartile ≤ 0.45(mg/dL)		II quartile 0.46 – 0.55(mg/dL)		III quartile 0.56-0.65(mg/dL)		IV quartile ≥0.66(mg/dL)	
	No.	%	No.	%	No.	%	No.	%
Controls (96)	1	1	0	0	0	0	95	99
T2DM (96)	0	0	0	0	6	6.2	90	93.8
Mild NPDR(36)	0	0	5	13.9	30	83.3	1	2.8
Moderate NPDR(29)	0	0	29	100	0	0	0	0
Severe NPDR(17)	5	29.4	12	70.6	0	0	0	0
PDR(14)	9	64.3	5	35.7	0	0	0	0

T2DM: Type 2 Diabetes Mellitus; NPDR: Non Proliferative Diabetic Retinopathy; PDR: Proliferative Diabetic Retinopathy

Diabetic Retinopathy (Mild NPDR), 29 had moderate Non-Proliferative Diabetic Retinopathy (Moderate NPDR), 17 had severe Non-Proliferative Diabetic Retinopathy (Severe NPDR), and 14 had Proliferative Diabetic Retinopathy (PDR). Based on the serum total bilirubin levels, the study groups were divided into four quartiles[18]. I quartile (< 0.45), II quartile (0.46 -0.55), III quartile (0.56-0.65), and IV quartile (> 0.66) as represented in Table 2, with the first quartile representing the lowest and the fourth quartile denoting the highest.

We observed the highest percentage of PDR cases in the I quartile, moderate and severe NPDR cases in the II quartile, mild NPDR cases in the III quartile, and T2DM and control subjects in the IV quartile, indicating an inverse relation of serum total bilirubin levels with severity of DR ($p<0.001$).

Table 3 depicts the biochemical and oxidant-antioxidant parameters of the study groups. Serum total bilirubin levels significantly decreased in DR cases compared to control and T2DM subjects ($p<0.001$). Results on the levels of GSH showed a significant decrease in T2DM and DR patients compared to the control group ($p<0.001$). However, significantly increased serum MDA levels and GGT in the T2DM and DR groups compared to the control ($p<0.001$). We did not observe significant differences between the three groups, comparing urea, creatinine, SGPT, SGOT, total proteins, albumin, and alkaline phosphatase.

Post Hoc analysis using Bonferroni correction for significance indicates that Group I Vs. Group II, Group II Vs. Group III and Group I Vs. Group III showed increased FBS, HbA1c, GGT, and MDA levels and decreased GSH levels

Table 3. ANOVA comparing HbA1c, FBS, Renal function test, Liver function test, Lipid profile, GSH and MDA in Group I (Controls), Group II (T2DM) and Group III (DR)

Parameters	Group I	Group II	Group III	P value
FBS (mg/dL)	89.01 ± 9.81	152.32 ±29.51	206.34 ±42.46	p<0.001*a, b, c
HbA1c %	5.49±0.58	8.37±1.16	10.68±1.80	p<0.001* a, b, c
Total Bilirubin (mg/dL)	1.02±0.20	1.00±0.01	0.63±0.48	p<0.001* b, c
Direct Bilirubin (mg/dL)	0.2±0.02	0.12±0.01	0.02±0.01	p=0.062
GSH(mg/Gm of Hb)	16.14±0.90	9.07±1.13	5.97±1.14	p<0.001* a, b, c
MDA(nmol/mL)	1.90+0.57	6.43 ± 1.72	10.88 ± 1.36	p<0.001* a, b, c
Total Cholesterol(TC) (mg/dL)	160.89±20.27	192.59 ± 25.99	199.80 ± 29.47	p<0.001* a, c
Triglycerides(TG) (mg/dl)	136.72±27.59	222.81±45.40	239.22±57.17	p<0.001* a, c
HDL - C(mg/dL)	40.49±7.44	40.15±7.04	39.63±7.41	p=0.717
LDL -C(mg/dL)	95.00±21.39	114.32±22.70	108.27±31.06	p<0.001* a, c
AST/ SGOT (IU/L)	28.75±7.83	27.05±7.0	25.04±5.94	p=0.071
ALT /SGPT(IU/L)	31.69±7.45	30.05±6.72	30.96±6.82	p=0.271
ALP(IU/L)	160.01±30.82	159.96±34.59	165.20 ± 54.93	p=0.603
Total protein (g/dL)	7.27±0.73	7.28±0.73	7.36±0.79	p=0.642
Albumin (g/dL)	4.33±0.51	4.44±0.61	4.81±0.60	p=0.081
Globulin (g/dL)	2.86±0.57	2.86±0.4	2.61±0.48	p=0.073
A/G ratio	1.56±0.32	1.58±0.26	1.82±0.33	p=0.062
GGT (IU/L)	24.01±7.79	40.60±6.20	51.33±6.66	p<0.001* a, b, c
Blood Urea (mg/dL)	22.43±7.66	23.41±8.00	21.44±6.85	p=0.195
Serum Creatinine (mg/dL)	0.83±0.25	0.90±0.19	0.90±0.22	p=0.711

Values are expressed as Mean ± SD. *p-value <0.001 is highly significant:
a for Group I Vs. Group II, b for Group II Vs. Group III and c for Group III Vs. Group I.
FBS: Fasting blood sugar; HbA1c: Glycated haemoglobin; MDA: Malondialdehyde; GSH: Glutathione; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; GGT: Gamma glutamyl transferase

Table 4. Correlation of serum total bilirubin with FBS, HbA1c, GGT, GSH, MDA and Lipid Profile in T2DM (Group II)

		FBS	HbA1c	GGT	GSH	MDA	TC	TG	HDL-C	LDL-C
Serum total bilirubin	Pearson Correlation (r value)	-0.338	-0.533	0.221	0.130	-0.308	0.130	-0.154	-0.154	0.130
	p- value Sig.(2-tailed)	0.001	0.001	0.031	0.206	0.002	0.206	0.134	0.134	0.206
	No.	96	96	96	96	96	96	96	96	96

*Correlation is highly significant at p value 0.001 level

FBS: Fasting blood sugar; HbA1c: Glycated haemoglobin; GGT: Gamma glutamyl transferase; GSH: Glutathione; MDA: Malondialdehyde; TC: Total Cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol

Table 5. Correlation of serum total bilirubin with FBS, HbA1c, GGT, GSH, MDA and Lipid Profile, in DR (Group III)

		FBS	HbA1c	GGT	GSH	MDA	TC	TG	HDL-C	LDL-C
Serum total bilirubin	Pearson Correlation (r value)	-0.375	-0.351	0.335	-0.323	-0.323	0.159	0.097	-0.056	0.104
	p- value Sig.(2-tailed)	0.001	0.001	0.001	0.001	0.001	0.122	0.347	0.587	0.313
	No.	96	96	96	96	96	96	96	96	96

*Correlation is significant at p- value 0.001 level

FBS: Fasting blood sugar; HbA1c: Glycated haemoglobin; GGT: Gamma glutamyl transferase; GSH: Glutathione; MDA: Malondialdehyde; TC: Total Cholesterol; TG: Triglycerides; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol

with highly significant $p<0.001$. Total Bilirubin in Group I Vs. Group II did not show any significance; however, the increase in Group II Vs. Group III and Group I Vs. Group III was highly significant with $p<0.001$. Increased total cholesterol, TG, and LDL showed a highly significant p-value <0.001 in Group I Vs. Group II and Group I Vs. Group III, however, increased levels did not show any significance ($p=0.355$, $p=0.336$, $p=0.300$) in Group II Vs. Group III.

Tables 4 and 5 depict the correlation analysis of serum total bilirubin in T2DM (Group II) and DR (Group III), which showed a significant positive correlation with GSH and a significant negative correlation with FBS, HbA1C, and MDA. On the other hand, there was no significant correlation of serum total bilirubin with lipid profile parameters. However, no significant correlation was observed between the duration of diabetes mellitus with fasting blood sugars, glycated hemoglobin, liver function test, lipid profile parameters, GSH, and MDA levels in T2DM and DR cases.

DR is caused by microangiopathy, leading to microvascular leakage and occlusion of the retinal veins, arteries, and capillaries. Prolonged hyperglycemia, dyslipidemia, aging, and oxidative stress are major risk factors associated with the progression of retinopathy in diabetic patients[19]. The present study demonstrated a male preponderance of 57.3% versus 42.7% females for early development of DR. Our findings are consistent with a study by Cherchi and his coworkers[20]. However, in a previous study reported female predominance[21] and Yau and his team documented equal distribution across both genders[22].

Our study showed an increase in the prevalence of DR correlating positively with the disease duration. These findings are consistent with the previous study[23]. We observed the mean duration of diabetes at 5.31 years and retinopathy following diabetes at 12.79 years (Table 1). This finding implies the importance of regular fundus examinations and tight diabetic control in T2DM.

The frequency quartile distribution of DR subjects documented in Table 2 predicted that 64.3% of PDR cases were in the I quartile, with severe NPDR of 70.6 % and 100% of moderate NPDR in the II quartile. A mild NPDR of 83.3% was observed in the III quartile. We observed serum bilirubin values of 93.8% in the IV quartile in T2DM cases. The IV quartile values correlate well with the control group, indicating strict diabetes control shall enable delay in developing either NPDR or PDR or both. Observed findings are on par with previous studies which demonstrated that serum total bilirubin levels are inversely proportional to the severity of DR[24,25].

The present study showed a significant increase in FBS and HbA1c levels in Group III and Group II compared to Group I (Table 3) and is similar to the conducted study by Hadeel in 2020[26]. In T2DM, the early development and progression of micro and macrovascular complications is mainly due to chronic hyperglycemia. HbA1c has a unique affinity for oxygen, leading to tissue anoxia, and plays a vital role in causing micro and macroangiopathy[27].

Bilirubin, intended as a toxic substance, is an end product of heme breakdown. Studies have demonstrated that a higher total bilirubin level within the biological reference interval protects against cardiovascular diseases, stroke, and peripheral vascular disease[28,29]. Our results of the serum total bilirubin revealed significantly decreased levels in DR subjects compared to T2DM subjects, which concords with the study by Yasuda and coworkers and our previous in-house study[30,8]. A study conducted in Netherlands population demonstrated an increase in serum total bilirubin level interrupts the pathways leading to the progression of DR by inhibiting inflammation processes and oxidative stress[31]. Possible mechanisms of the protective role of bilirubin may be through its cytoprotective, anti-inflammatory, and antioxidant action on retinal vasculature[31].

Vital factors considered in the pathogenesis of DR are oxidative stress and inflammation. Studies have suggested the critical role of oxidative stress in the pathogenesis of diabetic retinopathy. Chronic hyperglycemia plays a vital role in the formation of Reactive Oxygen Species (ROS) due to the activation of the secondary pathways viz, polyol, protein kinase C (PKC) pathways, and overactivity of hexosamine pathways, leading to structural and functional changes in the retinal microvasculature[32,33,34].

ROS damages crucial biomolecules such as DNA, proteins, and lipid membranes. Lipids are one of the primary targets of ROS, and oxidized lipids generate MDA[35,36]. Increased MDA in plasma, serum, and other tissues observed in diabetic patients[37]. In the present study, there was increased lipid peroxidation, expressed as significantly increased levels of MDA in T2DM and DR compared with clinically proven controls. Our results are on par with few studies, who have demonstrated higher MDA levels in the DR compared with DM and controls[38,39]. The biochemical mechanisms for increased levels of MDA in DR are mainly based on the degree of lipolysis, with peroxidative damage of the membrane lipids resulting in increased levels of free fatty acids in the blood, leading to increased production of MDA levels and suggesting it as lipid peroxidation marker for retinal complications of diabetes[18].

The body has natural antioxidant systems to protect against the harmful effects of ROS. These systems include enzymes such as catalase, glutathione peroxidase, superoxide dismutase, and non-enzymatic antioxidants such as glutathione and vitamin E[40]. In the present study, there was a statistically significant decrease in levels of GSH in DR and T2DM groups compared with that of clinically proven healthy controls. Similar findings were found in the study by Kundu and his coworkers[41].

A study conducted in 2018 observed the elevation in circulating levels of pro-inflammatory cytokines, reactive oxidative species, and a decrease in GSH levels. The possible mechanism is that an increase in polyol pathway activity

in DR causes increased usage of nicotinamide adenine dinucleotide phosphate (NADPH) by the enzyme aldose reductase (AR), which further reduces the availability of NADPH for regenerating the intracellular antioxidant GSH and thereby decreasing the antioxidant capacity of the cells[40]. Irreversible loss and diminished GSH synthesis may reduce the concentration of GSH[42,43]. From these findings, it is proposed that upper levels of serum total bilirubin levels in the physiological range may inhibit inflammation processes, decrease oxidative stress, and thereby interrupt or delay the development of DR.

We observed elevated total cholesterol, LDL-cholesterol, and triglyceride values in Group II and Group III and HDL-C values in biological reference intervals. Our findings are on par with few studies [44,45]. An International study showed no significant association between hyperlipidemia and DR[46]. Hyperlipidemia is found in poorly controlled diabetes and causes increased viscosity of blood with alterations in the fibrinolytic system, leading to the formation of hard exudates. There may also be an assimilation of serum triglycerides into the cell membrane, which causes changes in membrane fluidity, leading to plasma leakage into the retina and resulting in hemorrhage and edema in the retina[46]. Few studies demonstrated that decreased serum lipids due to oral statins may help prevent retinal hard exudate formation and loss of vision[47,48]. Gamma-glutamyl transferase (GGT) is a recognized marker of alcohol intake and liver-related diseases. The present study showed a significant increase in serum GGT levels in DR subjects compared to clinically proven healthy controls and T2DM. Similar observations found in the study conducted in Pakistan & Iran population[49,50].

A study in 2019 demonstrated that serum GGT levels were inversely proportional to glutathione and glutathione reductase in people with T2DM and DR, showing decreased antioxidant defenses[51].

A cross-sectional study conducted in the third U.S. National Health and Nutrition Examination Survey demonstrated that serum GGT values elevated along with serum MDA levels and further indicates that GGT is potentially a pro-oxidant, and its effect is expressed in the presence of transition metals or iron. The cysteinyl glycine, a product of GGT, reduces ferric ions to ferrous, which promotes free radical production[52].

We observed no significant differences between the three Groups when comparing non-nitrogenous substances and hepatic markers. Previous study reported that increased levels of urea and creatinine, which were associated with an increased risk for progression to DR[53]. Decreased serum albumin levels in DR cases were demonstrated in a previous study[54]. A study by Gupta and his team showed deranged levels of SGOT, SGPT, and ALP in DR cases[55].

The correlation of duration of diabetes with fasting blood sugars, glycated hemoglobin, lipid profile parameters, renal and liver function test parameters, GSH, and MDA in Groups II and III did not show any significant positive or negative association.

In Groups II and III, we observed a significant negative correlation of serum total bilirubin with FBS and glycated hemoglobin. These findings are concurrent with two international studies conducted in Japan population and our previous study[56,57,8]. In Groups II and III, we observed a significant positive correlation of serum total bilirubin levels with GSH and a negative correlation with MDA. Our observations are inconsistent with our previous study and a study conducted in 2017 by Shumaila and coworkers [8,58]. A study conducted in 2014 showed no correlation between GSH and other parameters in all three groups[41].

CONCLUSION

This study demonstrated that increased levels of MDA and decreased levels of GSH and serum total bilirubin were associated with increased risk of T2DM to DR development. Serum total bilirubin in upper levels in the physiological range may protect against the development of retinopathy in subjects with T2DM, and these findings suggest that serum total bilirubin levels may be used as a biomarker to expect the risk of development of retinopathy. Estimating serum total bilirubin in T2DM on regular check-ups helps the physician to predict DR and initiate early treatment.

Limitation

The fundus examination and one-time measurement of serum total bilirubin served as

the foundation for our investigation. Concordant readings of total bilirubin within the physiological range are relevant considering the rheological variations. Insulin estimation to assess the resistance in the subjects would have been better. Dietary habits or medications that may alter liver function or bilirubin levels are pertinent information. As this study was carried out in a semi-urban tertiary care hospital in a Kolar population of Karnataka state and our findings apply to other ethnic groups, it has to be considered and proved with prospective multi-centric studies.

ACKNOWLEDGMENT

The authors would like to express their gratitude to Sri Devaraj Urs Academy of Higher Education and Research, Kolar, for funding this project

Conflict of Interest

The authors have no conflicts of interest to declare.

Funding Sources

Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Funded this project. Reference No. SDUAHER/KLR/R&D/261/2013-14 Dated 18-12-2013.

Ethical clearance

No. SDUAHER/KLR/R&D/242/2013-14 dated 03-12-2013.

Approval of university research project for funding

Project no. SDUAHER/Res.Proj/89/2013-14

REFERENCES

1. Sun H, Saeedi P, Karuranga S, et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045 [published correction appears in Diabetes Res Clin Pract. 2023 Oct;204:110945]. *Diabetes Res Clin Pract*. 2022;183:109119. doi:10.1016/j.diabres.2021.109119
2. Pradeepa R, Mohan V. Epidemiology of type 2 diabetes in India. *Indian J Ophthalmol*. 2021;69(11):2932-2938. doi:10.4103/ijo.IJO_1627_21
3. Kumar A, Vashist P. Indian community eye care in 2020: Achievements and challenges. *Indian J Ophthalmol*. 2020;68(2):291-293. doi:10.4103/ijo.IJO_2381_19
4. Cecilia O M, José Alberto C G, José N P, Ernesto Germán, C. M., Ana Karen, L. C., Luis Miguel, R. P., Ricardo Raúl, R. R., & Adolfo Daniel, R. C. Oxidative Stress as the Main Target in Diabetic Retinopathy Pathophysiology. *J Diabetes Res*. 2019:8562408. Published 2019 Aug 14. doi:10.1155/2019/8562408
5. Shawki HA, Elzehery R, Shahin M, Abo-Hashem EM, Youssef MM. Evaluation of some oxidative markers in diabetes and diabetic retinopathy. *Diabetol Int*. 2020;12(1):108-117. Published 2020 Jun 27. doi:10.1007/s13340-020-00450-w
6. Cheriyath P, Gorrepati VS, Peters I, Nookala, V., Murphy, M. E., Srouji, N., & Fischman, D. High Total Bilirubin as a Protective Factor for Diabetes Mellitus: An Analysis of NHANES Data From 1999 - 2006. *J Clin Med Res*. 2010;2(5):201-206. doi:10.4021/jocmr425w
7. Cho HC. The Relationship among Homocysteine, Bilirubin, and Diabetic Retinopathy. *Diabetes Metab J*. 2011;35(6):595-601. doi:10.4093/dmj.2011.35.6.595
8. Prabhavathi K, Kunder M, Shashidhar KN, et al. Serum total bilirubin levels in diabetic retinopathy—a case control study. IOSR J Pharm. 2013;4(8):1–6. https ://doi.org/10.9790/3013-04080 106.
9. Trinder P. Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor. *Annals of Clinical Biochemistry*. 1969;6(1):24-27. doi:10.1177/000456326900600108
10. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem*. 1974;20(4):470-475.
11. Trinder, P. Enzymatic Calorimetric Edmund Lamb, David J, Cristopher P. Estimation of Creatinine and Urea. In. Carl A Burtis, Edward R Ashwood and David E Bruns, Tietz text book of Clinical Chemistry 4th ed, Saunders. New Delhi: Elsevier Co.:798-803(2006).
12. Trinder P. Enzymatic Calorimetric Determination of Triglycerides by GOP-PAP Method. *Annals of Clinical Biochemistry*,1969; 6:24-27. https://doi.org/10.1177/000456326900600108
13. Naito, H.K. HDL Cholesterol. Kaplan A et al. *Clin Chem*. The C.V. Mosby Co., St Louis, Toronto, Princeton.1984; 437 :1207-1213.
14. Weiss JS, Sundberg MW, Dappen GM, et al. Diazo-based assay for total bilirubin in a coated thin film evaluated. *Clin Chem*. 1984;30(8):1310-1313.
15. Panteghini M, Bais R and Van soling WW. Enzymes In. Carl A Burtis , Edward R Ashwood and David E Bruns, *Tietz text book of Clinical*

Chemistry 4th ed, Saunders.NewDelhi: ElsevierCo.: 604-613(2006).
16. Beutler E, Duron O. Kelly B.M. Improved method for the determination of blood glutathione. *J Lab Clin Med*. 1963; 61: 882-888.
17. Gutteridge JM, Quinlan GJ. Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: the role of lipid radicals, iron salts, and metal chelators. *J Appl Biochem*. 1983;5(4-5):293-299. PMID: 6679543
18. Dave A, Kalra P, Gowda BH, Krishnaswamy M. Association of bilirubin and malondialdehyde levels with retinopathy in type 2 diabetes mellitus. *Indian J Endocrinol Metab*. 2015;19(3):373-377. doi:10.4103/2230-8210.152777
19. Aldosari DI, Malik A, Alhomida AS, Ola MS. Implications of Diabetes-Induced Altered Metabolites on Retinal Neurodegeneration. *Front Neurosci*. 2022;16:938029. Published 2022 Jul 13. doi:10.3389/fnins.2022.938029
20. Cherchi S, Gigante A, Spanu MA, Contini P, Meloni G, Fois MA, Pistis D, Pilosu RM, Lai A, Ruiu S, et al. Sex-Gender Differences in Diabetic Retinopathy. *Diabetology*. 2020; 1(1):1-10. https://doi.org/10.3390/diabetology1010001
21. Li M, Wang Y, Liu Z, et al. Females with Type 2 Diabetes Mellitus Are Prone to Diabetic Retinopathy: A Twelve-Province Cross-Sectional Study in China. *J Diabetes Res*. 2020;2020:5814296. Published 2020 Apr 21. doi:10.1155/2020/5814296
22. Yau JW, Rogers SL, Kawasaki R, et al. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care*. 2012;35(3):556-564. doi:10.2337/dc11-1909
23. Mehta R , Punjabi S , Bedi N , Prevalence of diabetic retinopathy: A tertiary care centre based study. *Indian J Clin Exp Ophthalmol.* 2020;6(3):383-386
24. Ghaffar T, Marwat ZI, Ullah F, Khan S, Hassan Aamir AU. Association Of Serum Total Bilirubin Level With Diabetic Retinopathy In Type 2 Diabetes Mellitus. *J Ayub Med Coll Abbottabad*. 2016;28(3):537-541.
25. Wan H, Zhu H, Wang Y, et al. Associations between different bilirubin subtypes and diabetic microvascular complications in middle-aged and elderly individuals. *Ther Adv Endocrinol Metab*. 2020;11:2042018820937897. Published 2020 Jul 11. doi:10.1177/2042018820937897
26. Shawki HA, Elzehery R, Shahin M, Abo-Hashem EM, Youssef MM. Evaluation of some oxidative markers in diabetes and diabetic retinopathy. *Diabetol Int*. 2020;12(1):108-117. Published 2020 Jun 27. doi:10.1007/s13340-020-00450-w
27. Sinclair H S, Luttrull K J. Diabetes Mellitus Associated Progressive Neurovascular Retinal Injury: Recommendations for Imaging and Functional Testing and Potential Role for Early Intervention with Modern Retinal Laser Therapy. *Journal of Ophthalmology Research Reviews and Reports*. 2022;3:2-17. Doi 10. 47363/ JORRR/ 2022(3) 130.
28. Gazzin S, Vitek L, Watchko J, Shapiro SM, Tiribelli C. A Novel Perspective on the Biology of Bilirubin in Health and Disease. *Trends Mol Med.* 2016;22(9):758-768. doi:10.1016/j.molmed.2016.07.004
29. Ding Y, Zhao J, Liu G, et al. Total Bilirubin Predicts Severe Progression of Diabetic Retinopathy and the Possible Causal Mechanism. *J Diabetes Res*. 2020;2020:7219852. Published 2020 Jul 31. doi:10.1155/2020/7219852
30. Yasuda M, Kiyohara Y, Wang JJ, et al. High serum bilirubin levels and diabetic retinopathy: the Hisayama Study. *Ophthalmology*. 2011;118(7):1423-1428. doi:10.1016/j.ophtha.2010.12.009
31. Abbasi A, Deetman PE, Corpeleijn E, et al. Bilirubin as a potential causal factor in type 2 diabetes risk: a Mendelian randomization study. *Diabetes*. 2015;64(4):1459-1469. doi:10.2337/db14-0228
32. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res*. 2010;107(9):1058-1070. doi:10.1161/CIRCRESAHA.110.223545
33. Safi SZ, Qvist R, Kumar S, Batumalaie K, Ismail IS. Molecular mechanisms of diabetic retinopathy, general preventive strategies, and novel therapeutic targets. *Biomed Res Int*. 2014;2014:801269. doi:10.1155/2014/801269
34. Rodríguez ML, Pérez S, Mena-Mollá S, Desco MC, Ortega ÁL. Oxidative Stress and Microvascular Alterations in Diabetic Retinopathy: Future Therapies. *Oxid Med Cell Longev*. 2019;2019:4940825. Published 2019 Nov 11. doi:10.1155/2019/4940825
35. Juan CA, Pérez de la Lastra JM, Plou FJ, Pérez-Lebeña E. The Chemistry of Reactive Oxygen Species (ROS) Revisited: Outlining Their Role in Biological Macromolecules (DNA, Lipids and Proteins) and Induced Pathologies. *Int J Mol Sci*. 2021;22(9):4642. Published 2021 Apr 28. doi:10.3390/ijms22094642
36. Nita M, Grzybowski A. The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults. *Oxid*

Med Cell Longev. 2016;1-23:2016:3164734. doi:10.1155/2016/3164734

37. Shalash M, Badra M, Imbaby S, Elbanna E. Malondialdehyde in type 2 diabetics and association with cardiovascular risk factors. *Journal of the Medical Research Institute*, 2020; 41(2):21-30. Doi:10.21608/jmalexu.2020.147116
38. de Souza Bastos A, Graves DT, de Melo Loureiro AP, et al. Diabetes and increased lipid peroxidation are associated with systemic inflammation even in well-controlled patients. *J Diabetes Complications*. 2016;30(8):1593-1599. doi:10.1016/j.jdiacomp.2016.07.011
39. Hou Y, Lin M, Qiu X , He M. Zhang Y, Guo F. Effect of Type-2 diabetes mellitus in retinopathy patients on MDA, SOD activity and its correlation with HbA1c.Brazilian Archives of Biology and Technology. 2021; 64. Doi:10.1590/1678-4324-2021200075.
40. Prabhakar PK. Pathophysiology of Diabetic Secondary Complication and their Management. *Curr Diabetes Rev*. 2021;17(4):395-396. doi:10.2174/1573399817 04210326092455
41. Kundu D, Mandal T, Nandi M, Osta M, Bandyopadhyay U, Ray D. Oxidative stress in diabetic patients with retinopathy. *Ann Afr Med*. 2014;13(1):41-46. doi:10.4103/1596-3519.126951
42. Lutchmansingh FK, Hsu JW, Bennett FI, et al. Glutathione metabolism in type 2 diabetes and its relationship with microvascular complications and glycemia. *PLoS One*. 2018;13(6):e0198626. Published 2018 Jun 7. doi:10.1371/journal.pone.0198626
43. Zhu B, Wu X, Ning K, Jiang F, Zhang L. The Negative Relationship between Bilirubin Level and Diabetic Retinopathy: A Meta-Analysis. *PLoS One*. 2016;11(8):e0161649. Published 2016 Aug 29. doi:10.1371/journal.pone.0161649
44. Badikillaya V U, Potluri R, Venkataramana G, Pernenkil SR. (2013). Serum Gamma Glutamyl Transferase as a marker of oxidative stress in Type 2 Diabetic Retinopathy. *Ijpbs*.2013; 3(2) :496-503.
45. Khubchandani A S. Sanghani H. Study of serum magnesium and HbA1C in diabetic patients along with changes in their lipid profiles. *IJCP*.2013;23(11):717-719.
46. Chou Y, Ma J, Su X, Zhong Y. Emerging insights into the relationship between hyperlipidemia and the risk of diabetic retinopathy. *Lipids Health Dis*. 2020;19(1):241. Published 2020 Nov 19. doi:10.1186/s12944-020-01415-3
47. Kang EY, Chen TH, Garg SJ, et al. Association of Statin Therapy With Prevention of Vision-Threatening Diabetic Retinopathy. *JAMA Ophthalmol*. 2019;137(4):363-371. doi:10.1001/jamaophthalmol.2018.6399
48. Kawasaki R, Konta T, Nishida K. Lipid-lowering medication is associated with decreased risk of diabetic retinopathy and the need for treatment in patients with type 2 diabetes: A real-world observational analysis of a health claims database. *Diabetes Obes Metab*. 2018;20(10):2351-2360. doi:10.1111/dom.13372
49. Javaid H, Haroon MA, Javaid M, Abidin FU, Masud H, Khalid UB. Association of serum gamma glutamyl transferase as biomarker of oxidative stress in type-II diabetics with and without diabetic retinopathy. *Pak J Pathol*. 2021; 32(4): 165-169.
50. Valizadeh N, Mohammadi R, Mehdizadeh A, Motarjemizadeh Q, Khalkhali H R. Evaluation of serum gamma glutamyl transferase levels in diabetic mellitus patients with and without retinopathy. *Shiraz E- med J.* 2018;19(7):e 64073.https:doi.org/10.5812/semj.64073.
51. R Divya, V Ashok. Evaluation of serum gamma glutamyl transferase levels as a marker of oxidative stress in type 2 diabetes patients with and without retinopathy. *MedPulse International Journal of Physiology*. 2019; 9:30–34. DOI:10.26611/103935.
52. Lim JS, Yang JH, Chun BY, Kam S, Jacobs DR Jr, Lee DH. Is serum gamma-glutamyltransferase inversely associated with serum antioxidants as a marker of oxidative stress?. *Free Radic Biol Med*. 2004;37(7):1018-1023. doi:10.1016/j.freeradbiomed.2004.06.032
53. Hsieh YT, Tsai MJ, Tu ST, Hsieh MC. Association of Abnormal Renal Profiles and Proliferative Diabetic Retinopathy and Diabetic Macular Edema in an Asian Population With Type 2 Diabetes [published correction appears in JAMA Ophthalmol. 2019 Feb 1;137(2):233]. *JAMA Ophthalmol*. 2018;136(1):68-74. doi:10.1001/jamaophthalmol.2017.5202
54. Wang GX, Fang ZB, Li JT, et al. The correlation between serum albumin and diabetic retinopathy among people with type 2 diabetes mellitus: NHANES 2011-2020. *PLoS One*. 2022;17(6):e0270019. Published 2022 Jun 16. doi:10.1371/journal.pone.0270019
55. Gupta R, Bhat SPS, Gangadhar PR, et al. Study of liver function tests in patients with long standing type 2 diabetes mellitus in comparison to healthy individuals. *J Evolution Med Dent Sci* 2021;10(05):289- 293, DOI: 10.14260/jemds/2021/64

56. Ohnaka K, Kono S, Inoguchi T, et al. Inverse associations of serum bilirubin with high sensitivity C-reactive protein, glycated hemoglobin, and prevalence of type 2 diabetes in middle-aged and elderly Japanese men and women. *Diabetes Res Clin Pract*. 2010;88(1):103-110. doi:10.1016/j.diabres.2009.12.022

57. Shichiri M, Kishikawa H, Ohkubo Y, Wake N. Long-term results of the Kumamoto Study on optimal diabetes control in type 2 diabetic patients. *Diabetes Care*. 2000;23 Suppl 2:B21-B29.

58. Shaikh S, Memon A, Ata M A, Hina, Khoharo HK. Association of Serum Bilirubin, Serum Malondialdehyde and Glycemic Control with Retinopathy in Type 2 Diabetic Subjects. *Int J Diabetes Endocrinol*. (2017);2(1):10-14. doi: 10.11648/j.ijde.20170201.13