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Increased serum level of homocysteine correlates with retinal nerve fiber layer thinning in diabetic retinopathy

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Purpose: To study the correlation between serum levels of vitamin B_{12}, folic acid, and homocysteine and the severity of diabetic retinopathy and the correlation with retinal nerve fiber layer (RNFL) thinning on spectral domain optical coherence tomography (SD-OCT).

Methods: In a tertiary care center–based prospective cross-sectional study, 60 consecutive cases and 20 healthy controls in the age group of 40–65 years were included. The eyes of the cases were divided into three groups according to Early Treatment Diabetic Retinopathy Study (ETDRS) classification: diabetes mellitus without retinopathy (n = 20), non-proliferative diabetic retinopathy with macular edema (n = 20), and proliferative diabetic retinopathy with macular edema (n = 20). The serum levels of vitamin B_{12} and folic acid were measured using a standard protocol. The serum homocysteine assay was performed using an enzyme-linked immunosorbent assay (ELISA) kit. Average RNFL thickness was measured using SD-OCT. Statistical analysis was used to assess the correlations between the study variables.

Results: Increased severity of diabetic retinopathy was found to correlate with an increase in the serum levels of homocysteine ($F = 53.79$; $p<0.001$). The mean serum levels of vitamin B_{12} and folic acid were found to be within the normal reference range. A positive correlation was found between retinal nerve fiber layer thinning and serum levels of homocysteine ($p<0.001$).

Conclusions: This study, for the first time, demonstrated a correlation between increased homocysteine with a decrease in RNFL thickness and increased severity of diabetic retinopathy.

Diabetic retinopathy reflects a compromise of the metabolic, endocrine, and hematological systems. It is estimated that 382 million people all over the world had diabetes mellitus in 2013. This number is expected to rise to 592 million by 2035 [1].

Diabetic retinopathy was considered solely a disease of retinal vasculature in the past. Neurodegeneration is a component of diabetic retinopathy. Chronic loss of retinal neurons occurs due to increased frequency of apoptosis and activation of glial cells. Vulnerability to neurons exists before any sign of vascular damage [2-5]. Retinal neurodegeneration causes early microvascular changes that include the breakdown of the blood–retinal barrier, vasoregression, and impairment of neurovascular interaction [6-10].

Homocysteine is a by-product of transmethylation reactions and is detoxified by methionine synthetase, which depends on vitamin B_{12} and folate as coenzymes for proper function. Several studies were conducted to find correlations between retinal vascular disease and homocysteine. Elevated total plasma levels of homocysteine has been found to be an independent risk factor for retinal vascular occlusive disease [11]. Homocysteine has been found to be involved in a complex and dynamic way in vascular injury and repair, thus contributing to the development of diabetic microangiophathy. Therefore, strategies for controlling the level of homocysteine by supplementation with folic acid or vitamin B_{12} may be potential treatment strategies to ameliorate neurodegeneration. The present study was conducted to evaluate the status of serum levels of vitamin B_{12}, folic acid, and homocysteine in diabetic retinopathy and the correlation with retinal nerve fiber layer (RNFL) thinning on spectral domain optical coherence tomography (SD-OCT).

METHODS

Institutional review board approval was obtained, and the study was performed in accordance with the tenets of the Declaration of Helsinki. Sample size was calculated to be 80 according to the standard sample size calculation formula. The study was conducted in adherence to the ARVO guidelines regarding the ethical use of human subjects in research.

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In this tertiary care center–based cross-sectional study, 60 consecutive cases of type 2 diabetes mellitus and 20 healthy controls (presenting for refraction) aged between 40 and 65 years were included after informed voluntary consent was obtained. The right eye of the healthy controls was taken into consideration. The 60 cases of type 2 diabetes mellitus were divided into three groups: patients with diabetes without retinopathy (n = 20), patients with non-proliferative diabetic retinopathy with macular edema (NPDR; n = 20), and patients with proliferative diabetic retinopathy with macular edema (PDR; n = 20), based on the Early Treatment Diabetic Retinopathy Study (ETDRS) classification. Level of diabetic retinopathy was graded according to the eye with the more severe form of disease. The diagnosis of diabetes mellitus was made using American Diabetes Association guidelines [12].

The duration of illness was defined as the duration from the time of the diagnosis of diabetes mellitus given to the participant until the time of the examination. Exclusion criteria included ocular or systemic diseases affecting the retinal vasculature (hypertension), nervous system (Alzheimer disease, peripheral neuropathy, glaucoma, age-related macular degeneration, end stage renal disease), previous intravitreal injection(s), ophthalmic surgical or laser interventions, cases on medications, vitamin, or antioxidant supplements, and cases with signal strength 5 or below on the optical coherence tomography examination.

The examination consisted of an explanation of the study, measurement of the blood pressure, refraction and assessment of the logMAR best-corrected visual acuity (LogMAR is expressed as the decadic logarithm of the minimum angle of resolution with 20/20 line equivalent to LogMAR 0.00 and the 20/200 line to LogMAR 1.0), and slit-lamp biomicroscopy of the anterior segment. Further, Goldmann applanation tonometry, gonioscopy, and computerized static perimetry using the Full Threshold 24–2 program of the Humphrey perimeter (Humphrey Field Analyzer, Zeiss/Humphrey Systems, Dublin, CA) were performed.

Fundus examination was performed with slit-lamp biomicroscopy with a 90-diopter lens and indirect ophthalmoscopy. Fundus photography was performed in all cases using a Zeiss fundus camera FF 450 Plus with pixel width of 0.0054 and image size 2588×1958. Cases showing retinal changes were subjected to fundus fluorescein angiography. Subsequently, all the study subjects were evaluated using SD-OCT (CIRRUS High Definition OCT; Carl Zeiss Meditec Inc., Dublin, CA). Every study subject underwent RNFL thickness analysis using the optic disc cube 200×200 feature (Figure 1). The average retinal nerve fiber layer thickness was noted. Macular thickness analysis using the macular cube 512×128 feature was also performed. The central subfield thickness (µm) was noted.

Blood samples were collected from the study subjects in the morning after an overnight fasting interval of 6–8 h. The samples were drawn by vein puncture using a 5 ml metal-free plastic syringe fitted with a 24-gauge stainless steel needle (Nirlife; Nirma Limited, Sachana, India) under contamination-controlled conditions, and the blood samples were collected in a 4 ml vacutainer (VAKU-8; Hindustan Syringes and Medical Devices Limited; Faridabad, India) that contained heparin as an anticoagulant. The volume of the samples ranged from 4 ml to 5 ml. For separation of plasma, blood was transferred into tubes containing 3.89% trisodium citrate in the ratio of 9:1. The citrated blood was centrifuged at 200 ×*g* for 10 min, and the supernatant platelet-rich plasma was aspirated out into another plastic tube. All samples were stored at −20 °C until the assay was performed. The standard protocol was maintained during sample collection and storage.

Fasting and post-prandial blood glucose levels was estimated using the glucose oxidase method [13]. Glycated hemoglobin was measured using the Bio-Rad D-10 (Bio-Rad Laboratories, Inc., Hercules, CA) that employs the high-power liquid chromatography cation exchange chromatography method. The coefficient of variation (CV%) was 1.93%.

SERUM FOLIC ACID AND VITAMIN B12 ASSAY

The serum folic acid assay was performed using Elecsys Folate III (Roche Diagnostics; Indianapolis, IN) assay method that employs a competitive test principle using natural folate binding protein (FBP) specific for folate. The serum vitamin B_{12} assay was performed using the Elecsys Vitamin B_{12} (Roche Diagnostics) assay method that employs a competitive test principle using the intrinsic factor specific for vitamin B_{12}.

Serum homocysteine assay: Assay of homocysteine was performed using the human homocysteine enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, Inc., San Diego, CA). The reagents in the kit were prepared following the standard protocol provided with the kit. Human homocysteine standard samples were added to corresponding wells (100 µl for each well), and the 0 nmol/ml well was filled with standard diluent. Serial dilutions of the homocysteine standard were performed using 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 nmol/ml concentrations to the standard curve following the manufacturer's instructions and run in parallel. The serum sample was diluted ten times with the sample dilution for the best detection range of the ELISA kit, and further results of the serum levels of homocysteine multiplied by 10.

Standard sample diluent (300 µl) was added to each of the seven microtiter wells. Diluent (300 µl) was pipetted out from one tube to another tube sequentially. The standard sample dilution in tube 8 was the negative control. Reaction wells were sealed with adhesive tape and incubated at 37 °C for 90 min. Biotinylated human homocysteine antibody liquid was prepared 30 min in advance. The ELISA plate was washed three times. Biotinylated human homocysteine antibody liquid was added to each well (100 µl for each). Reaction wells were sealed and incubated at 37 °C for 60 min. Enzyme-conjugate liquid was prepared 30 min in advance. The ELISA plate was washed three times. Enzyme-conjugate liquid was added to each well except the blank wells (100 µl for each). The reaction wells were sealed and incubated at 37 °C for 30 min. The ELISA plate was washed five times. Color reagent liquid (100 µl) was added to individual wells (also to the blank well) and incubated at 37 °C. When the color of the high concentration of the standard curve became darker and the color gradient appeared, the hatching was stopped. Color Reagent C (100 µl) was added to individual wells (also to the blank well) and mixed well. The intensity of the color was read over the ELISA plate reader (Synergy HT; BioTek, Winooski, VT) at 450 nm. The calibration curve of the standard homocysteine was plotted against the homocysteine on the y-axis and the concentration on absorbance on the x-axis. The homocysteine concentration in the serum sample was

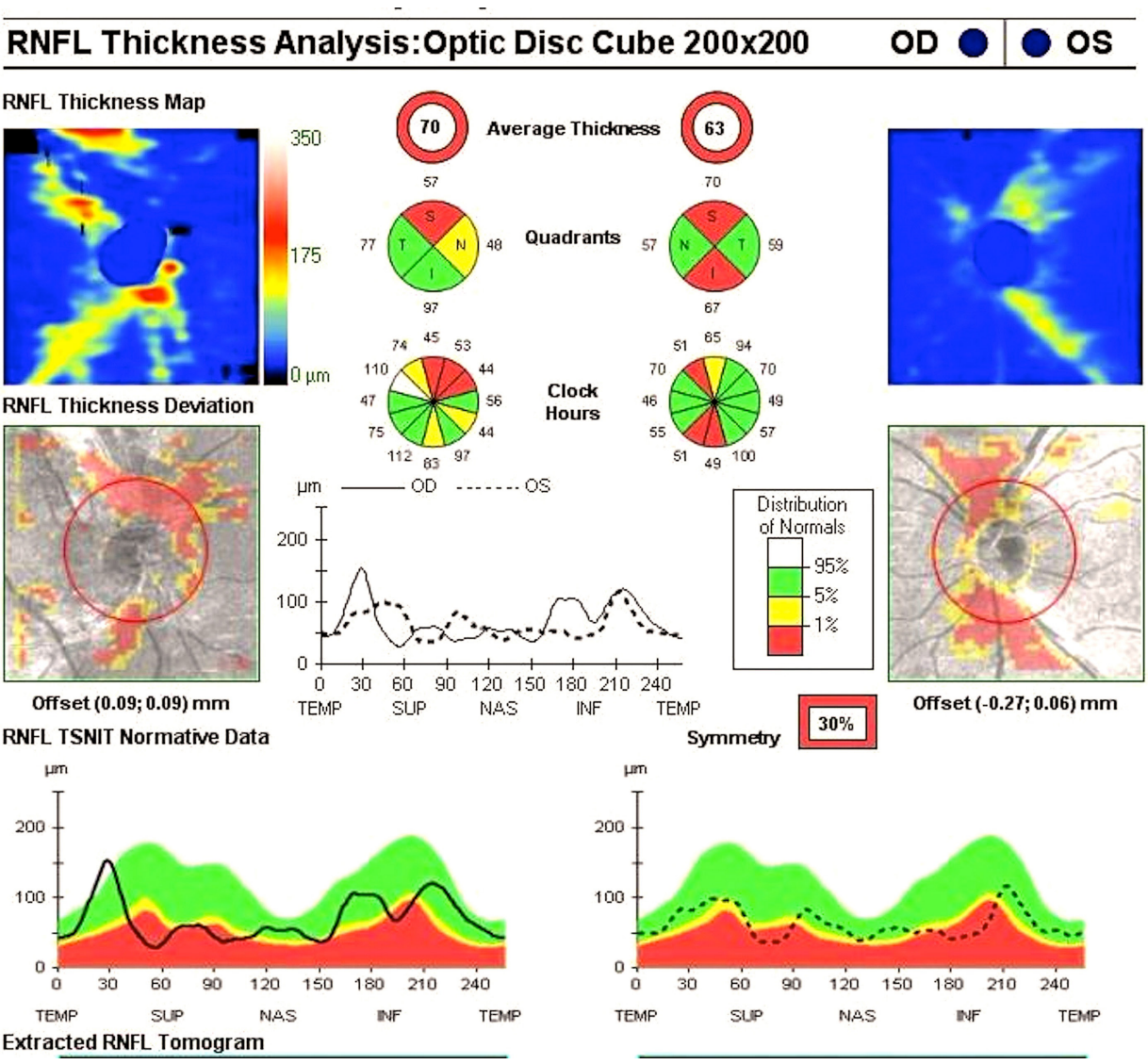

Figure 1. Retinal nerve fiber thickness analysis using optic disc cube 200x200 feature depicting thickness map, thickness deviation, TSNIT (Temporal, Superior, Nasal, Inferior, Temporal) normative data and extracted tomograph of both eyes. The quadrant map shows thinning in the superior quadrant of both eyes and thinning in inferior quadrant of left eye.

Table 1. Summary (Mean±SD) of the mean age, duration of diabetes mellitus, glycated hemoglobin, visual acuity (logMAR), serum homocysteine, serum vitamin B 12, serum folic acid, average retinal nerve fiber layer thickness, central sub field thickness, fasting and post prandial blood glucose levels among the study groups.

Variable	Group			
	Controls	No DR	NPDR	PDR
Age(years)	53.26±9.81	56.0±6.71*a	54.3±6.91*a,b	51.02±7.21*a,b,c
Duration(years)	0±0	5.98±6.96	11.7±6.84	11.7±6.35
Visual acuity (LogMAR)	0.081±0.09	0.262±0.25	0.649±0.36	1.278±0.51
Fasting blood sugar (mg/dl)	95.10±8.19	107.64±21.49*c	153.25±80.23*a,b	163.33±42.62*a,b,c
Post prandial blood glucose (mg/dl)	139.60±17.13	164.95±31.53*c	223.80±96.31*a,b	249.38±50.89*a,b
S. homocysteine (nmol/ml)	17.12±7.0	27.22±1.05*a,	28.9±8.3*a,b	30.8±2.1*a,b,c
S. vitamin B12 (pg/ml)	944.75±206.62	330.86±94.40	286.97±46.11	194.17±84.16
S. folic acid (ng/ml)	18.26±4.87	10.74±4.26	7.05±2.52	7.81±3.38
Average RNFL thickness (µm)	96.75±10.30	93.15±9.86*a,c	86.95±14.14*a,b	65.90±115.98*a,b,c
Central sub field thickness (µm)	249.85±12.62	233.15±31.09*a	330.10±90.02*a,b	421.60±93.55*a,b,c
Glycated hemoglobin (%)	6.21±1.02 (33 to 56 mmol/mol)	6.39±0.41*c (42 to 51 mmol/mol)	8.35±2.53*a,b (40 to 95 mmol/mol)	7.95±1.69*a,b,c (45 to 82 mmol/mol)

Statistical analysis was performed by one way ANOVA (ANOVA) involving Newman–Keuls test for posthoc comparisons. The level of significance was accepted at $p<0.05$. *a compared to control group, b compared to NoDR group, c compared to NPDR group.

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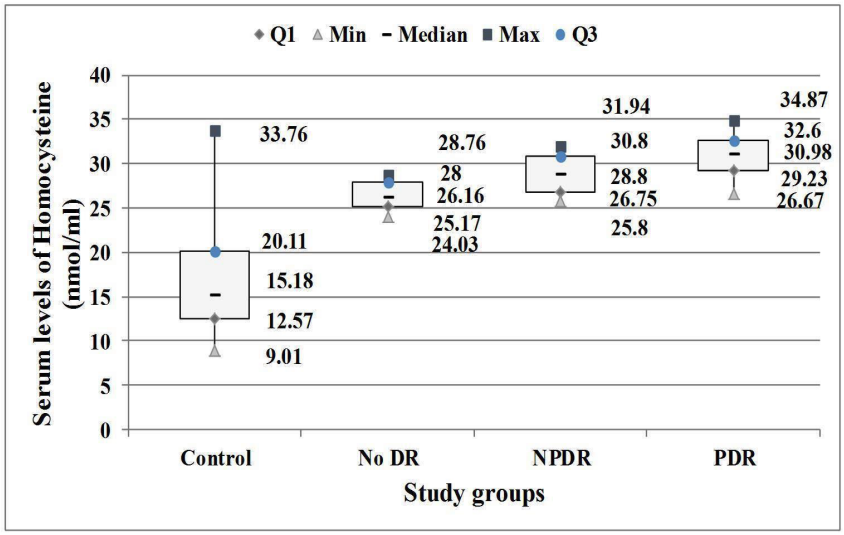

Figure 2. Box and whisker plot graphically representing the descriptive statistics for serum levels of homocysteine among the study groups. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values. An increase in serum level of homocysteine with increase in severity of diabetic retinopathy is observed.

calculated based on the standard curve. The standard curve was drawn using professional curve software (CurveExpert 1.3; Hyams Development; Madison, AL) to analyze and compute the result. The values are expressed as nmol/ml.

Data are summarized and presented as mean ± standard deviation (SD). The continuous variables of the study groups were compared with one-factor ANOVA (ANOVA). The Kolmogorov–Smirnov test was used to check the normality of the data. Distribution of variables such as visual acuity, homocysteine, and vitamin B_{12} was skewed. Therefore, log and antilog conversions were made to analyze these variables. For pair-wise comparison between the groups, the Newman–Keuls test for post hoc multiple comparison was used. The discrete (categorical) variables were compared

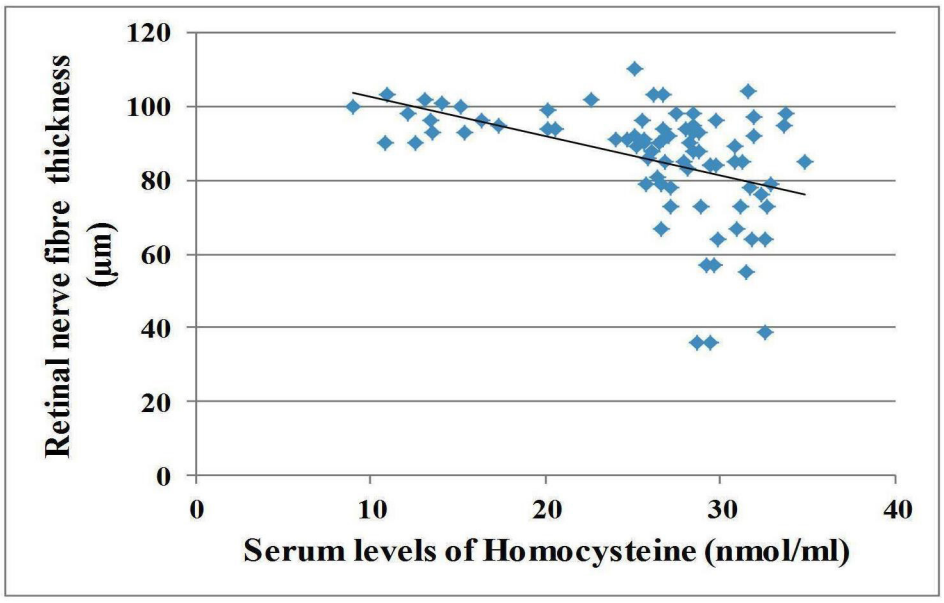

Figure 3. Scatter plot showing a negative correlation between the serum levels of homocysteine and the average retinal nerve fiber layer thickness observed on Pearson correlation analysis.

Table 2. Summary of Pearson correlation analysis of serum homocysteine with average retinal nerve fiber layer thickness.

Study variables	Pearson correlation analysis
Average RNFL thickness	(r=-0.315; p=0.004)

with a chi-square ($\chi 2$) test. The logMAR vision score of two groups (NPDR and PDR) was compared with an independent Student *t* test. Pearson correlation analysis was used to assess correlations between the variables. A p value of less than 0.05 was considered statistically significant. All analyses were performed using STATISTICA 6.0 software package (Dell, Inc., Round Rock, TX).

RESULTS

Table 1 summarizes the mean age, duration of type 2 diabetes mellitus, visual acuity (logMAR), fasting and post-prandial blood glucose levels, serum levels of homocysteine, vitamin B_{12}, and folic acid, average RNFL thickness, and central sub-field thickness in the study groups. No statistically significant difference in the mean age of the study groups was observed (F = 1.404, p = 0.248). The chi-square test revealed similar sex proportions among all four groups (male/female: 12/8 versus 12/8 versus 13/7 versus 10/10, $\chi 2$ = 1.152; p = 0.334). A statistically significant difference in the mean duration of diabetes mellitus of the study groups was observed (F = 25.95, p<0.001).

The mean glycated hemoglobin in the Control, NoDR, NPDR, and PDR groups was 6.21±1.02% (33 to 56 mmol/mol), 6.39±0.41% (42 to 51 mmol/mol), 8.35±2.53% (40 to 95 mmol/mol), and 7.95±1.69% (45 to 82 mmol/mol), respectively. The difference in the glycated hemoglobin levels was statistically significant between the study groups (F = 8.95; p<0.001). Pearson correlation analysis revealed a statistically significant correlation between glycated hemoglobin and homocysteine (r = 0.231; p=0.039), vitamin B_{12} (r = –0.363; p = 0.001), folic acid (r = –0.358; p = 0.001), and average RNFL thickness (r = –0.357; p = 0.001). The mean blood pressure (systolic/ diastolic) levels in the Control, No DR, NPDR, and PDR groups were 114/74, 126/78, 134/82, and 144/86 mmHg, respectively.

In the comparison of visual acuity, ANOVA revealed a statistically significant difference in visual acuity in each group (F = 47.192, p<0.0001).

The ANOVA showed that the difference in the serum levels of homocysteine was statistically significant between the study groups (F = 53.79; p<0.001; Figure 2). The ANOVA also showed that the difference in average RNFL thickness and central sub-field thickness was statistically significant between the study groups (p<0.001 and p<0.001, respectively). The serum levels of folic acid and vitamin B_{12} were observed to be within the normal reference range (2–20 ng/ml and 200–900 pg/ml, respectively).

Pearson correlation analysis revealed a statistically significant negative correlation between homocysteine with average RNFL thickness (r = –0.315; p = 0.004; Figure 3; Table 2) and a positive correlation with central sub-field thickness (r = 0.240; p = 0.032).

DISCUSSION

This study was conducted to determine the correlation between the serum levels of homocysteine, vitamin B_{12}, and folic acid and alterations in RNFL thickness in cases of diabetes mellitus. A previous study by Corrêa et al. [14] found an association between a risk factor such as the duration of diabetes mellitus and the severity of retinopathy. In the present study, a statistically significant difference existed among the study groups in the comparison of the duration of diabetes. It was concluded that increased duration of diabetes mellitus is associated with the increased severity of diabetic retinopathy. In this study, visual acuity was found to decrease with the increase in the severity of retinopathy. This finding is in accordance with Alkuraya et al.'s findings [15].

Homocysteine is a by-product of transmethylation reactions and is detoxified by methionine synthetase, which depends on vitamin B_{12} and folate as coenzymes for proper function. The role of homocysteine has been induced in several microvascular complications of diabetes mellitus, including diabetic retinopathy.

Impaired activity of the enzyme methylenetetrahydrofolate reductase raises the plasma levels of homocysteine [16]. Vaccaro et al. found significantly raised fasting plasma levels of homocysteine in patients with diabetes mellitus with microalbuminuria or proliferative retinopathy that were not attributable to confounders, such as age, sex, smoking, or dissimilar plasma folate and vitamin B_{12} concentrations [17]. Several other studies also reported a statistically significant increase in the total plasma level of homocysteine and a statistically significant decrease in the serum levels of vitamin B_{12} and folic acid in diabetic retinopathy [18-28]. However, other studies found a statistically insignificant association [29,30].

Our study demonstrated that the difference in the serum level of homocysteine was statistically significant between the study groups. Although the mean serum levels of folic acid and vitamin B_{12} among the cases were found to be within the normal reference range, the values were on the lower side

of normal. The mean serum levels of folic acid and vitamin B_{12} in the controls were found to be on the higher side of the normal reference range. It can be concluded that increased serum levels of homocysteine are associated with increased severity of diabetic retinopathy.

The mechanism of action of homocysteine leading to retinal neurodegeneration has been studied in several animal models. Several studies suggested that homocysteine induces apoptosis in retinal ganglion cells with the expression of Bax (Bcl-2-associated X protein), a proapoptotic protein, as it was found in increased levels in diabetic retinas [31,32]. In addition, increased levels of terminal nick-end labeling positive cells that correlated with increased levels of caspases (the enzymes involved in apoptosis) in the neural retinas of diabetic rats have been found [33]. Ganapathy et al. concluded that homocysteine-induced ganglion cell loss involves the dysregulation of mitochondrial dynamics, in vivo and in vitro [34].

Several studies proposed another mechanism. Glutamate is the major excitatory amino acid in the brain and the retina. Disruption of glutamate homeostasis in the diabetic retina initiates the development of diabetic retinopathy [35-37]. The major cause of neuronal cell death following glutamate-induced activation of N-methyl D-aspartate (NMDA) receptors is generation of free radicals that induce apoptosis [38]. Therefore, strategies for decreasing the level of extracellular glutamate or inhibiting the activation of NMDA receptors may decrease neurotoxicity and cell death [37]. In vitro studies of retinal ganglion cells and in vivo studies in the brain suggest that homocysteine acts as an agonist at the glutamate site of NMDA receptors [39,40].

Multifocal electroretinography, flash electroretinography, contrast sensitivity, color vision, and short-wavelength automated perimetry have demonstrated functional deficits in the neuronal component of the diabetic retina [41,42]. Our study for the first time provides clinical evidence of a correlation between increased serum levels of homocysteine and RNFL damage in an in vivo human diabetic retina. The difference in the average RNFL thickness levels was statistically significant between the study groups. Increased severity of retinopathy was associated with decreased average RNFL thickness that can be attributed to increased levels of homocysteine.

The severity of diabetic retinopathy has been reported to correlate with macular thickness parameters on SD-OCT. Mean macular thickness, retinal thickness, foveal thickness, and central macular thickness have been shown to correlate with the severity of diabetic retinopathy [43,44]. A recent study we conducted demonstrated that central sub-field thickness is associated with the severity of diabetic retinopathy [45]. Results of the present study are also in concordance with the findings of this previous research.

Conclusions: The novelty of our study lies in the demonstration of a correlation between increased serum levels of homocysteine and in vivo retinal nerve fiber layer thinning in the human diabetic retina. A correlation between increased serum levels of homocysteine and increased severity of retinopathy was also found.

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