

Correlation between vitamin D serum levels and severity of diabetic retinopathy in patients with type 2 diabetes mellitus

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Purpose: To study the correlation of serum vitamin D levels with quantitative (central subfield thickness [CST], cube average thickness [CAT]), cross-sectional (disorganisation of retinal inner layer [DRIL] and ellipsoid zone [EZ]) and topographic parameters (retinal pigment epithelium [RPE]) on spectral domain optical coherence tomography (SD-OCT) in diabetic retinopathy (DR), for the first time.

Methods: Eighty-eight consecutive cases of type 2 diabetes mellitus with no retinopathy (No DR; $n = 22$); non-proliferative DR (NPDR; $n = 22$); proliferative DR (PDR; $n = 22$) and healthy controls ($n = 22$) were included, after sample size calculation. On SD-OCT, physician-friendly grading systems were created for DRIL, EZ disruption and RPE alterations. Serum vitamin D was analysed using a standard protocol. Statistical analysis was done using Pearson correlation, Student's *t*-test, ANOVA, Newman-Keuls test, chi-square test and univariate ordinal logistic regression analysis.

Results: Mean serum vitamin D levels (ng/ml) were: No DR = 23.36 ± 2.00 , NPDR = 17.88 ± 1.86 , PDR = 14.07 ± 1.21 , and controls = 25.11 ± 1.59 . Low vitamin D levels correlated significantly with severity of retinopathy, VA ($r = 0.50$), CST ($r = 0.36$), CAT ($r = 0.41$), DRIL ($r = 0.35$), EZ disruption ($r = 0.40$) and RPE alterations ($r = 0.37$), respectively ($p < 0.01$). Significantly low vitamin D levels were observed in subjects with DRIL present versus DRIL absent; EZ disruption, focal versus global versus intact; RPE alterations, focal versus global versus none, respectively ($p < 0.05$).

Conclusions: Low serum vitamin D levels correlate with the presence of DRIL, EZ disruption and RPE alterations and increased severity of DR.

Keywords: Diabetic retinopathy (DR), disorganization of retinal inner layer (DRIL), spectral domain optical coherence tomography (SD-OCT), vitamin D

Introduction

Diabetes mellitus will be the seventh leading cause of death in 2030, as proposed by the World Health Organization (WHO).¹ There are approximately 93 million people with diabetic retinopathy (DR), 17 million with proliferative DR, 21 million with diabetic macular oedema, and 28 million with vision-threatening DR. The overall prevalence of proliferative DR is 6.81% (6.74–6.89) worldwide.²

Vitamin D insufficiency has reached pandemic proportions with nearly half of the world's population at risk.^{3–5} Vitamin D, a fat-soluble vitamin, has antioxidant, anti-inflammatory and anti-angiogenic role in DR.^{6,7} Vitamin D receptors (VDR) are expressed in the retinal ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer and photo receptor layer.⁸ Recently it was shown that adult retinal pigmentary epithelium-19 expresses mRNA and protein for all vitamin D3 synthesising and metabolising components.⁹

Cross-sectional imaging of the retina and topography of the retinal pigment epithelium (RPE) can be non-invasively and

precisely evaluated on spectral-domain optical coherence tomography (SD-OCT).¹⁰ Disorganisation of retinal inner layers (DRIL) has been found to be associated with a decrease in visual acuity (VA).¹¹ Early change in DRIL prospectively identifies eyes with a high probability of subsequent VA improvement or decline. Disruption of the ellipsoid zone (EZ) has been found to be associated with a decrease in VA.^{12–17} RPE topographic alterations have been found to be associated with a decrease in VA and EZ disruption.^{18–21}

The present study was undertaken to evaluate the correlation of serum vitamin D levels with central subfield thickness (CST), cube average thickness (CAT), DRIL, EZ disruptions and RPE topographic alterations, for the first time.

Material and methods

All procedures performed in this study were in accordance with the ethical standards of the institutional review board and with the Helsinki Declaration and its later amendments. Written informed voluntary consent was obtained from all study

subjects. The study was a tertiary care centre based cross-sectional study. Diagnosis of type 2 diabetes mellitus was made according to American Diabetes Association (ADA) guidelines, which include fasting plasma glucose level ≥ 126 mg/dl, and two-hour plasma glucose level ≥ 200 mg/dl during an oral glucose tolerance test.²² Mean duration of diabetes mellitus, in years, was 7.48 ± 5.59 in No DR, 9.78 ± 5.62 in NPDR, and 10.81 ± 4.54 in PDR groups respectively. Based on the statistical power of the study, more than 80% with alpha at 0.05, sample size was calculated as 88. Sixty-six consecutive cases of type 2 diabetes mellitus, in the age group 40–70 years, were included. Cases were divided into three groups based on the early treatment diabetic retinopathy study (ETDRS) classification:²³ diabetes patients without retinopathy (NO DR, $n = 22$), non-proliferative diabetic retinopathy (NPDR, $n = 22$) and proliferative diabetic retinopathy (PDR, $n = 22$). Twenty-two healthy controls were also included. Subjects with any ocular or systemic disease affecting retinal vascular pathology, age-related macular degeneration, subjects with previous intravitreal injection(s), surgical or laser interventions were excluded. Subjects with media haze at any level giving signal strength of less than 7, on SD-OCT, were also excluded from the study. Subjects with systemic diseases like cardiovascular disease, tuberculosis, chronic liver disease and cancer were excluded. Any prior disease that suggested baseline alterations in vitamin D and calcium metabolism, such as hyperparathyroidism or hypoparathyroidism, or recent nephrolithiasis and subjects taking vitamin supplements, calcium supplements, dietary supplements, antioxidants, anti-inflammatory therapy or on any medications causing change in vitamin D metabolism such as rifampin, phenobarbital and phenytoin were also excluded. None of the study subjects were confined to bed or their home and all were mobile. The cases and controls had similar dietary habits to those popular in the geographic region.

The best-corrected visual acuity was documented on the logMAR scale. Age and gender of subjects were documented. All study subjects underwent detailed fundus evaluation using stereoscopic slit lamp biomicroscopy and indirect ophthalmoscopy. Digital fundus photography and fluorescein angiography were done using a Zeiss fundus camera FF 450 Plus (Carl Zeiss Meditec, Jena, Germany). Study subjects underwent SD-OCT using the macular cube 512×128 feature of Cirrus High Definition SD-OCT (Carl Zeiss Meditec Inc, Dublin, CA, USA). Central subfield thickness and cube average thickness were documented in μm .²⁴ Central subfield thickness was defined as thickness of the central circle in the circular map known as the ETDRS Grid. Cube average thickness was defined as an overall average thickness for the internal limiting membrane-retinal pigment epithelium tissue layer over the entire 6×6 mm square scanned area. The authors created physician-friendly SD-OCT based grading systems for DRIL, EZ and RPE. Disorganisation of inner retinal layers was defined as the inability to delineate the inner retinal layer boundaries; any of the boundaries of the ganglion cell layer–inner plexiform (GCL-IPL) complex, inner nuclear layer and outer plexiform layer. DRIL was graded as: grade 0, DRIL absent; and grade 1, DRIL present (Figure 1). If intraretinal cysts were observed in the outer nuclear layer, resulting in overall retinal thickening, but the inner retinal layers could be still demarcated, then DRIL was not considered to be present. Loss of the normal foveal contour did not constitute DRIL by itself unless there was concurrent loss of retinal layer boundaries. The ellipsoid zone was defined as the second hyper-reflective band of the

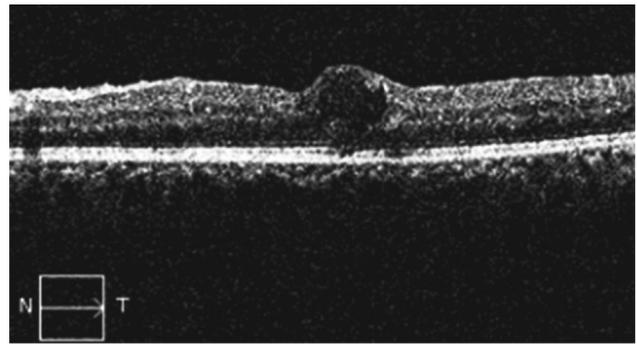


Figure 1: SD-OCT showing presence of disorganisation of retinal inner (DRIL) layers.

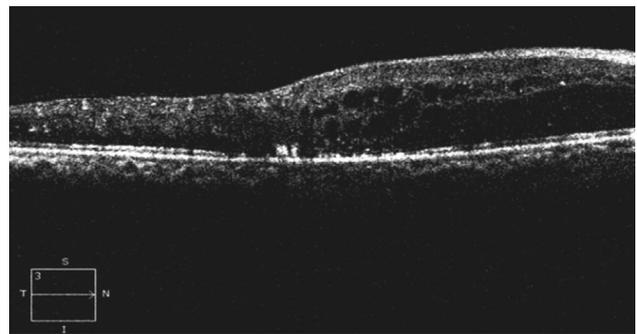


Figure 2: SD-OCT showing focal ellipsoid zone (EZ) disruption.

outer retinal layers, on horizontal and vertical SD-OCT scans. Ellipsoid zone disruption was graded as:¹⁶ Grade 0: intact photoreceptor ellipsoid zone; Grade 1: focal disruption (photoreceptor ellipsoid zone disruption indicating subfoveal localised involvement; Figure 2); and Grade 2: global disruption (photoreceptor ellipsoid zone disruption indicating generalised involvement within the macular cube). Retinal pigment epithelium topographic alterations were defined as undulations on a single-layer retinal pigment epithelial (SL-RPE) map. RPE topographic alterations were graded as:²⁰ grade 0, no RPE alterations; grade 1, RPE alterations in up to two quadrants; and grade 2, RPE alterations in more than two quadrants (Figure 3). Two experienced observers masked to the status of diabetic retinopathy assessed the grades of DRIL, EZ disruption and RPE alterations. The interobserver correlation was computed using Spearman's rank correlation.

Blood samples were collected from study groups using a standard protocol. Fasting blood samples were drawn by vein puncture using a 5 ml metal-free plastic syringe fitted with a 24-gauge stainless steel needle under sterile conditions and blood samples were collected in a 4 ml vacutainer. The volume of the samples ranged from 7 ml to 8 ml. Blood was transferred into glass tubes for separation of serum. The tubes containing blood were set on to the stand and left for 30 minutes to allow the blood to clot. Subsequently, the samples were centrifuged at $1000 \times g$ for 10 minutes and serum was carefully removed to another tube. Haemoglobin, blood sugar fasting and post-prandial, glycated haemoglobin (HbA_{1c}) and serum vitamin D were analysed. Haemoglobin A_{1c} was measured using high-performance liquid chromatography.

Serum vitamin D levels were measured by a chemiluminescence delayed, one-step assay on the Abbott Architect i-1000SR

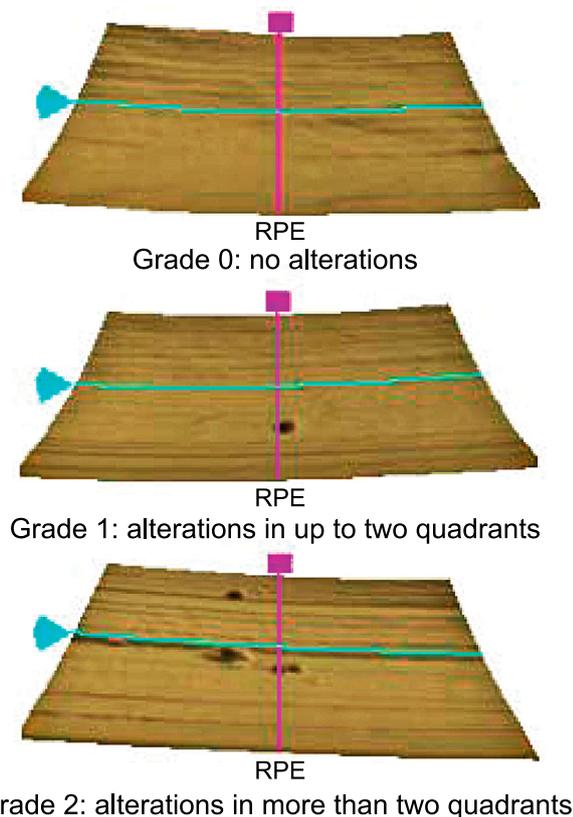


Figure 3: Segmentation map of retinal pigment epithelium (RPE) showing gradings of topographic alterations.

analyzer (Abbott Diagnostics, Wiesbaden, Germany). The 25-OH vitamin D assay was performed using chemiluminescence immunoassay (CMIA) technology with flexible assay protocols referred to as Chemiflex.

Statistics

Data were summarised as Mean \pm SE (standard error of the mean). Continuous 2 independent groups were compared by Student's *t* test. Continuous more than 2 independent groups were compared by one-way analysis of variance (ANOVA) and the significance of mean difference between groups was done by Newman–Keuls post hoc test after ascertaining normality by Shapiro–Wilk test and homogeneity of variance between groups by Levene's test. Categorical (discrete) groups were compared by chi-square (χ^2) test. Pearson correlation analysis was done to assess association between the variables. Independent predictor(s) for severity of diabetic retinopathy was assessed by univariate ordinal logistic regression analysis. A two-tailed ($\alpha=2$) $p < 0.05$ was considered statistically significant. Analyses were performed on SPSS software (Windows version 17.0; SPSS Inc, Chicago, IL, USA).

Results

Demographic, clinical and optical coherence tomography-based quantitative, cross-sectional and topographic parameters in the study subjects are shown in Table 1.

Analysis of variance showed no statistically significant difference in age among the study groups ($F=0.03$, $p=0.993$). A chi-square test showed similar sex frequency among the groups ($\chi^2=4.89$, $p=0.180$). Interobserver correlation for DRIL, EZ grades and RPE grades was observed to be $\rho=0.78$ ($p < 0.001$). Inter-correlation of different variables in the study

subjects is shown in Table 2. Pearson correlation analysis showed that low serum vitamin D levels significantly correlated with HbA1c ($r=0.38$, $p < 0.001$), VA ($r=0.50$, $p < 0.001$) (Figure 4), CST ($r=0.36$, $p < 0.001$) (Figure 5), CAT ($r=0.41$, $p < 0.001$) (Figure 6), DRIL ($r=0.35$, $p < 0.01$), EZ grades ($r=0.40$, $p < 0.001$) and RPE grades ($r=0.37$, $p < 0.001$).

Student's *t*-test showed significantly lower Vitamin D levels in subjects with DRIL present as compared with DRIL absent ($p=0.001$) (Table 3). Similarly, ANOVA showed significant positive correlation between low serum vitamin D levels and EZ disruption grading ($p < 0.001$) (Table 3). Further, the Newman–Keuls test showed significant lower mean serum vitamin D levels in focal and global disruption as compared with intact EZ ($p < 0.01$). Similarly, ANOVA showed significant positive correlation between low serum vitamin D levels and RPE alterations ($p=0.002$) (Table 3). Further, the Newman–Keuls test showed significantly lower mean vitamin D level in both focal and global alterations as compared with no RPE alterations ($p < 0.05$). Univariate logistic regression analysis showed significant association of severity of retinopathy with HbA1c, VA, vitamin D, CST, CAT, CV, DRIL, grades of EZ disruption and RPE alterations ($p < 0.001$) (Table 4).

Discussion

Low serum vitamin D levels have been found to be associated with increasing severity of DR, as revealed by recent meta-analyses.^{25,26} The present study also highlighted significant positive correlation between low serum vitamin D levels and severity of diabetic retinopathy. Significant correlation between serum vitamin D levels with CST, CAT, DRIL and grades of EZ disruption and RPE alterations on SD-OCT were observed for the first time. Also, a positive correlation was observed between low serum vitamin D levels and VA.

Vitamin D, a fat-soluble secosteroid and a multifunctional hormone, has many diverse functions including effects on immune regulation, cellular inflammation pathways, endothelial cell proliferation, angiogenesis and apoptosis, in addition to its well-established traditional role in regulating calcium homeostasis.^{27–29} Our recent study highlighted that increased serum ionised calcium induces retinal photoreceptor apoptosis resulting in increased EZ disruption in DR.³⁰

Vitamin D deficiency has been shown to impair insulin synthesis and secretion in animal models of diabetes.³¹ Active vitamin D mediates its biological actions by binding to vitamin D receptors. Vitamin D receptors have been identified in the human retina and have been implicated in the pathogenesis of DR.^{8,9} Vitamin D receptor expression plays a significant role during retinal vascular development, especially during maturation of retinal vasculature, by promoting pericyte quiescence and endothelial survival. Vitamin D deficiency leads to thickened basement membrane and reduced number of pericytes, which are believed to increase the permeability and incompetence of the retinal vasculature.³²

Vitamin D plays a role in the pathogenesis of diabetic retinopathy through its effects on the immune system. Several inflammatory cytokines (TNF- α , TNF- β , IL-6, and plasminogen activator inhibitor-1) are upregulated in patients with type 2 diabetes. Vitamin D decreases the production of several pro-inflammatory cytokines (IL-2, IL-6, IL-8, IL-12 and TNF- α).^{6,33} Vitamin D also exerts an anti-inflammatory effect by decreasing

Table 1: Demographic, clinical and optical coherence tomography-based quantitative, cross-sectional and topographic parameters in the study subjects (mean \pm SE)

Variables	Controls (n = 22) (%)	No DR (n = 22) (%)	NPDR (n = 22) (%)	PDR (n = 22) (%)	F/ χ^2 value	p-value
Age (years)	53.45 \pm 1.57	53.18 \pm 1.20	53.77 \pm 1.42	53.64 \pm 1.66	0.03	0.993
Sex (%):						
Female	8 (36.4)	13 (59.1)	6 (27.3)	9 (40.9)		
Male	14 (63.6)	9 (40.9)	16 (72.7)	13 (59.1)	4.89	0.180
Hb (gm/dl)	11.60 \pm 0.31	11.72 \pm 0.35	10.91 \pm 0.49	11.38 \pm 0.37	0.84	0.474
HbA1c (%)	5.35 \pm 0.11	7.99 \pm 0.49	8.22 \pm 0.43	8.84 \pm 0.55	12.65	< 0.001
Blood sugar F (mg/dl)	83.14 \pm 1.96	141.70 \pm 10.14	161.44 \pm 10.52	181.43 \pm 9.95	22.75	< 0.001
Blood sugar PP (mg/dl)	104.90 \pm 2.09	217.18 \pm 15.17	251.03 \pm 13.37	260.97 \pm 12.30	36.30	< 0.001
Vitamin D (ng/ml)	25.11 \pm 1.59	23.36 \pm 2.00	17.88 \pm 1.86	14.07 \pm 1.21	8.95	< 0.001
VA (logMAR)	0.09 \pm 0.02	0.36 \pm 0.04	0.68 \pm 0.07	1.18 \pm 0.02	112.64	< 0.001
CST (μ m)	247.91 \pm 2.62	256.55 \pm 12.20	328.27 \pm 23.80	500.09 \pm 23.86	42.34	< 0.001
CAT (μ m)	255.64 \pm 1.08	282.23 \pm 8.94	298.55 \pm 7.87	389.23 \pm 10.54	52.89	< 0.001
CV (mm ³)	9.56 \pm 0.03	9.91 \pm 0.23	10.75 \pm 0.29	13.68 \pm 0.43	43.38	< 0.001
DRIL (%):						
Absent	22 (100.0)	22 (100.0)	12 (54.55)	0		
Present	0	0	10 (45.45)	22 (100.0)	52.00	< 0.001
EZ grade (%):						
Intact EZ	22 (100.0)	22 (100.0)	10 (45.5)	0	67.20	< 0.001
Focal disruption	0	0	6 (27.3)	2 (9.1)		
Global disruption	0	0	6 (27.3)	20 (90.9)		
RPE grade:						
No alteration	22 (100.0)	22 (100.0)	15 (68.2)	0		
Focal alteration	0	0	4 (18.2)	12 (54.5)		
Global alteration	0	0	3 (13.6)	10 (45.5)	59.14	< 0.001

the proliferation of helper T-cells, cytotoxic T-cells and natural killer cells.³² Vitamin D deficiency has been found to be associated with vascular endothelial dysfunction in middle-aged and elderly adults.³⁴

This compromise of the blood–retinal barrier leads to leakage of plasma constituents, resulting in intra-retinal oedema with resultant increase in CST, CAT and DRIL. Our earlier studies demonstrated that CST and CAT increased with severity of diabetic retinopathy.^{17–19}

Vitamin D may also contribute via angiogenesis mechanisms. Vitamin D inhibits ischemia-mediated retinal neovascularisation.³¹ An active metabolite of vitamin D, calcitriol, is a potent in vivo inhibitor of retinal neovascularization. It inhibits in

vitro retinal endothelial cell capillary morphogenesis.⁵ Furthermore, calcitriol also downregulates hypoxia-inducible factor-1 (HIF-1) transcriptional activity, as well as HIF-1 target genes, such as vascular endothelial growth factor (VEGF).³⁵ As several of the complications in diabetic retinopathy, such as macular oedema and neovascularisation, are driven by VEGF production, vitamin D could exert its positive role via calcitriol-mediated VEGF reduction.^{36–38}

Our earlier studies have showed that increased grades of EZ disruption are associated with increased serum VEGF levels and severity of DR.¹⁷ Vitamin D receptors have been detected in the photoreceptor layer of retina. Therefore, low vitamin D levels result in increased EZ disruption through calcitriol-mediated VEGF upregulation.

Table 2: Inter-correlation of different variables in the study subjects (n = 88)

Variables	Vitamin D	Hb	HbA1c	VA	CST	CAT	CV	DRIL	EZ grades	RPE grades
Vitamin D	1.00									
Hb	0.12 ^{ns}	1.00								
HbA1c	−0.38 ^{***}	0.12 ^{ns}	1.00							
VA	−0.50 ^{***}	−0.10 ^{ns}	0.44 ^{***}	1.00						
CST	−0.36 ^{***}	−0.21 ^{ns}	0.17 ^{ns}	0.71 ^{***}	1.00					
CAT	−0.41 ^{***}	−0.19 ^{ns}	0.31 ^{**}	0.72 ^{***}	0.87 ^{***}	1.00				
CV	−0.42 ^{***}	−0.19 ^{ns}	0.29 ^{**}	0.68 ^{***}	0.88 ^{***}	0.95 ^{***}	1.00			
DRIL	−0.35 ^{**}	−0.27 [*]	0.24 [*]	0.78 ^{***}	0.78 ^{***}	0.76 ^{***}	0.70 ^{***}	1.00		
EZ grades	−0.40 ^{***}	−0.22 [*]	0.27 [*]	0.82 ^{***}	0.84 ^{***}	0.81 ^{***}	0.77 ^{***}	0.94 ^{***}	1.00	
RPE grades	−0.37 ^{***}	−0.18 ^{ns}	0.32 ^{**}	0.71 ^{***}	0.75 ^{***}	0.74 ^{***}	0.76 ^{***}	0.74 ^{***}	0.84 ^{***}	1.00

ns = non-significant, $p > 0.05$, ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$.

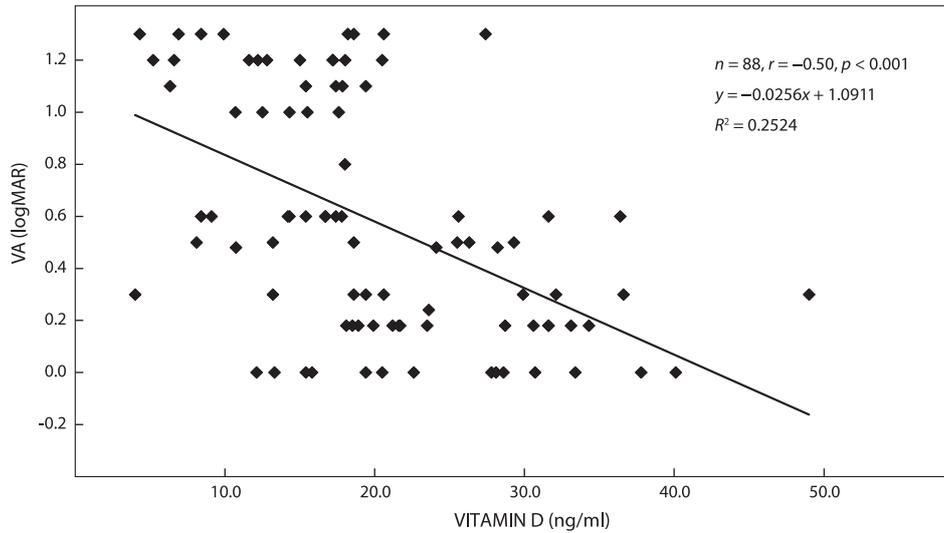


Figure 4: Correlation between serum vitamin D and visual acuity (VA).

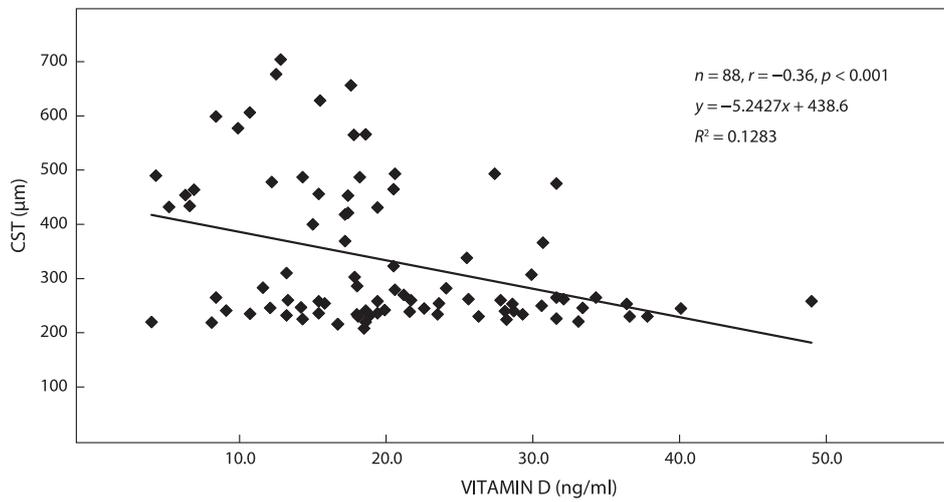


Figure 5: Correlation between serum vitamin D and central subfield thickness (CST).

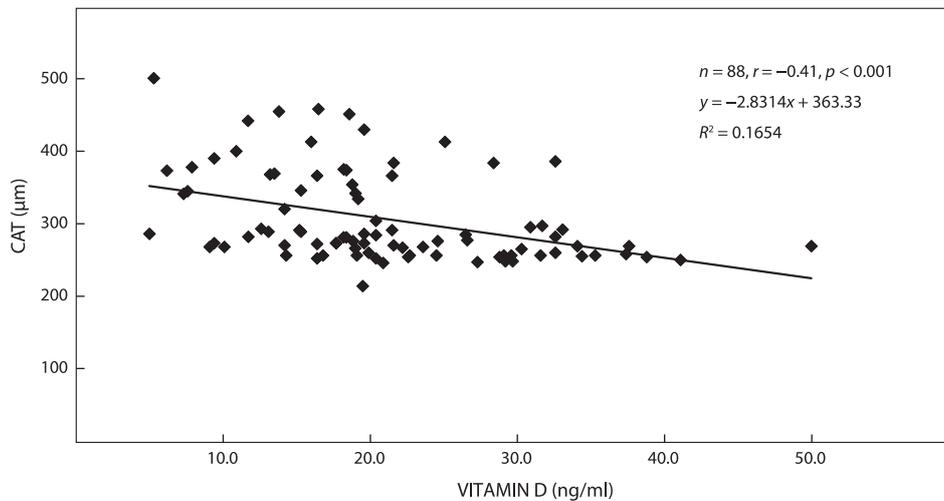


Figure 6: Correlation between serum vitamin D and cube average thickness (CAT).

RPE secretes VEGF (an angiogenic factor) and PEDF (pigment epithelium derived factor; anti-angiogenic factor). ARPE-19 expresses the machinery for vitamin D₃ and can produce an

active form of vitamin D. Vitamin D exerts its anti-angiogenic action by interruption of the angiogenesis signalling pathway.³⁹ Our earlier studies have shown significant increase

Table 3: Correlation of vitamin D with SD-OCT-based cross-sectional and topographic parameters in study subjects (n = 88)

Cross-sectional and topographic parameters	n	Vitamin D (ng/ml) mean ± SE	t/F-value	p-value
DRIL:				
Absent	56	22.77 ± 1.32	3.47	0.001
Present	32	16.43 ± 1.14		
EZ grade:				
Intact EZ (grade 0)	54	22.96 ± 1.33	7.95	<0.001
Focal disruption (grade 1)	8	19.56 ± 2.67		
Global disruption (grade 2)	26	15.04 ± 1.06		
RPE grade:				
No alteration (0)	59	22.47 ± 1.23	6.97	0.002
Focal alteration (1)	16	17.17 ± 1.52		
Global alteration (2)	13	14.02 ± 1.78		

Table 4: Independent predictors of severity of diabetic retinopathy using univariate logistic regression analysis (n = 88)

Predictors	Univariate	
	OR (95% CI)	p-value
Age	0.99 (0.94–1.05)	0.848
Sex:		
Female	Ref	0.699
Male	0.86 (0.40–1.84)	
Hb	1.09 (0.88–1.34)	0.439
HbA1c	0.62 (0.51–0.76)	< 0.001
Blood sugar F	0.97 (0.96–0.98)	< 0.001
Blood sugar PP	0.98 (0.97–0.99)	< 0.001
Vitamin D	1.11 (1.06–1.16)	< 0.001
VA	0.00 (0.00–0.00)	< 0.001
CST	0.98 (0.98–0.99)	< 0.001
CAT	0.95 (0.94–0.97)	< 0.001
CV	0.26 (0.17–0.41)	< 0.001
DRIL:		
Absent		
Present	0.02 (0.01–0.07)	< 0.001
EZ grade:		
Intact EZ		
Focal disruption	0.07 (0.02–0.29)	< 0.001
Global disruption	0.00 (0.00–0.02)	< 0.001
RPE grade:		
No alteration		
Focal alteration	0.01 (0.00–0.05)	< 0.001
Global alteration	0.01 (0.00–0.05)	< 0.001

Ref = reference group, OR = odds ratio, CI = confidence interval. In discrete group, odds ratio was evaluated against reference group.

in RPE alterations with severity of DR.^{19,20} Low serum vitamin D levels, through the effect on these receptors, result in RPE topographic alterations.

Conclusion

To conclude, low serum vitamin D levels correlate with the presence of DRIL, EZ disruption and RPE alterations and increased severity of diabetic retinopathy.

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