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Pro- and antiangiogenic VEGF and its receptor status for the severity of diabetic retinopathy

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Purpose: Alteration of pro- and antiangiogenic homeostasis of vascular endothelial growth factor (VEGF) isoforms in patients with hyperglycemia seems crucial but substantially unexplored at least quantitatively for diabetic retinopathy (DR). Therefore, in the present study we aimed to estimate the difference between the pro- ($VEGF_{165}$a) and antiangiogenic ($VEGF_{165}$b) VEGF isoforms and its soluble receptors for severity of DR.

Methods: The study included 123 participants (diabetic retinopathy: 81, diabetic control: 20, non-diabetic control: 22) from the Regional Institute of Ophthalmology, Kolkata. The protein levels of $VEGF_{165}$a (proangiogenic), $VEGF_{165}$b (antiangiogenic), VEGF receptor 1 (VEGFR1), VEGFR2, and VEGFR3 in plasma were determined with enzyme-linked immunosorbent assay (ELISA).

Results: An imbalance in VEGF homeostasis, a statistically significant concomitant increase ($p<0.0001$) in the level of $VEGF_{165}$a and a decrease in the level of $VEGF_{165}$b, was observed with the severity of the disease. Increased differences between $VEGF_{165}$a and $VEGF_{165}$b i.e. $VEGF_{165}$a-b concomitantly increased statistically significantly with the severity of the disease ($p<0.0001$), patients with diffuse diabetic macular edema (DME) with proliferative DR (PDR) had the highest imbalance. The plasma soluble form of VEGFR2 concentration consistently increased statistically significantly with the severity of the disease ($p<0.0001$).

Conclusions: The increased difference or imbalance between the pro- ($VEGF_{165}$a) and antiangiogenic ($VEGF_{165}$b) homeostasis of the VEGF isoforms, seems crucial for an adverse prognosis of DR and may be a better explanatory marker compared with either VEGF isoform.

The current global scenario for diabetes seems alarming as it is increasing exponentially with economic progression [1]. Around 8.5% of the world's population is affected by the disease, and 93 million people around the globe are affected by vision-threatening retinopathy due to diabetes [2,3]. Diabetic retinopathy (DR) develops either from increased permeability of retinal vessels (diabetic macular edema) or from the proliferation of new retinal vessels after persistent exposure to hyperglycemia and hypoxemia [3]. Prolonged uncontrolled diabetes has been associated with the severity of DR [4]. Multiple mediators, such as oxidative stress, polyol, hexosamine pathway activation, advanced glycation product (AGE) accumulation, and inflammation, have been shown for the disease pathogenesis [5-7]. After Michaelson's postulations on retinal angiogenesis mediated by vascular endothelial growth factor (VEGF) [8], several studies, including a previous study published by our lab, established the pathogenic potentiality of VEGF for DR [9-11]. A subtle genetic variation in the VEGF promoter can predispose an individual to DR suggesting transcriptional regulation of the *VEGF* (Gene ID: 7422; OMIM 192240) gene [11]. Studies postulate that proangiogenic VEGF is one of the crucial factors for the development of proliferative DR [12]. Therapeutic intervention with steroids as an alternative to the anti-VEGF, anti-inflammatory molecule has been documented with limited success [13-15]. Identifying the role of each molecule for the pathogenesis of DR is difficult because they can act alone but more often in synteny. Multiple interactive mechanisms have hypothesized that cellular damage and adaptive changes lead to the development of this ocular complication of diabetes [11,16]. Recently, it was established that alternative splicing and proteolytic processing of VEGF transcripts produce two families of polypeptides with opposite characteristics: One is proangiogenic (activates angiogenesis), and the other is anti-angiogenic (inhibits angiogenesis), maintaining the similar receptor binding domains [17,18]. This knowledge invites the scientific community to estimate pro- and antiangiogenic VEGF homeostasis for a deeper understanding of several angiogenic disorders, including cancer [19]. Downregulation of antiangiogenic VEGF has been documented qualitatively (relative quantification) in the diabetic retina [20]. Pro- and antiangiogenic homeostasis of VEGF isoforms in patients with persistent hyperglycemia seems crucial but has not been explored quantitatively. Therefore, in the present study we

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aimed to estimate the differences between the pro ($VEGF_{165}a$) and antiangiogenic ($VEGF_{165}b$) VEGF isoforms i.e. $VEGF_{165}a$-b and the soluble form of the receptor for different severity of DR among patients with type 2 diabetes.

METHOD

Study subjects: The study included 123 individuals (cases: 81, diabetic controls: 20, non-diabetic controls: 22). Patients with DR were recruited at the retina clinic at the Regional Institute of Ophthalmology, Kolkata, India, and the diabetic controls were recruited at the center's diabetes clinic. The present study was restricted to individuals with type 2 diabetes. Non-diabetic controls were enrolled from among volunteers who work at the clinic. Approval from the institutional ethics board (Regional Medical Research Centre, North East Region, Indian Council of Medical Research, Dibrugarh) and written informed consent from each participant were obtained in accordance with the Declaration of Helsinki along with the ARVO guideline for utilizing human samples..

Diagnosis of diabetes mellitus (DM) was done according to the World Health Organization (WHO) criteria [21]. Diabetic macular edema (DME) and the severity of DR were measured by two trained ophthalmologists using dilated fundus examination with slit-lamp biomicroscopy with +90 D and three-mirror lens, seven-field digital fundus photography with fluorescence angiography and optical coherence tomography (OCT). Grading and severity of retinopathy assessed through modified Early Treatment Diabetic Retinopathy Study (ETDRS) and letter scores were documented according to the Snellen chart [22]. The patients with DR were further cross-classified into focal DME with mild to moderate nonproliferative DR (NPDR; n = 27), focal DME with severe NPDR (n = 12), diffuse DME with severe NPDR (n = 18), and diffuse DME with proliferative DR (n = 24).

Individuals with coronary artery disease (CAD), peripheral vascular diseases, history of any thrombotic event, acute infection, or any other ocular disorder, such as glaucoma, branch retinal venous occlusion, and Eales disease) were excluded from the study. To exclude patients with overt diabetic nephropathy, patients with a microalbumin-creatinine ratio >30 mg/gm and urinary microalbumin level >300 mg/day were excluded from the study [23]. Demographic characteristics, duration of diabetes (DOD), HbA1, and urinary microalbumin-creatinine ratio data were collected from the clinic on a structured questionnaire.

Sample collection and laboratory investigation: Blood samples were collected by venipuncture in an EDTA vial. Plasma was separated by centrifugation (2000 ×g for 5 min) and stored at −80 °C until the assay was performed. Plasma $VEGF_{165}a$ (proangiogenic), $VEGF_{165}b$ (antiangiogenic), soluble VEGF receptor1 (sVEGFR1), sVEGFR2, and sVEGFR3 concentrations were determined in replicate with enzyme-linke immunosorbent assay (ELISA) using a commercially available kits (My Biosourse, San Diego, CA and Raybiotech, Norcross, GA) according to the manufacturers' instructions. Details of the commercially used kits are presented in Appendix 1.Commercially available ELISA kits confirmed that they do not have any cross reactivity with its close analog.

Statistical analysis: Demographic characteristics, such as age, DOD, urinary microalbumin level, and glycemic and nutritional status (total protein), were compared among the study groups using the two-tailed Student *t* test and one-way ANOVA where applicable (Table 1A). Clinical phenotype in terms of severity and visual acuity among the patients with DR is presented in Table 1B.

We compared the protein level of $VEGF_{165}a$, $VEGF_{165}b$, VEGFR1, VEGFR2, and VEGFR3 among the patients with DR, the diabetic controls and the non-diabetic controls with one-way ANOVA. The protein levels of $VEGF_{165}a$, $VEGF_{165}b$, difference of $VEGF_{165}a$ and $VEGF_{165}b$, and VEGFR2 were further analyzed with one-way ANOVA and Tukey's test among the cross-classified DR group according to the clinical stratification. Distribution of the protein levels among the different clinical phenotypes of DR is presented in a box whisker plot. Data were presented in mean ±Standard Deviation (SD). P value considered significant at less than 0.05 and further it was adjusted with multiple testing correction where ever applicable. All statistical analysis was performed using R3.1 [24]. Figures and graphs were generated in Prism (Graph Pad: La Jolla, CA) [25].

RESULTS

Age, sex, and nutritional status (total protein) were matched among the non-diabetic controls, diabetic controls, and DR groups ($p>0.05$). Glycemic levels (HbA1c) were matched among the diabetic controls and the patients with DR ($p = 0.06$). The DOD was statistically significantly higher among the patients with DR (10.4 ± 3.6 years) compared with the diabetic controls (6.5 ± 2.5 years; $p<0.0001$). The demographic and clinical characteristics and visual acuity for the different phenotypes of DR are presented in Table 1.

The plasma $VEGF_{165}a$, $VEGF_{165}b$, and soluble form of VEGFR2 (sVEGFR2) concentrations were statistically significantly elevated among the patients with DR compared to the diabetic controls and the non-diabetic controls ($VEGF_{165}a$, difference of $VEGF_{165}a$ and $VEGF_{165}b$, and VEGFR2: $p<0.0001$; $VEGF_{165}b$: $p = 0.0002$) Table 2. The

Table 1. Represents the demographic and clinical characteristics among the study groups.

A. Demographic Features among cases and controls

Characteristics	DR (n=81)	DC (n=20)	NDC (n=22)	P-value
Age	57.6±7.17	55.5±7.85	57.3±7.8	0.75
Male	60	17	14	
Female	21	3	8	
HbA1c (%)	7.6±0.6	7.9±0.7	-	0.06
Urinary microalbumin creatinin ratio	21.6±6.2	18.8±5.9	-	0.08
Duration of diabetes	10.4±36	6.5±2.5	-	<0.001

Data presented here in mean ± SD

B. Clinical Characteristics among the cases

Visual acuity		
DR Phenotype	ETDRS CHART	SNELLS CHART
Focal DME with MNPDR (n=27)	20/50 – 20/40	80 - 85
Focal DME with SNPDR (n=12)	20/100 – 20/63	65 - 75
Diffuse DME with SNPDR (n=18)	20/100 – 20/63	65 - 75
Diffuse DME with PDR (n=24)	20/200 – 20/160	50 – 55

study demonstrated that the plasma $VEGF_{165}b$ ($p = 0.002$) and sVEGFR2 ($p = 0.0003$) concentrations were statistically significantly elevated among the diabetic controls compared with the non-diabetic controls (Appendix 1).

The levels of $VEGF_{165}a$ concomitantly increased with the severity of the disease ($p_{anova}<0.0001$). Further, Tukey's test across the subgroup revealed that the elevation level was not constantly statistically significant across all clinical phenotypes of DR. We did not find a statistically significant difference among focal DME with moderate NPDR, severe NPDR, and diffuse DME with severe NPDR (Appendix 1).

We observed a concomitant decreased trend of $VEGF_{165}b$ with the severity of DR ($p_{anova} = 0.0002$) although Tukey's test among the different phenotypes of DR revealed that the decreased trend is not significant among focal and diffuse DME with mild and severe nonproliferative DR (Appendix 1).

The differences between $VEGF_{165}a$ and $VEGF_{165}b$ concomitantly increased with the severity of the disease statistically significantly ($p<0.0001$) as the patients with diffuse DME with PDR showed the highest difference ($VEGF_{165}a$ and $VEGF_{165}b$: 232.1 ± 119.4 pg/ml). The patients with diffuse DME with severe NPDR had a higher imbalance between the pro- and antiangiogenic isoforms of VEGF (difference of $VEGF_{165}a$ and $VEGF_{165}b$; 180.2 ± 62.59 pg/ml) compared with the patients with focal DME with moderate NPDR (146.3±46.48 pg/ml; $p<0.01$). The difference was lowest (81.65 ± 62.24 pg/ml) among the patients with focal DME with mild to moderate NPDR ($p<0.0001$; Figure 1). Further, Tukey's test demonstrated that the levels of $VEGF_{165}a$

Table 2. Distribution of growth factors (VEGF) and its receptors among cases and controls.

Study phenotype variables	Non diabetic control (n=22)	Diabetic control (n=20)	Diabetic retinopathy (n=81)	P value
VEGF165a (pg/ml)	108.9±39.9	123.6±45.09	208.5±93.23	<0.0001*
VEGF165b (pg/ml)	26.75±14.35	45.94±22.86	56.27±33.08	0.0002*
VEGF165a-b (pg/ml)	52.16±40.06	61.38±42.54	157.70±101.2	<0.0001*
VEGFR1 (ng/ml)	26.97±10.38	21.00±16.75	19.81±16.14	0.15
VEGFR2 (pg/ml)	26.75±14.35	49.94±22.86	56.27±33.08	0.002*
VEGFR3 (pg/ml)	23.45±19.99	17.34±15.02	17.68±14.46	0.3
CMT (um)	-	213±102	319±181	<0.0001*

Data presented in Mean±SD among the study groups. *p value significant *after multiple testing correction (Bonferroni's correction). Adjusted p value after correction was 0.007.

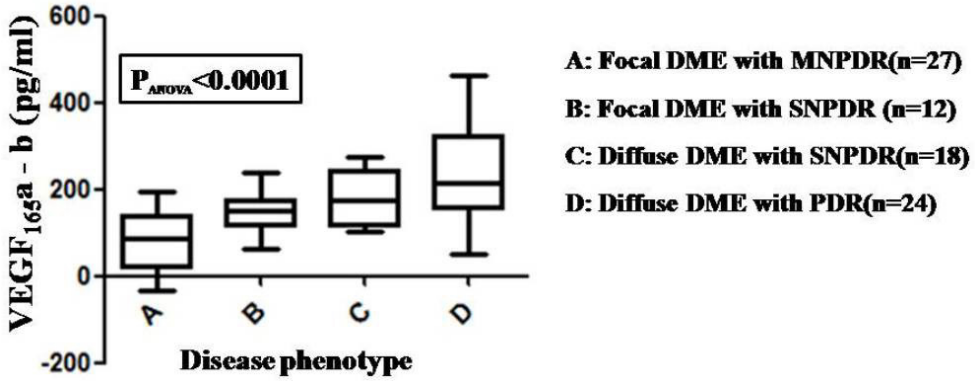

Figure 1. Box whisker plot represents the distributional difference of $VEGF_{165}$a-b (pg/ml) among the different phenotypes (grades or severity) of DR. Plasma concentration of $VEGF_{165}$a-b consistently increased during severity of the disease in significant manner (P_{anova}<0.0001). Further Tukey's Multiple Comparison Test revealed that $VEGF_{165}$a-b significantly elevated among Diffuse DME with SNPDR (**C**) and PDR (D) compared to Focal DME with MNPDR (**A**), P_{tukeys} <0.001 (**A** vs **C**) and P_{tukeys} <0.0001 (**A** vs **D**). Diffuse DME with PDR (**D**) further significantly elevated compared to Focal DME with SNPDR(B); P_{tukeys} <0.001 (**B** vs **D**). Level of $VEGF_{165}$a-b among Diffuse DME with PDR (**D**) significantly elevated compared to Diffuse DME with SNPDR (**C**), P_{tukeys} <0.01 (**C** vs **D**).Data are mean± SD, sample size (n) as indicatedin figure legend.

and $VEGF_{165}$b differed statistically significantly among the patients with focal and diffuse moderate NPDR and severe NPDR (Figure 1 and Appendix 1).

The plasma sVEGFR2 concentration consistently increased with the severity of the disease statistically significantly (p<0.0001). The patients with mild to moderate NPDR had lowest amount of sVEGFR2 (43.56 ± 36.65 pg/ml) whereas the patients with diffuse DME with PDR had highest level of sVEGFR2 (76.6 ± 31.71 pg/ml; Figure 2). Tukey's test revealed that VEGFR2 crucially differentiates the patients with PDR and severe NPDR from those with diffuse DME and focal moderate NPDR (Appendix 1). Additionally, sVEGFR2 was statistically significantly elevated among the diabetic controls compared with the non-diabetic controls (p = 0.002). We did not find a statistically significant alteration in the plasma VEGFR1 (p = 0.16) and VEGFR3 (p = 0.3) concentrations between the patients and the controls.

The difference between the pro- and antiangiogenic VEGF isoforms ($VEGF_{165}$a and $VEGF_{165}$b) was moderately correlated with the central macular thickness (p = 0.00003, Pearson; r = 0.54). The study did not reveal any significant correlation between VEGFR2 and CMT (p = 0.23) and $VEGF_{165}$a and $VEGF_{165}$b (p = 0.13; see Appendix 1).

DISCUSSION

VEGF has been documented as a potent angiogenic factor for the genesis of several angiogenic disorders, including DR [10,26]. There is a substantial lack of knowledge about

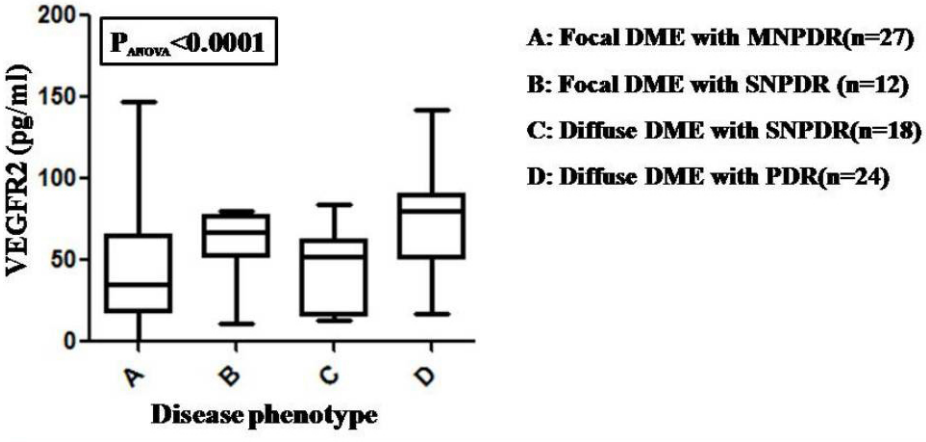

Figure 2. Box whisker plot represents the distributional difference of sVEGFR2 (pg/ml) among the different phenotypes (grades or severity) of DR. Plasma concentration of sVEGFR2 consistently increased during severity of the disease in significant manner (P_{anova} : 0.001). Further Tukey's Multiple Comparison Test revealed that VEGFR2 significantly elevated among Diffuse DME with PDR (**D**) compared to Focal DME with MNPDR (**A**), P_{tukeys} <0.001 (**A** vs **D**) Diffuse DME with SNPDR P_{tukeys} : 0.001 (**C** vs **D**). Data are mean± SD, sample size (n) as indicatedin figure legend.

the pro- and antiangiogenic imbalance between the VEGF isoforms for understanding the DR pathomechanism although it has been postulated that VEGF plays a pivotal role in the severity of DR.

The present study showed that increased differences between plasma $VEGF_{165}$a and $VEGF_{165}$b and elevated levels of sVEGFR2 are associated with the severity of retinopathy among individuals with type 2 diabetes (Figure 1, Figure 2, and Appendix 1). Increased differences between the pro- and antiangiogenic VEGF isoforms ($VEGF_{165}$a and $VEGF_{165}$a b) among the diabetic control group compared with the non-diabetic control group supports that the pro- and anti-angiogenic switch of VEGF via alternative splicing depends on host cellular physiology, and hyperglycemia may favor angiogenic switching (Table 2). We observed a concomitant increased trend of the plasma $VEGF_{165}$a (the proangiogenic isoform) concentration along with a consistent decreased level of $VEGF_{165}$b (the antiangiogenic isoform) for the adverse prognosis of the disease (Table 2, Figure 1, and Appendix 13). The observations support that persistent hyperglycemia (duration of diabetes: 10.4 ± 3.6 years) may favor an adverse prognosis of retinal angiogenesis or vascular permeability via inactivation of the antiangiogenic VEGF isoform ($VEGF_{165}$b) with the persistent activation of the angiogenic isoform ($VEGF_{165}$a) and its downstream signaling cascades. The study further demonstrated that the proangiogenic $VEGF_{165}$a isoform concomitantly increased with the severity but alone cannot explain the entire phenotypes of DR during the adverse prognosis of the disease at least for focal and diffuse DME with severe nonproliferative DR (Appendix 1). The plasma $VEGF_{165}$b concentration also was not able to explain the clinical phenotypes of DR, but the difference or imbalance between the plasma $VEGF_{165}$a (angiogenic) and $VEGF_{165}$b (antiangiogenic) concentrations seems to be a better explanatory marker for an adverse prognosis of DR compared to either isoform (pro- or antiangiogenic VEGF) of VEGF alone (Figure 1 and Appendix 1). The imbalance between the VEGF isoforms $VEGF_{165}$a and $VEGF_{165}$b differed statistically significantly among the phenotypes focal and diffuse moderate NPDR and severe NPDR.

The exact mechanism for splicing (i.e., pro- and anti-angiogenic switching of VEGF) is still enigmatic [27]. The identification of two opposite families of VEGF isoforms due to differential sequence selection on exon 8 was overlooked, and these findings require comprehensive revision of the understanding of VEGF-mediated pathobiology, including DR [27,28]. The *VEGF* gene is unusually polymorphic [29], and several loci have been associated with DR among different populations [11,30]. Until now, no polymorphic loci have been identified in exon 8b of the *VEGF* gene that implies its conserved nature across the population with immense functional impact [26]. Studies demonstrated that the anti-angiogenic isoform of VEGF inhibits ocular angiogenesis in mice models of retinopathy, age-related macular degeneration, and cancer [30-33]. Endogenous antiangiogenic VEGF seems cytoprotective for endothelial, epithelial, and neuronal cells [34]. Antineovascular therapies (i.e., anti-VEGF therapy in cancer and eye diseases) have prompted interest in the mechanisms behind the initiation, development, and refinement to vasculature [35,36]. Previously, the semiquantitative approach (relative quantification) revealed that $VEGF_{165}$b (antiangiogenic VEGF isoforms) was downregulated in the vitreous and the retina in DR [20]. In the present study, for the first time we quantitatively documented the plasma $VEGF_{165}$b level, as well as the imbalance between pro- and antiangiogenic VEGF homeostasis, namely, the differences between the pro- and antiangiogenic VEGF isoforms ($VEGF_{165}$a and $VEGF_{165}$b) for an adverse prognosis of DR.

This study showed that the plasma sVEGFR2 concentration is statistically significantly elevated in severe DR ($p_{anova<}$0.0001), but plasma sVEGFR1 and sVEGFR3 remained unaltered among the study patients and controls (Table 2). It has been documented that neovascularization and vascular permeability are centrally modulated by VEGF along with its three receptors [37]. A previous study showed that the pro- and antiangiogenic $VEGF_{165}$ isoforms ($VEGF_{165}$a and $VEGF_{165}$b) have similar binding affinity to VEGFR2, but $VEGF_{165}$b loses its angiogenic property due to the loss of the formation and phosphorylation of the VEGFR2/NRP1 complex. The reduced level of $VEGF_{165}$b may be crucial for impairment of antiangiogenic downstream signaling that may ultimately favor the adverse prognosis for disorders related to neovascularization. Previous reports [20] and the present observation postulated that the reduced level of $VEGF_{165}$b and the increased differences between the pro- and antiangiogenic VEGF isoforms ($VEGF_{165}$a and $VEGF_{165}$b) may favor the formation and phosphorylation of the VEGFR2/NRP1 complex. The scenario may activate angiogenesis due to the increased abundancy of the proangiogenic VEGF isoform ($VEGF_{165}$a) through its receptors, such as VEGFR2. The imbalance between pro- and antiangiogenic VEGF homeostasis ($VEGF_{165}$a and $VEGF_{165}$b) further crucially explains the anatomic alteration of the diabetic eye that may ultimately lead to vision impairment as the present study revealed that $VEGF_{165}$a and $VEGF_{165}$b were moderately correlated in the positive direction with central macular thickness (Appendix 1). Increased imbalance between pro- and antiangiogenic homeostasis of the VEGF isoforms ($VEGF_{165}$a and $VEGF_{165}$b) seems crucial for adverse prognosis of DR and may be a

better explanatory marker compared with either isoform of VEGF (proangiogenic $VEGF_{165}$a or antiangiogenic $VEGF_{165}$b) or VEGFR2 alone for different phenotypes of DR. The in vitro study demonstrated that hypoxia-regulated overexpression of sVEGFR2 controls angiogenesis, namely, tumor growth. In contrast, studies showed that increased levels of sVEGFR2 are associated with breast cancer [38] and vitreoretinal lymphoma [39]. Details of the functional interaction of sVEGFR2 on vascular permeability or angiogenesis are enigmatic, but our observation suggests that the increase in plasma sVEGFR2 is statistically significantly associated with an adverse prognosis for DR.

The present study provides a new horizon for a deeper understanding of the pathophysiology of DR in light of alternative splicing of VEGF to find better biomarkers to explain the disease phenotypes. Detailed functional studies on VEGF splicing and identification of splice regulators are required to understand the disease pathology. In addition, other pro- ($VEGF_{xxx}$a) and antiangiogenic ($VEGF_{xxx}$b) VEGF isoform interaction seems crucial for disease etiology. Recently, another VEGF isoform (VEGFAx) was identified with contradictory functional capability as it has been documented as a negative regulator of tumor angiogenesis [40]. Another report established VEGFAx is a weak stimulator of mitogenesis and vascular permeability on endothelial cells [41]. The recent developments in understanding of the biology of VEGF may encourage the scientific community to revisit the VEGF-mediated pathophysiology of DR to quantitate the VEGF isoforms (pro- and antiangiogenic) and their interaction with VEGF receptors that is lacking to understand the disease biology. The present study for the first time quantitatively measured the imbalance between the pro- and antiangiogenic VEGF isoforms to understand DR pathobiology after semiquantitative observations in the diabetic retina. Randomized controlled trials of anti-VEGF therapy revealed that the vision of only 31% of the patients improved >2 lines on the ETDRS scale. Limited success and significant cost seem to be a crucial barrier for this promising anti-VEGF therapy as an effective biomarker that could predict outcomes. Future studies focused on pro- and antiangiogenic VEGF isoforms and their homeostasis may be crucial to identify pretherapeutic markers of anti-VEGF therapy. $VEGF_{165}$b is a promising therapeutic candidate for several angiogenic disorders in animal models. Therefore, we believe our observation (i.e., pro- and antiangiogenic difference between the $VEGF_{165}$ isoforms among different disease phenotypes of DR in a quantitative manner) is crucial for future research on therapeutic agents.

The study was limited due to its sample size. Another replicative study in a larger cohort is required. The duration of diabetes among the diabetic control and DR groups may act as a confounder as it was not matched. It is well-established that prolonged hyperglycemia leads to complications, such as DR. Thus, it is difficult to match the duration of diabetes among the patients and controls. Further, the protein data generated by ELISA had less than 15% inter- and intra-assay coefficient variation (CV). Isoforms of VEGF molecules other than $VEGF_{165}$a and $VEGF_{165}$b were not analyzed; however, previous studies demonstrated that $VEGF_{165}$ is the most dominant isoform of the VEGF family to explain angiogenic disorders [42].

APPENDIX 1. SUPPLEMENTARY DATA.

To access the data, click or select the words "Appendix 1."

ACKNOWLEDGMENTS

The author sincerely thanks Dr. Analabha Basu, the associate professor of National Institute of Biomedical Genomics for his assistance in study design and analysis. The fund was obtained from Indian Council of Medical Research, Govt. of India and grant ID is ICMRPDF319. **Author Contribution:** The grant for the study was received by SKP and was involved in study design, data generation and analysis. JM acts as co-corresponding author and responsible for study design and overall coordination of the study. Clinical phenotyping and diagnosis was done by LKM. PKB and CB were responsible for sampling, data generation and assisted in analysis. All the authors actively involved in manuscript preparation. **Declaration of funding**: This work was financially supported by Indian Council of Medical Research, New Delhi. **Statement**: Existence of pro and anti angiogenic isoforms insist the scientific community to revisit several angiogenic disease mechanism including cancers in light of alternative splicing of VEGF transcripts. Studies on Diabetic Retinopathy (DR) are extremely limited though previous study demonstrated the downregulation (relative quantification) of anti angiogenic VEGF isoforms in diabetic eye. The present study tried to explain pro and anti angiogenic VEGF homeostasis for different degrees of DR in quantitative manner. Increased difference of pro and anti angiogenic homestasis of VEGF isoforms ($VEGF_{165}$a-b) seems crucial for adverse prognosis of DR and may play better explainable marker compared to either isoform of $VEGF_{165}$. Suman K Paine(painesuman01@gmail.com) and Jagadish Mahanta (jmahanta@gmail.com) are co-corresponding authors for this paper.

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