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Original Research Article

Role of VEGF in Prediction and Diagnosis of Severe Dengue Viral Illness

Rohit Jagar[1], Ankita Singh[2], Champa Kumari Saini[3], Prakash Keswani[4]

[1,2,3]Resident, Department of General Medicine, SMS Medical College and Attached Hospitals, Jaipur, Rajasthan.

[4]Senior Professor & Unit Head, Department of General Medicine, SMS Medical College and Attached Hospitals, Jaipur, Rajasthan.

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Corresponding author: Dr Champa Kumari Saini

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Abstract

Detectable VEGF levels in plasma before, during, and after the identification of severe dengue, could potentially offer various clinical usage. This protein can serve as predictive markers for determining patient's need for admission, as a diagnostic marker or as a prognostic marker to determine the likelihood of death.

Method: The present study is a hospital-based cross-sectional study conducted at for the period of 15 months. A convenient sample size of 80 cases and 10 controls was chosen by using simple random sampling. Confirmed cases of dengue were taken for study as per the inclusion and exclusion criteria. Patients were evaluated by taking complete history of the present febrile illness, patient demographics, presence of WHO warning signs for severe dengue, associated co-morbidities, and length of hospital stay. Complete blood count, hematocrit, platelet count and platelet volume were noted at the time of admission and at 48 hours along with ferritin and triglyceride levels at admission were noted. Serum VEGF level was measured in cases at day 5 of hospitalization after the diagnosis.

Results: The mean age of cases was 39.86 ± 14.83 years, while that of controls was 40.25 ± 13.30 years with male : female ratio of 1.14:1 while that of controls was 1.1. 3.42% patients had non-severe dengue, while the remaining 26.58% patients had severe dengue. The most common complication in our patients included bleeding manifestations. There was an insignificant difference in over hematocrit of our patients from day 1 to day 3 and day 5. The mean VEGF level was 863.301 ± 316.673 ng/ml. While the mean VEGF level in controls was 287.830 ± 187.612 ng/ml and the difference between cases and controls was significant (p value=0.0001). The mean VEGF of non-severe cases was 658.373 ± 146.166 ng/ml, while that of severe cases was 1053.087 ± 458.384 ng/ml. Similarly, cases with severe illness had significantly higher VEGF than non-severe cases (p value<0.0001). Overall, the sensitivity and specificity of serum VEGF in predicting the severity of dengue was is 95.24% and 77.58%, respectively. The positive and negative predictive value of VEGF was 66.66% and 97.8%, respectively. The accuracy was 76.76%. There was a negative correlation between platelet count and serum VEGF in patients, the correlation coefficient being R= -0.29 (p value=0.001).

Conclusion: In conclusion, plasma leakage is a cardinal hallmark of severe dengue. We demonstrated that plasma VEGF levels in cases with severe dengue were significantly higher than values from a mild illness. The increased plasma levels of VEGF in patients with dengue illness were correlated positively with haematocrit levels, and negatively with low platelet count. These findings suggest that VEGF, as a contributory factor of increasing vascular

permeability, contributes to the pathogenesis of dengue. VEGF may serve as an adjunct investigation to support the diagnosis.

Keywords: VEGF Levels, Dengue, Co-Morbidities, Ferritin and Triglyceride.

Introduction

Dengue is one of the most prevalent arthropod-borne diseases and is a major public health problem worldwide. It is an important cause of morbidity and mortality in tropical and subtropical areas with around 2.5 billion people living in areas at risk [1]. Frequency of dengue infection has increased in India, and it has now become a major public health problem [2].

Dengue clinical manifestations are divided into three phases, namely, febrile, critical and recovery phase [3]. Critical phase is the most important phase where the patient can either recover or succumb to the illness [4,5]. Unfortunately, the critical phase is often difficult to predict even with the usage of the latest classification recommended by the WHO 2009 Clinical Practice Guideline [6,7]. The warning signs listed in the WHO 2009 classification are predictors for a severe course of dengue. Hence, patients presenting with these warning signs must be admitted to the hospital for close monitoring. However, both the specificity and the positive predictive value of the listed signs are poor which could sometimes lead to high false positive rates [6]. In addition, the clinicians are at times unable to predict the severity accurately, leading to unnecessary hospital admissions for intensive monitoring [8]. Hence, this can pose an impact on the healthcare cost and workforce.

To reduce the burden of the disease, an optimally accurate predictive or screening method is essential [9]. This method should be easily performed and cheap enough to be applied in a dengue-endemic population. This will allow safe triaging of the patients to reduce unnecessary hospital admissions.

In response to the illness there are various pathophysiological changes in the body [10]. The disturbance in haemostasis involving thrombocytopenia, coagulopathy and vascular changes are seen frequently during progressive stage of disease [11-19].

Various biomarker tests have also emerged as a reliable indicator for diagnosing severe dengue [19,20]. These biomarkers for dengue severity are generally divided into two techniques: enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). VEGF is amongst the most accepted potential biomarkers in differentiating severe dengue from non-severe dengue cases. Both VEGF and PTX-3 are involved in the pathogenesis of dengue virus infection. VEGF is specifically involved in plasma leakage when the endothelial cells in the blood vessels are damaged, releasing VEGF as a response to repair the damaged cells. VEGF and cytokines are released into the circulation from dengue-infected mast cells, vascular endothelium, platelet, and macrophages. VEGF and other similar cytokines could stimulate other biochemicals such as platelet-activating factor (PAF) to increase the vascular permeability through the activation of receptors on the endothelial cells. It is a member of growing family of related proteins that includes VEGF B, VEGF C, VEGF D and placental growth factor. At least two types of VEGF receptors are expressed on endothelial cells. Both the receptors are transmembrane receptors tyrosine kinases. VEGF promotes angiogenesis and vascular integrity [13].

Detectable VEGF levels in plasma before, during, and after the identification of severe dengue, could potentially offer various clinical usage. This protein can serve as predictive markers for determining patient's need for admission, as a diagnostic marker or as a prognostic marker to determine the likelihood of death. However, many studies conducted till date could not substantiate its use as predictive, diagnostic, and prognostic markers because of the lack of generalizability and inappropriate study design [13].

Owing to the complex role of VEGF in the pathogenesis of dengue, we conducted this study to evaluate VEGF as a predictive and diagnostic marker in differentiating severe dengue from non-severe dengue.

Methods

The present study was a hospital-based cross-sectional study conducted at for the period of 15 months from

The study was commenced after obtaining clearance from both the Institutional Ethics Committee and the Scientific Review Committee.

Sample size: A convenient sample size of 80 cases and 10 controls was chosen by using simple random sampling.

Case definition

Inclusion criteria

1. Confirmed cases of Dengue IgM positive, NS1 Ag positive later confirmed by ELISA (Age > 18 yrs)
2. Patients with symptoms of dengue fever based on WHO criteria.
3. Those willing to provide informed consent and comply with protocol requirements.

Exclusion Criteria

1. <18 years of age
2. Unwilling to provide consent.
3. Concomitant illness like malaria, enteric fever, chikungunya, and other inflammatory disorders.
4. Patient with pre-existing chronic disease.
5. Patient with severe anaemia (<7mg/dl) or iron overload

Laboratory Analysis

For all patients, the following clinical and biochemical data was collected as per our study protocol. History of the present febrile illness, patient demographics, presence of WHO warning signs for severe dengue, associated co-morbidities, and length of hospital stay.

Complete blood count, hematocrit, platelet count and platelet volume were noted at the time of admission and at 48 hours along with ferritin and triglyceride levels at admission were noted. Serum VEGF level was measured in cases at day 5 of hospitalization after the diagnosis. Clinical and platelet count monitoring were monitored daily. Patients were classified as severe and non-severe according to 2009 WHO criteria [60].

Statistical Analysis

Descriptive statistics was performed by calculating mean and standard deviation for the continuous variables. Categorical variables were presented as absolute numbers and percentage. Nominal categorical data between the groups were compared. Chi-square test was used to compare categorical variables. Student T test (paired and unpaired) and Z test to compare mean of quantitative variables.

The p-value was taken significant when less than 0.05 ($p<0.05$) and a confidence interval of 95% was taken. Data was entered in Microsoft Excel and was subsequently imported to Statistical package for the social sciences (SPSS) 26.

Results

In the present study we included 80 cases and 10 controls in our study. The general characteristics of cases and controls is shown in table no. 1.

In our study, we categorized cases as severe and non-severe according to the 2009 WHO criteria. Out of total 80 patients, 71.25% patients had non-severe dengue, and 26.58% patients had severe dengue. [Image 2] Most patients with severe dengue had fever for 1-2 days at the time of admission (95.2%), while most non-severe cases had fever for 5-6 days (41.4%). This shows statistical significance (p-value = 0.001). Average number of days of fever at admission was 4.5 ± 1.9 days. The most common manifestations in patients were bleeding manifestations in 22.5% cases, followed by oedema in 18.75% cases, dehydration in 17.50% cases, and shock in 5.0% cases. [Image 3] Bleeding manifestations were present in 22.5% cases. The most common form of bleeding manifestation was purpura and petechiae seen in 21.5% cases, followed by gum bleeding in 15.2%, epistaxis seen in 11.4%, subconjunctival haemorrhage in 8.9%, melena in 5.1%, hematemesis in 3.8%, while 2.5% cases had haematuria. [Image 4]

Table 1: General Charaterstics Of Cases And Controls

		Cases (N=80)		Controls (N=10)		
		No. of patients	**Percent**	**No. of patients**	**Percent**	**p-value**
Age in years	< 20	4	5.00%	1	10.00%	0.94
	20-29	18	22.50%	1	10.00%	
	30-39	18	22.50%	1	10.00%	
	40-49	19	23.75%	3	30.00%	
	50-59	13	16.25%	2	20.00%	
	60-69	5	6.25%	1	10.00%	
	≥ 70	3	3.75%	1	10.00%	
Gender	Females	37	46.25%	6	60.00%	0.78
	Males	43	53.75%	4	40.00%	
Demography	Rural	29	36.25%	4	40.00%	0.53
	Urban	51	63.75%	6	60.00%	

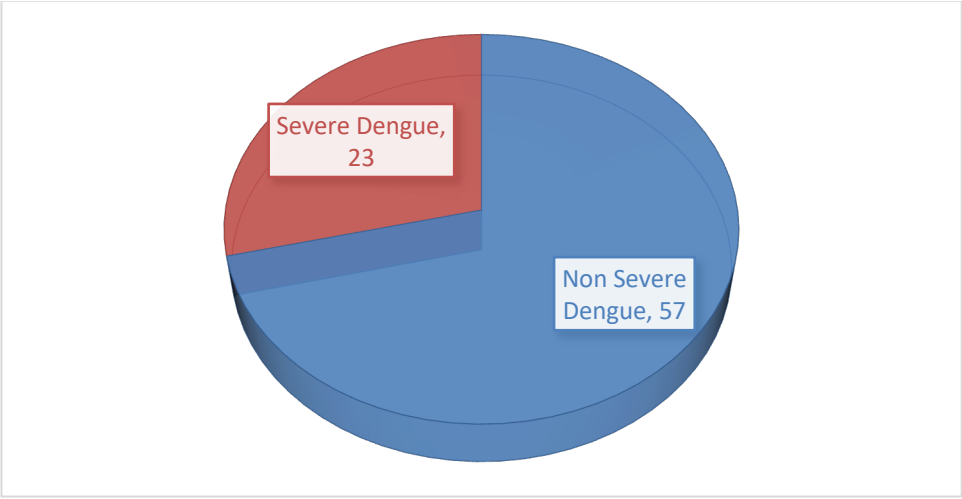

Figure 1: Distribution of cases according to disease severity

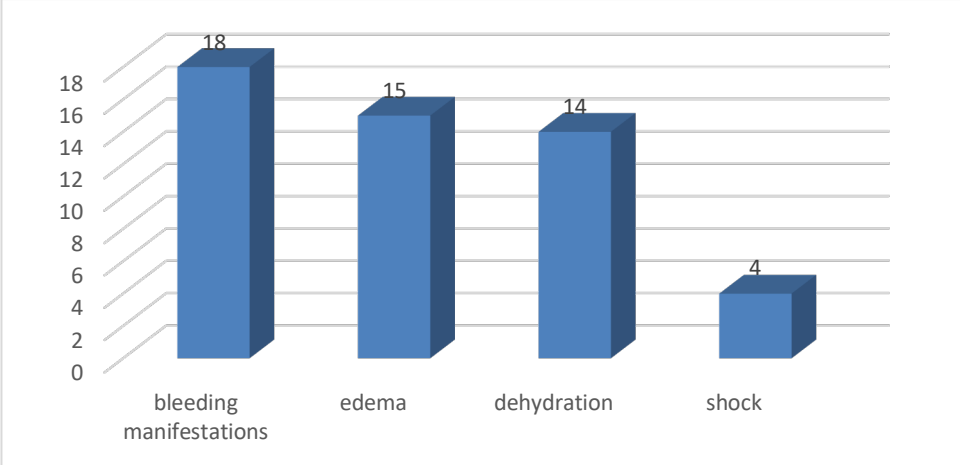

Figure 2: Manifestations of severe dengue

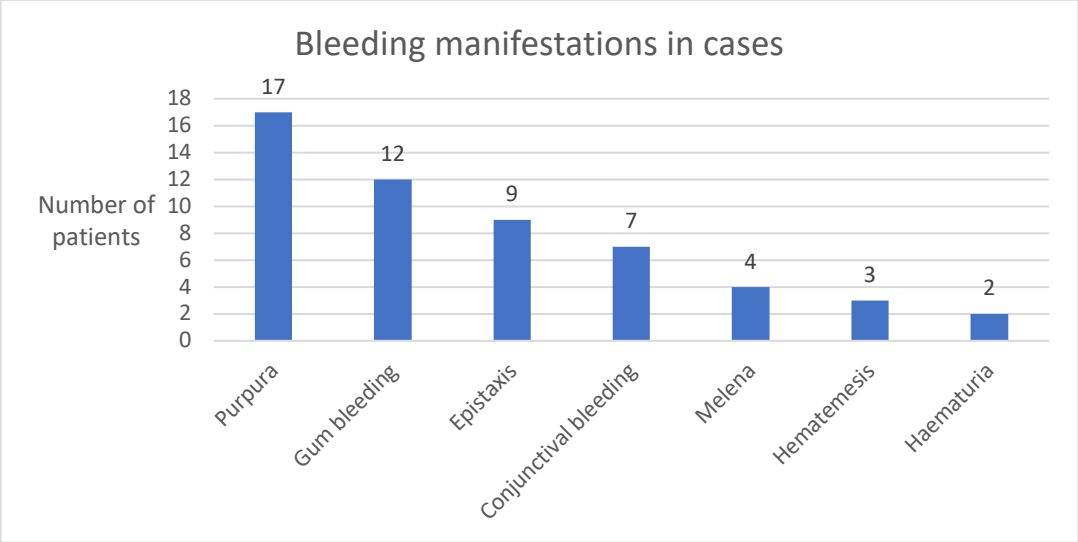

Figure 3: Bleeding manifestations of severe dengue

For comparing the kinetics of haematological profile in our subjects, the parameters on Day 3 and Day 5 were compared with the baseline (day 1 or admission). The mean total leukocyte count (TLC), the mean platelet count of patients on day 1 and day 3 showed statistically nonsignificant variation whereas there was statistically significant change on day 5. There was a significant difference in haematocrit of patients from day 1 to day 3 (p-value=0.01), and day 5 (p-value=0.02). While the change in haemoglobin from day 1 to day 3 and day 1 to day 5 was insignificant (p-value=0.91 and 0.08).

Table 2: Comparison Of Haematological Parameters Of Study Group On Day 1, Day 3 And Day 5 Of Hospitalization

	MeanTLC (per mm3)	**Mean platelet count (10^3/ml)**	**Mean platelet volume (fL)**	**Hematocrit (%)**	**Hb (g/dL)**
Day 1	5417.7 ± 1023.3	99.8 ± 24.7	9.05 ± 0.79	54.2 ± 3.4	11.5 ± 2.1
Day 3	4627.2 ± 786.3	108.3 ± 43.6	10.09 ± 0.80	56.2 ± 3.3	11.7 ± 1.9
p-value	0.061	0.131	0.558	0.01	0.91
Day 5	3164.62 ± 537.1	125.3 ± 52.1	10.34 ± 0.94	57.5 ± 4.1	12.1 ± 2.5
p-value	0.032	0.001	0.478	0.02	0.08

The levels of VEGF ranged between 482.854 pg/ml to 2382.070 pg/ml in dengue cases with a mean level 852.301 ± 305.673 ng/ml. While the level of VEGF in control ranged from 69.513 pg/ml to 567.786 pg/ml, with a mean level of 287.830 ± 187.612 ng/ml. [Table no. 5] We observed that cases had a significantly higher VEGF level than controls (p-value=0.0001). In our study the mean VEGF of non-severe cases was 658.373 ± 146.166 ng/ml, while that of severe cases was 1053.087 ± 458.384 ng/ml. Similarly, cases with severe illness had significantly higher VEGF than non-severe cases (p-value<0.0001). [Table no. 5] The median value of VEGF in non-severe cases was 660.96 ng/ml, in severe cases was 967.77 ng/ml, and in controls was 262.427 ng/ml. [Image 5]

Table 3: Serum VEGF Of Cases And Controls On Day 5 Of Hospitalization

Serum VEGF (pg/ml)	Cases		Controls	
	N=80		N=10	
	No. of patients	percent	No. of patients	percent
<400	0	0.00%	5	50.00%
400-700	21	26.25%	4	40.00%
701-1000	48	60.00%	1	10.00%
1001-1300	6	7.50%	0	0.00%
>1300	5	6.25%	0	0.00%
Total	80	100.00%	10	100.00%
Mean	852.301 ± 305.673 ng/ml		287.830 ± 187.612 ng/ml	
p-value=0.0001.				

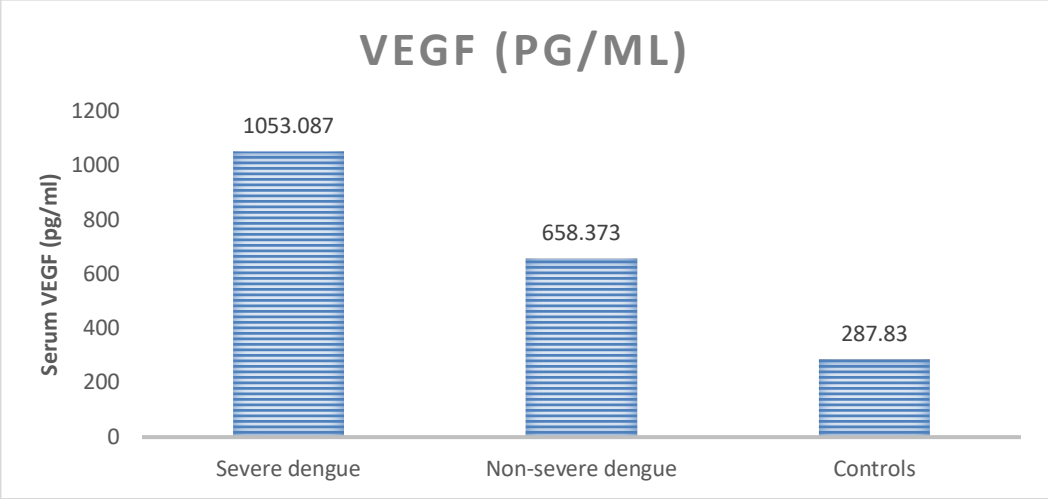

Image 4: Mean VEGF level in severe cases, non-severe cases and controls.

The mean platelet count on day 1 was 101.1 ± 12.4 (10^3/ml), on day 3 was 109.8 ± 17.8 (10^3/ml), while that on day 5 was 137.2 ± 21.9 (10^3/ml). There was a significant rise from day 1 to day 5 (p-value=0.04). The platelet count of patients on day 1 ranged from 75 to 332 10^3/ml with mean 98.8 ± 24.7 x 10^3/ml. On day 3 the counts ranged from 75 to 382 x 10^3/ml with mean 108.3 ± 43.6 x10^3 /ml. While on day 5 it ranged from 550 to 417 x 10^3/ml on day 5 with mean of 129.2 ± 56.3 x 10^3/ml

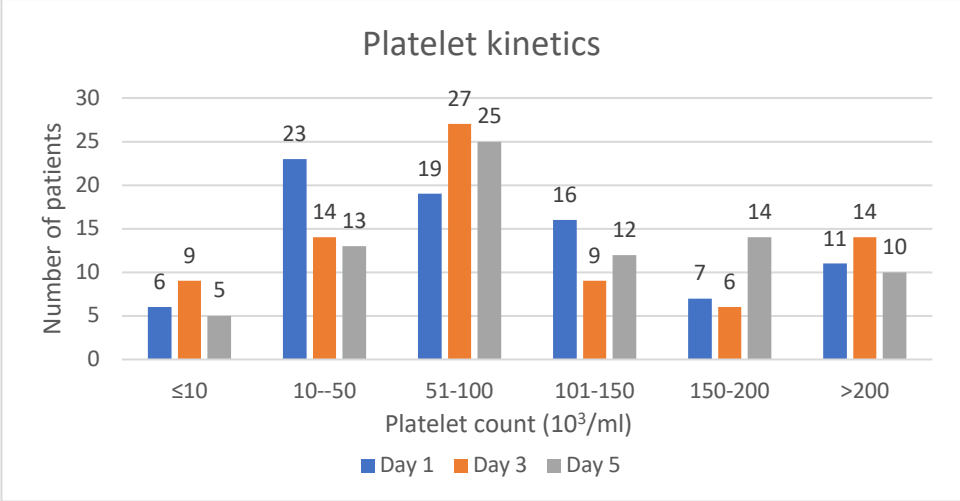

Image 5: Comparative analysis of platelet count on day 1, day 3 and day 5

Using the median values in severe and non-severe cases, a cut off value of 750 ng/ml was taken to further categorize the patients. The mean Platelet count, and hematocrit of patients showed statistically significant association with high VGEF levels (>750ng/ml) or to say severe dengue cases. There was an insignificant difference between the mean TLC of patients with and without high VEGF on day 1, however, on day 3 and day 5 patients with high VEGF had significantly lower TLC (p-value=0.03, and 0.01).

Table 4: Comparison Of Mean Parameters Of Patients On Day 1, Day 3, And Day 5 Of Hospitalization

Parameters	Day 1	Day 3	Day 5
MEAN PLATELET COUNT			
VEGF <750	114.3 ± 28.5	148.9 ± 33.2	161.5 ± 44.7
VEGF ≥ 750	84.3 ± 14.5	73.1 ± 12.2	66.3 ± 9.6
p-value	**0.001**	**0.0001**	**0.0001**
HEMATOCRIT			
VEGF ≥ 750	55.74 ± 8.32	57.33 ± 5.61	60.15 ± 3.78
VEGF <750	31.82 ± 6.40	32.53 ± 3.80	31.63 ± 3.15
p-value	**0.01**	**0.001**	**0.01**
TLC			
VEGF ≥ 750	4095 ± 2376.1	2935 ± 2723.3	2015 ± 2346.8
VEGF <750	4135.7 ± 958.7	4015 ± 1083.4	3325 ± 1872.5
p-value	**0.13**	**0.03**	**0.01**

In our study 20 (25%) cases with severe dengue had VEGF >750 pg/ml, while 13 (16.25%) non-severe cases had VEGF >750. Similarly, 1 (1.25%) case with severe dengue had low VEGF levels, while 45 (56.25%) non-severe cases had normal VEGF levels. The incidence of high VEGF was significantly higher in cases than controls (p-value=0.0001).

Overall, the sensitivity and specificity of serum VEGF in predicting the severity of dengue was 95.24% and 77.58%, respectively. The positive and negative predictive value of VEGF was 66.66%% and 97.8%, respectively. The accuracy was 76.76%. There was a negative correlation between platelet count and serum VEGF in patients, the correlation coefficient being R= -0.29. Patients with low platelet count had high serum VEGF levels. The correlation was statistically significant (p-value=0.001). There was a positive correlation between serum SGOT and

SGPT values with serum VEGF in patients. The correlation coefficient was 0.58 and 0.51 respectively which was statistically significant.

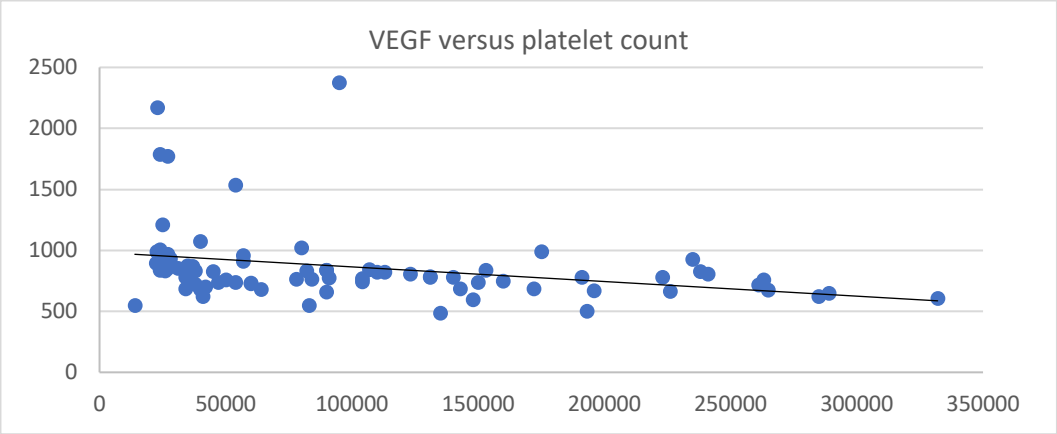

Image 5: Correlation between serum VEGF and platelet count

Discussion

In our study, VEGF levels were able to predict the complications and severity of dengue fever in combination with routine lab parameters. In fact, VEGF as a single marker was also able to diagnose severe dengue illness in our study with 95.24% sensitivity. VEGF is involved in the pathophysiological mechanism of many diseases including malignancies and diabetic retinopathy, especially during the healing process of damaged vasculature [14]. However, serum VEGF has also been involved in the pathophysiology of plasma leakage seen during dengue virus infection [15]. Hence, a higher VEGF level is also expected in a dengue fever patient developing circulatory shock [13].

In our study we included 80 cases and 8 controls in our study. The mean age of cases was 39.86 ± 14.83 years, while that of controls was 40.25 ± 13.30 years. Similar distribution of age was observed by Gary Kim Kuan Low *et al* [16]. The mean age of patients in Awan *et al*.'s study was 36 ± 2.9 years [17]. In our study the most affected age group was between 40-49 years. Similar observation was made by Saeed *et al* [18]. Whereas Awan *et al*.'s [17] reported 20-29 years with more than 40% cases. In our study the male to female ratio of cases was 1.14:1. A male preponderance for dengue has also been reported by Saeed *et al* [18]. however, Tseng *et al*.'s reported female preponderance [13]. We categorized the cases of dengue fever in our as severe and non-severe according to the 2009 WHO criteria [19]. Out of total 80 patients, 71.25% patients had non-severe dengue, while the remaining 26.58% patients had severe dengue. Our findings were in striking agreement with Tseng *et al* [13]. results who had observed 73.6% severe cases and 26.4% non-severe cases. While in Patra *et al*.'s [20]. study there were 85.7% severe and 14.3% non-severe cases. In our study the most common complication included bleeding manifestations in the form of hematemesis, haematuria, purpura, epistaxis, and subconjunctival haemorrhage in 21.5% cases. This was followed by oedema in 18.75% cases, dehydration in 17.50% cases, and shock in 5.0% cases. Zhang *et al* [21]. has reported bleeding, abdominal pain, skin rashes, and dehydration as the four symptoms which demonstrate an increased risk for severe dengue. Ahmed *et al* [22]. and Carlos *et al* [23]. have also made similar observations.

The levels of VEGF in our cases ranged between 482.854 pg/ml to 2382.070 pg/ml in dengue cases. The mean VEGF level was 852.301 ± 305.673 ng/ml and in cases mean

VEGF in severe dengue was 1053.087±458.384 while in non severe dengue it was 658.373±146.166. While the level of VEGF in control ranged from 69.513 pg/ml to 567.786 pg/ml, with a mean VEGF level of 287.830 ± 187.612 ng/ml. We observed that cases had a significantly higher VEGF level than controls (p-value=0.0001). The VEGF levels detected by us were consistent with the observations of Thakur *et al* [24], Tseng *et al* [13], and Young *et al* [25].

Results by Thakur *et al* [24]. had reported the mean levels of VEGF in control sera as 3.493 ± 1.982 pg/ml compared to patients with DF (mean value of 290.407 ± 167.17 pg/ml) having significantly higher levels of VEGF (p=0.001). On the other hand, patients with severe dengue had mean VEGF of 428.170 ± 224.61 pg/ml in their study. Tseng *et al* [13]. reported a highly significant difference in the VEGF levels of control versus non severe cases (p-value< 0.05), versus severe cases (p-value<0.01). We observed that high levels of VEGF, over 750 pg/ml, as a biomarker could predict the presence of complications in dengue patients, thereby reducing the chance of early treatment to prevent death. Overall, the sensitivity and specificity of serum VEGF in predicting the severity of dengue was 95.24% and 77.58%, respectively. The positive and negative predictive value of VEGF was 66.66% and 97.8%, respectively. The accuracy was 76.76%. The sensitivity of the prediction model of VEGF and other clinical parameters including thrombocytopenia, and haematocrit was higher than the warning signs employed by the WHO 2009 guideline. The WHO 2009 guideline has a sensitivity around 90% and specificity of up to 50% [69,70]. (Leo *et al*., 2013; Thein *et al*., 2013) in predicting severe disease. Similar sensitivity and specificity of VEGF have been reported by Low GKK *et al* [16].

Other studies also developed models to predict severe dengue with both sensitivity and specificity obtained like our prediction model, with sensitivity and specificity of 78.2% and 80.2% reported by Tanner *et al* [26], sensitivity and specificity of 97% and 48% by Potts *et al* [27], and sensitivity and specificity of 87.5% and 74.1% by Soundravally *et al* [28]. However, our diagnostic model and VEGF levels as a single marker have comparatively lower accuracy than the WHO 2009 severe dengue.

The hematological profile revealed that thrombocytopenia was present in 60.7% cases on day 1, 63.29% on day 2, and 54.44% on day 3. This fluctuation in platelet count was attributed to the fact that while patients with non-severe dengue had a rising level of platelet count from day 1 to day 3 and 5, in severe cases, the platelet count fell lower from day 1 to day 3 and 5. In our study, patients with high VEGF had significantly lower platelet count on all 3 days compared to those with low VEGF. Additionally, while in patients with low VEGF the platelet count improved from day 1 to day 3 and 5, in cases with high VEGF the platelet count fell lower from day 1 to day 3 and day 5. Similar observation was made by Thakur *et al* [24]. In their study 67.52% cases had thrombocytopenia. They observed that mean levels of platelet count were significantly lower in cases with high VEGF as compared to cases with low VEGF levels (p-value=0.05). We chose a logistic regression model to predict the accuracy of VEGF levels in predicting the derangement of various lab parameters seen consistently with dengue. In our study we observed that there was a negative correlation between platelet count and serum VEGF in patients, the correlation coefficient being R= -0.29. We inferred that patient with low platelet count had high serum VEGF levels. The correlation was statistically significant (p-value=0.001). Patra *et al* [20]. also observed that VEGF had a negative correlation with platelets in severe dengue patients (r=-0.331, p=0.001).

We observed an significant difference between the mean haematocrit levels of patients with and without high VEGF on day 1, day 3 and day 5 patients with VEGF >750 pg/ml had significantly higher hematocrit (p-value=0.01, 0.001, and 0.01). These findings indicate that rising VEGF levels correlate with haemoconcentration seen in dengue. Thakur *et al* [24]. reported haemoconcentration in 47 patients (44.33 %). In their study, the mean haematocrit of patients with low VEGF levels was 24.22 ± 5.41%, while those with raised VEGF levels was 50.31 ± 40.33% (p-value=0.0001).

In addition, based on the patient's blood report, it was challenging to identify severe dengue at the febrile phase. Hence, serum level of VEGF in different categories of dengue infection was measured on day 5. We measured and established a specific value of threshold VEGF over the fifth day of infection which would provide a distinct indication of dengue severity.

Conclusion

In conclusion, plasma leakage is a cardinal hallmark of severe dengue. We demonstrated that plasma VEGF levels in cases with severe dengue were significantly higher than values from a mild illness. The increased plasma levels of VEGF in patients with dengue illness were correlated positively with haematocrit levels, and negatively with low platelet count. These findings suggest that VEGF, as a contributory factor of increasing vascular permeability, contributes to the pathogenesis of dengue. VEGF may serve as an adjunct investigation to support the diagnosis.

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