



## The Role of Volume, Conductivity, Scatter Changes of Neutrophils and Monocytes in Diagnosis of Megaloblastic Anemia

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### ABSTRACT

**Background:** Megaloblastic anemias are macrocytic normochromic anemia with mean corpuscular volume (MCV) of 100 fl-140 fl and caused by deficiency of either cobalamin (vitamin B12) or folate. However, increased MCV is not specific for megaloblastic anemia, nor is Vitamin B12 assay by chemiluminescence. We undertook this study to evaluate the possible role of Volume, Conductivity and Scatter (VCS) of WBCs derived from standard hematology analyzer to indicate megaloblastic anemia.

**Methods:** We performed a case control study comparing data of 60 patients with low serum vitamin B12 or folate levels with 60 healthy volunteers. Comparison of the volume, conductivity and scatter parameters for neutrophils and monocytes of cases and control were done.

**Result:** The mean neutrophil volume of cases (158.37±18.13fl) was significantly higher (p= 0.0001) compared to controls (141.26±4.22fl). Similarly, mean monocyte volume of cases (183.34±16.90fl) was significantly (p=0.0001) higher compared to controls (166.55±8.66fl).

The difference in the mean conductivity of both neutrophils and monocytes between cases and controls were insignificant (p=0.43).

**Conclusion:** Our study suggests analysis of VCS parameters for neutrophils and monocytes was a simple and objective method that substantiates the existence of subclinical deficiency of vitamin B 12 and folate with fair degree of certainty.

**Keywords:** Megaloblastic Anemia, Automated Hematology Analyzer, Neutrophils, Monocytes.

### Introduction

Megaloblastic anemias are a group of disorders characterized by deficiency of either cobalamin (vitamin b12) or folate, thereby imparting distinctive morphologic appearances in the developing hematopoietic cells in the bone marrow.<sup>[1]</sup> This is reflected in the peripheral smear as macro-ovalocytes and macropolycyte. Megaloblastic anemia is typically a macrocytic normochromic anemia. The MCV should be 100 fl-140 fl. However increased MCV is not specific for megaloblastic anemia. Moreover, the deficiency affects all labile cells including white blood cells(WBCs).<sup>[2,3]</sup>

The volume, conductivity, scatter (VCS) technology of the Beckman-Coulter LH 750 haematology analyzer obtain data directly from more than 8000 WBC's. Direct current impedance is used to measure cell volume. Radiofrequency opacity is used to characterize conductivity that indicates internal composition of each cell. Laser beam is used to measure light scatter for cytoplasmic granularity and nuclear structure. All these in conjunction identify each leucocyte as neutrophil, lymphocyte, monocyte, eosinophil or basophil generating automated differential count.

<sup>[4]</sup> Studies on the changes in VCS parameters in acute

bacterial sepsis has concluded that they can indicate sepsis in the patients with high accuracy.<sup>[5]</sup>

We undertook this study to evaluate the possible role of Coulter derived VCS parameters to detect megaloblastic anemia.

### Materials and Methods

#### Study design

In this prospective case-control study, we recruited patients having low serum vitamin B12 or folate levels. To ensure randomization, patients received on one particular day of the week i.e. Thursday were accepted. For all the patients, folate and vitamin B12 levels were estimated by electrochemiluminescence method on COBAS e411 analyzer. Patients with haemoglobin (Hb)<12mg/dl for men and 11mg/dl for women were considered anemic according to world health organization (WHO) criteria. Patients having c- reactive protein (CRP) >6 mg/L, total leukocyte count greater than 11,000 cells/mm<sup>3</sup> and other clinical features suggestive of sepsis/ inflammation were excluded. Any hormone abnormalities e.g. thyroid function test anomalies or other abnormal hormone test results, any liver function test abnormality or renal function test



abnormalities were also deemed unacceptable. Patients admitted as in-patients for any reason and children below 18 years as well as pregnant women (propensity for folate, vit. B12 and iron deficiency as well as physiological macrocytosis is high). Patients on treatment for any malignancy or any patient with any infection (proven on serology/ culture) or infestation (detected on antigen/ antibody tests) or with differential counts outside the normal range were barred from the study as well.

The peripheral smear in association with the red blood cell(RBC) indices were studied to identify prospective cases (with macrocytic anemia). MCV>100fl is accepted as macrocytosis in our study and we also expected few normal people to be harboring macrocytosis.<sup>[6, 7]</sup> Vitamin B12 and folate assay were done to confirm the cases.

Age and sex matched controls were also chosen carefully from the patients at health-check-camps organized by the hospital only on Thursdays who are young, fit and had nil complaints at the time of check-up. The laboratory parameters were checked for abnormalities as decided above for the patients. The Hb levels of the controls were within the normal range of 12-15.5 g/dl for women and 13.5-17.5 g/dl for men respectively according to WHO criteria. Further, vit. B12, folate and ferritin were estimated to rule out nutritional deficiencies. These patients were excluded of alcohol consumption and any abnormality in liver function tests.

Since the native inhabitants of this region are predominantly non-vegetarian with a minor vegetarian diaspora, deficiencies of Vit. B12 and folate were rare. Our sample size was calculated by the formula using the following formulas

$$nf = \frac{n}{1 + \frac{n}{N}}$$

where, nf = desired sample size if less than 10,000; n = desired sample size when population is more than 10,000; N = estimate of population size.

$$n = \frac{z^2 pq}{d^2}$$

Where, n = desired sample size if population is above 10,000, z = standard normal deviate usually at 1.96, p = proportion of normal population estimated to have a particular characteristic, q = 1.0 – p, d = degree of accuracy desired that is 0.05. Since the prevalence of megaloblastic anemia is 3.7% (3.5% for men and 3.9% for women,<sup>8</sup> the

desired sample size is minimum 55 and our sample size was 60. An Indian reference for prevalence of megaloblastic anemia was not available. We assumed the predominant non-vegetarian population of this region would generate prevalence rates approximating that of the western population. The number of controls were thus 60 too with normal haematological parameters and no vit. B12/ folate deficiency. The sample collection was continued till the desired sample size was met.

The study was commenced after the receipt of Institutional ethical clearance.

#### ***Instruments***

Beckman Coulter LH 780, automated 5-part differential cell counter was used to analyze the blood samples. LH 780 was calibrated half yearly using manufacturer's calibrator. A 3-level internal quality control run daily along with a quarterly external quality assurance scheme was used to ensure precision. Quality control samples from Biorad were used to check instrument precision for estimation of serum Vit. B12 and folate. Quality check was done before running every batch of samples which is run twice a week.

#### ***Data Collection***

The serum vitamin B12 and folate levels of cases and controls were recorded. Serum vitamin B12 < 180 pg/ml was considered 'below normal levels'. The EDTA blood sample was run through Beckman Coulter LH 780 hematology analyzer. Mean Volumes of Neutrophil and Monocyte (MNV and MMV respectively), Mean Conductivity of Neutrophil and Monocyte (MNC and MMC respectively) and Mean Scatter of Neutrophil and Monocyte (MNS and MMS respectively) were determined and recorded.

Other relevant data including age, sex, clinical features, complete blood count, peripheral smear and bone marrow findings were recorded wherever available. The maximum allowable limit for age of both cases and controls was kept below 50 years to obviate the possibility of age related macrocytosis and myelodysplastic syndromes, which are commoner in the elderly.

#### ***Statistical Analysis***

Data was analyzed using SPSS version 20, IBM. Comparison and determination of significance between the means of VCS parameters of the WBCs were performed using student t test. A 'p' value less than 0.05 was considered statistically significant. To determine cut offs of the VCS parameters to distinguish between cases and controls, Receiver Operation Characteristic (ROC) curves were constructed and the cut off values calculated

according to the highest Youden's index (YI)/accuracy or higher sensitivity depending on its particular importance.

## Results

A total of 60 cases and 60 controls were included in the study. Out of 60 cases of megaloblastic anemia, serum Vit B12 (vitamin B12) only was low in 35 cases, serum folate only was low in 11 cases and both were low in 14 cases. Males constituted 88% (n=53) and females 12% (n=7). Their Hb ranged from 4.4g/dl to 10.2 g/dl. Seven patients were non-anaemic but with high normal mean corpuscular volumes (MCVs) and were incidentally detected at a health check camp. Bone marrow examination was done in four patients and showed megaloblastic erythroid hyperplasia.

### VCS characteristics

The mean corpuscular volume (MCV) were also recorded, for the cases. The mean MCV was  $111 \pm 4.12$  (SD). The control showed a mean MCV of  $98 \pm 3.58$ . 76% of the cases had a higher MNV+MMV. Of these, 80% correlated with a higher MCV (greater than 100fl). The rest of the cases had MCVs within the normal range but towards the upper limit. Of the controls, 21 (36%) had a higher MNV and MMV. All these patients also showed borderline high mean MCV of  $103.2 \pm 3.12$ . The mean neutrophil volume of cases (MNV:  $158.37 \pm 18.13$ ) was significantly higher ( $p = 0.0001$ ) than that of controls (MNV:  $141.26 \pm 4.22$ ) (Table 1). Similarly mean monocyte volume of cases (MMV:  $183.34 \pm 16.90$ ) was significantly ( $p=0.0001$ ) higher compared to that of controls (MMV:  $166.55 \pm 8.66$ ). The MNV and MMV discriminatory ability is demonstrated in Figure 1 that shows the receiver operating characteristic (ROC) curve. The area under the curve (AUC) of MNV and MMV are respectively 0.839 and 0.837.

**Table 1: Mean volume of neutrophils and monocytes (in fl) cases and controls HS- Highly Significant (statistical method- student 't' test).**

	N	Mean volume	Std. Deviation	p
<b>NEUTROPHILS Cases</b>	60	158.37	18.13	0.000 (HS)
<b>Controls</b>	60	141.26	4.22	
<b>MONOCYTES Cases</b>	60	183.34	16.90	0.000 (HS)
<b>Controls</b>	60	166.55	8.66	

**Table 2: Mean conductivity for neutrophils and monocytes in cases and controls. (statistical method- student 't' test).**

	N	Mean conductivity	Std. Deviation	p
<b>NEUTROPHILS Cases</b>	60	138.58	4.67	0.043
<b>Controls</b>	60	141.35	4.73	
<b>MONOCYTES Cases</b>	60	114.66	4.79	0.036
<b>Controls</b>	60	117.62	4.93	

The mean conductivity for neutrophils for cases (MNC:  $138.58 \pm 4.67$ ) was lower compared to controls (MNC:  $141.35 \pm 4.73$ ) and was statistically significant ( $p=0.043$ ) (Table 2). Similarly the mean conductivity for monocytes for cases (MMC:  $114.66 \pm 4.79$ ) was lower than that of controls (MMC:  $117.62 \pm 4.93$ ) and was statistically significant ( $p=0.036$ ). ROC curve to confirm its differentiating capability shows an AUC of 0.690 for both MNC and MMC (Figure 2).

The mean scatter for neutrophils did not show significant difference between cases and controls. Mean scatter for monocytes in cases (MMS:  $87.91 \pm 4.49$ ) was significantly ( $p=0.014$ ) lower than that of controls (MMS:  $91.35 \pm 5.06$ ) (Table 3). The corresponding ROC curve is shown in Figure 3

The receiver operating characteristic (ROC) curve was constructed, and cut off values for volume and conductivity for neutrophils and monocytes to distinguish between megaloblastic anemia from normal were determined using Youden's index to select the maximum sensitivity and specificity. Similarly, cut off value for scatter of monocytes diagnostic of megaloblastic anaemia were determined. Accuracy, defined as number of samples correctly diagnosed, too was calculated at those cut-offs to check appropriateness. (Table 4, 5, 6).

MNV greater than 144.3 and MMV greater than 166.65 was suggestive of megaloblastic anemia with accuracy of 86% and 80 % respectively. Also MNC less than 140.65 and MMC less than 115.25 respectively were suggestive of megaloblastic anemia with an accuracy of 77% each.

MMS less than 90.05 was observed in cases of megaloblastic anemia with an accuracy of 75.8%.

**Table 3: Mean scatter for neutrophils and monocytes in cases and controls. (statistical method- student 't' test).**

	N	Mean scatter	Std. Deviation	p
<b>NEUTROPHILS Cases</b>	60	145.80	7.30	0.505
<b>Controls</b>	60	147.06	5.89	
<b>MONOCYTES Cases</b>	60	87.91	4.49	0.014
<b>Controls</b>	60	91.35	5.06	

**Table 4: Cut off values for neutrophil and monocyte volume.**

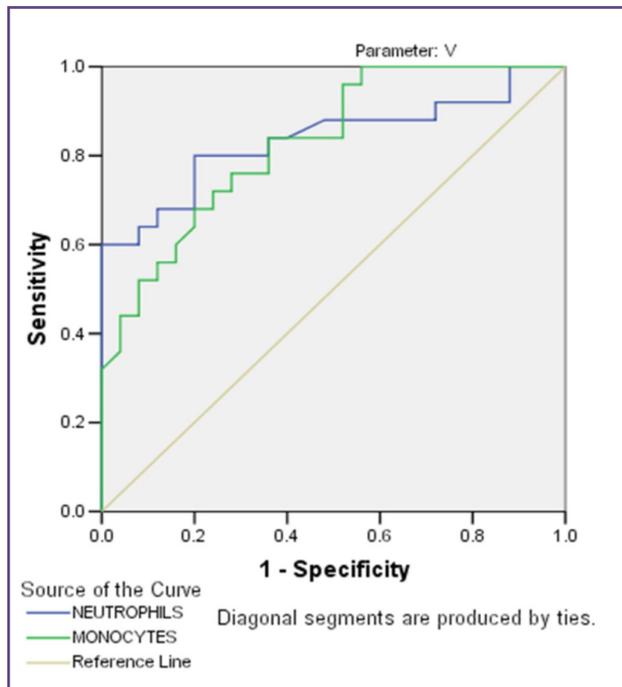
Cell	Cut off value volume	Sensitivity (%)	Specificity (%)	YI	Accuracy (%)
<b>Neutrophil</b>	>144.3	88	84	72	85.6wq
<b>Monocyte</b>	>166.65	86	74	60	80

**Table 5: Cut off values for neutrophil and monocyte conductivity.**

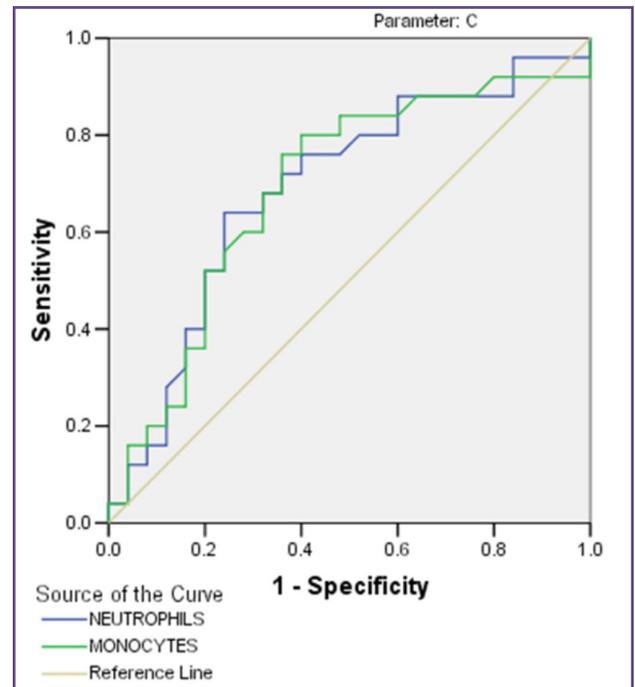
Cell	Cut off value conductivity	Sensitivity (%)	Specificity (%)	YI	Accuracy (%)
<b>Neutrophil</b>	<140.65	74	80	54	76.6
<b>Monocyte</b>	<115.25	84	70	54	76.6

**Table 6: Cut off values for neutrophil and monocyte scatter.**

Cell	Cut off value Scatter	Sensitivity (%)	Specificity (%)	YI	Accuracy (%)
<b>Neutrophil</b>	<142.6	84	42	26	63
<b>Monocyte</b>	< 90.05	82	70	52	75.8



**Fig. 1: ROC curve of the mean volumes of neutrophils (MNV) and monocytes (MMV).**



**Fig 2: ROC curve of the mean conductivity of neutrophils (MNC) and monocytes (MMC)**

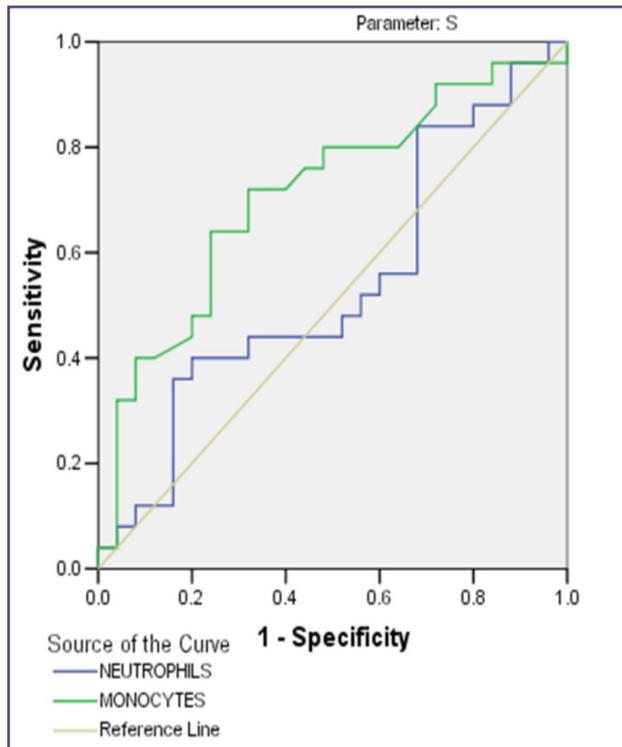


Fig. 3: ROC curve of the mean scatter of neutrophils (MNS) and monocytes (MMS).

## Discussion

High prevalence of vitamin B12 and folate deficiencies is due to many factors like insufficient intake, inadequate absorption, increased loss or increased need and affect all age groups.<sup>[8]</sup> Hematological findings that have been attributed conspicuously to vitamin B12 deficiency are, macrocytosis, hypersegmentation of neutrophils, and increased red cell distribution width (RDW).<sup>[9, 10]</sup> It presents with variable clinical manifestations including megaloblastosis in the bone marrow, macrocytosis in peripheral smear and a raised MCV. While pernicious anemia is the most common cause for vitamin B12 deficiency in the west, insufficient intake is the cause in India.<sup>[3]</sup>

At present, the MCV obtained by hematology analysers is used as a screening test for megaloblastic anemia. A raised MCV was suggestive of megaloblastic anemia.<sup>[3]</sup> However studies have shown that MCV alone as screening test may be unreliable due to concomitant iron deficiency anemia, anemia of chronic disease or thalassemia carrier state.<sup>[3,11]</sup>

A systematic review showed that the MCV is far from perfect in terms of detecting or excluding B12 deficiency, both in anaemic and non-anaemic patients. Even in retrospective series of patients with pernicious anaemia, the sensitivity of the MCV for B12 deficiency was far from

perfect, with a summary estimate of 77%. In studies where an admission or community screening was performed for B12 deficiency, the sensitivity of macrocytosis for B12 deficiency was found to be very low. Only 9 out of 33 anaemic patients had an elevated MCV.<sup>[9]</sup> Oosterhuis et al. proclaimed failure to detect 84% of cobalamin deficient patients using MCV as the sole discriminant.<sup>[10]</sup>

Estimation of vitamin B12 levels by chemiluminescence may show falsely normal values.<sup>[12-14]</sup> So, methyl malonic acid (MMA) is an early indicator of Vit. B12 deficiency and helps discriminate between cobalamin deficient patients from those with spuriously low levels, detected by chemiluminescence.<sup>[15-17]</sup> Unfortunately, MMA is not estimated in our laboratory. Since the cases taken into consideration into our study are supported by hematological, biochemical and peripheral smear findings, reservations against these cases may be safely overlooked. However, we might, in our routine testing miss out on some of the patients who are deficient on cobalamin. An indirect evidence may be obtained by high lactate dehydrogenase (LDH) levels in cobalamin deficient patients suffering from megaloblastic anemia, in our own experience. Malignancy or any other cause of high cell-turnover has to be ruled out. It works best when atypical/ dysplastic looking hematopoietic cells appear in the peripheral smear, but otherwise, LDH elevation is an invariable feature of megaloblastic anemia, both manifested as well as subtle. Thus, LDH testing is too non-specific to be of any use to diagnose cobalamin deficiency.

Sepsis and infection may be suggested by higher MMV, NMV and anisocytosis of neutrophils and monocytes. These patients show a higher mean volume and anisocytosis of neutrophils and monocytes than that of healthy controls.<sup>[18-20]</sup>

C. Risch et al. found an association between monocyte and neutrophil anisocytosis as well as mean lymphocyte volume with decreased serum holotranscobalamin levels thus predicting symptomatic megaloblastic anemia. They included subjects  $\geq 60$  years old in their study.<sup>[21]</sup> In our study, age range was between 19-59 years. Moreover, they studied relative anisocytosis among neutrophils and monocytes and found it to be a sensitive marker for Vitamin B12 deficiency. Monocyte anisocytosis, particularly at the outset, is a harbinger of cobalamin deficiency in incipient stages.<sup>[22]</sup>

In our study, we found a significant difference between the mean neutrophil and monocyte volume, their conductivity and scatter of patients with either vitamin B12 or folate deficiency and that of normal persons. The mean neutrophil and monocyte volumes were significantly

higher, conductivity significantly lower and monocyte scatter significantly lower than that of the normal subjects.

Presence of MNV greater than 144.3fl, MMV greater than 166.65 was highly accurate in indicating the presence of megaloblastic anemia. MNC and MMC greater than 140.65 and 115.25 respectively was associated with the disease. MMS greater than 90.05 was strongly associated with the disease. However, MNS is not a useful indicator of megaloblastic anemia.

Neutrophil scatter distribution width could be used to assess cobalamin status. To prove this hypothesis, Totoskovic et al divided patients at risk for cobalamin deficiency into three groups namely, group 1, with MMA <227nmol/L and much decreased possibility of Vitamin B12 deficiency and group 3, with MMA >367nmol/L and increased possibility of cobalamin deficiency. Group 2 represented indeterminate cobalamin deficiency. They proved with the help of ROC curves and AUC that high MMA levels correlate with low serum Vitamin B12 and vice versa and routine tests might engender spuriously low serum Vitamin B12 as evidenced by 14 patients with MMA <227 nmol/L and concomitant low vitamin B12 concentrations. The Neutrophil scatter-distribution width (Nes-dw) was confidently associated with MMA. Like the MMA, Nes-DW and was higher in patients with an increased prospect for vitamin B12 deficiency and vice-versa. Nes-DW above 3.51% cutoff detects increased MMA levels and thereby increased chances of cobalamin deficiency with 74.19% sensitivity and 68.87% specificity. The relative Nes-DW used in this study represents variation of nuclear morphology and the AUC of the ROC curve for detection was 0.761. [22]

An exact precedent to our study was not available when a pubmed/medline database search was made. Our study is one among the early attempts to delineate anemias related to deficiencies of vitamin B12 and folic acid from the volume, conductivity and scatter (VCS) perspective obtained routinely from hematology analysers as part of complete hemogram ordered very frequently by the physicians for their patients. There are, though, studies which are related to ours but not the same.

If the parameters meet all the criteria prescribed above (i.e. MNV >144.3fl, MMV >166.65fl, MNC <140.65, MMC <115.25 and MMS <0.05), we obtained a sensitivity of 36% and specificity of 28% and thus an accuracy of 32% and YI becomes -36. With MNV and MMV considered together at these cut-offs, sensitivity is 76%, specificity is 64%, accuracy 61.6% and YI becomes 40. With only monocyte scatter (MMS), sensitivity becomes 68%, specificity 72%, accuracy 70% and YI becomes 40.

The MCV was statistically insignificant ( $p < 0.05$ ) to indicate vitamin B12 and folate deficiencies. It correlated weakly (Pearson r correlation test) with MNV and MMV in both cohorts of cases and controls. Our findings were similar to that of Bhatia et al. [3]

Small sample size is a limitation of this study considering some bias associated with choosing the cases and controls. Also, more studies involving larger sample size might substantiate the findings in the study.

## Conclusion

To conclude, our study suggests that analysis of coulter derived VCS parameters for neutrophils and monocytes was a simple and objective method that indicate the existence of clinical/subclinical deficiency of vitamin B 12 and folate in adult patients. More similar studies on larger sample size are looked forward to, to substantiate and to determine age specific cut offs.

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## Competing Interests

None

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