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RESEARCH ARTICLE

Association of Hyponatremia, Hypochromia and Microcytosis in Sickle Cell Patients from Chhattisgarh: Possible Interplay Determining Less Severe Clinical Presentation.

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Abstract

Backgrounds and objectives:- Clinical presentation of sickle cell disease (SCD) in India shows reduced episode of crisis and severity unlike reported elsewhere. Sickle hemoglobin (HbS) polymerizes upon deoxygenation causing RBC dehydration and consequently increases corpuscular hemoglobin concentration which in turn accelerates more HbS polymerization precipitating episodes of crisis. Any factor affecting reduced haemoglobin synthesis or changes in low serum osmolality may affect disease outcome by altering cellular hemoglobin concentration. The aim of this study was to elucidate the peculiar disease presentation by evaluating hematological and blood electrolyte alterations in the SCD patients from Chhattisgarh, central India.

Methods:- For the present study 100 SCD, 100 traits and 100 normal were selected from CG, India. Genotypes were determined by Hb variant HPLC. Hematological and biochemical test were performed using auto-analyzer.

Results:- Mean MCV and MCHC in SCD group was significantly lowered compared to control and trait. Significant reduction of serum sodium and potassium level (125.6±3.4 and 3.2±0.86 mEq/l respectively) were observed in SCD group compared to control and trait (137.4±3.25, 4±0.37 and 134.0±5, 3.7±0.84mEq/l respectively), ($p<0.001$). Patients in stable and crisis state do not show any significant difference in serum sodium and potassium level.

Interpretation and Conclusion:- Hyponatremia in the SCD study group might rehydrate RBC and could be an additional contributing factor along with increase HbF response and co-existing microcytic anaemia for fewer vaso-occlusive episodes in this geographical region. Mechanism and significance of simultaneous Hyponatremia and hypokalaemia as well hypochromic microcytic anaemia, the unusual finding in the SCD study population, need to be elucidate further in future study.

Introduction:-

Sickle cell disease (SCD) is one of the commonest heamoglobinopathies in India. SCD is resulting from a homozygous point mutation in the β-globin gene that changes the sixth amino acid from glutamic acid to valine($\alpha_2 \beta_2^{6\ Glu \rightarrow Val}$)[**31**]. In India, sickle gene was first detected by Lehman and Cutbush (1952) among the tribes of Nilgiri Hills. Since then, more than 300 tribal groups have been screened to look for the presence of sickle cell Disease[**8]**.SCD has a high prevalence in India, especially in the central (Madhya Pradesh, Orissa and Chhattisgarh) and western regions(Gujarat and Maharashtra), and poses a considerable health burden. Chhattisgarh state which is geographically situated in the central part of India has high frequency of sickle cell heamoglobinopathies (3.2-22.5%) among the tribes **[24,4,5].** Due to peculiar biochemical properties, sickle haemoglobin undergoes polymerization upon deoxygenation. Polymerization of sickle haemoglobin is associated with changes in cell permeability and ionic flux leading to cell dehydration and increased corpuscular haemoglobin concentration. This increase haemoglobin concentration further accelerates HbS polymerization**.** Under mild hypoxia dehydrated erythrocyte are likely to undergo instant polymerization **[15,42,6,9]**.Deoxygenated HbS polymer leads RBC to take sickle shape and decreased deformability when it passes through tiny blood vessels. Following RBC sickling, a cascade of molecular events involving interplay of leukocytes, clotting factors and endothelial cells start leading to occlusion of small vasculature and end organ necrosis called vaso-occlusive crisis (VOC). VOC causes episodic pain, haemolytic anemia, organ injury, and early mortality **[7].**Most commonly affected organs due to crisis are lungs, spleen, kidney, liver and bone joints [**40**]. One of the important factors determining the clinical outcome of SCD is fetal hemoglobin (HbF) level. HbF within RBC interferes with HbS polymerization upon deoxygenation. Accordingly populations like those in Eastern Saudi Arabia, having increase HbF level in RBC have been reported to have less clinical manifestation and better survival [**10,32,33,43**].

RBC shrinkage in SCD has been uniformly observed in different part of the world by different investigators [**16,2,11].** Cause of RBC shrinkage following intracellular HbS polymerization is illusive. In normal physiology RBC volume is predominantly maintained by Ca^{2+}-sensitive K^+ channels (Gardos channel), activity of K-Cl co-transport, partly Na/K ATPase activity and red cell membrane integrity. It is proposed that HbS polymerization and changes in RBC shape leads to activation of a ill-defined mechanism called P_{sickle} involving Ca^{2+} entry and Mg^{2+} extrusion from RBC, resulting increase activity of different K channels, intracellular loss of K^+ and gain of Na^+ along the concentration gradient and simultaneously activation of Na/K ATPase pump which expel Na^+ in excess of K^+ intrusion (3:2) leads to RBC dehydration [**26, 35,22,12,37,18**] and shrinkage. General dehydration also may cause increase HbS polymerization due to increase corpuscular haemoglobin concentration (MCHC) as a result of plasma hyperosmolality and shrinkage of RBC volume. The high osmolality in kidney medulla contribute to the particular vulnerability of this tissue to sickle cell crisis **[2,11,26].** A study by RM Rosa in 1980 showed that chronic induced hyponatremia in Sickle cell patients could reduce episode and duration of sickle cell crisis by rehydrating sickled RBC and consequently preventing RBC shrinkage and increase of Hb concentration **[37,18]**.Though this observation was not verified by other investigators and also was proved to be difficult to maintain therapeutically chronic hyponatremia in SCD, few studies claim benefit of transient hyponatremic therapy to treat acute sickle cell crisis [**38,39**].

Clinical presentation of SCD patients in Chhattisgarh differ significantly from other part of the world in terms of reduced episode of vaso-occlusive crisis and consequently better prognosis and life expectancy as was seen in Indian population, associated with higher fetal haemoglobin (HbF) levels and fewer complications [**23,24].**Though there are few reports from India describing epidemiological distribution of SCD, but there is lack of comprehensive studies to illuminate undercurrent alteration of hematological and biochemical parameters which may interplay contributing to varied disease manifestation. So in the current study we evaluated few hematological and serum parameters in SCD(HbSS)patients and compared with trait (HbAS) and normal healthy volunteers (HbAA) to elucidate the milder clinical presentation of SCD patients from this geographical region.

Materials and method:-

Study subjects and sample collection:-

This study is approved by the IEC (Institutional Ethical Committee), Sickle cell institute Chhattisgarh (SCIC). The study included 300 subjects taken randomly, residing in Chhattisgarh aged between 5-55years. Total 5 ml of venous blood was drawn from the subjects. Out of 5 ml of blood, 2ml was collected in heparinized vials for HPLC and CBC and 3 ml was collected in plain vials for serum electrolyte estimation.

Exclusion and Inclusion criteria:-
Individuals with conformed homozygous SCD or trait and normal were included in this study. Individuals with any other hemoglobinopathy, SCD patients taking hydroxyurea or having any history of blood transfusion within 3 months were excluded in the present study. Crisis state and stable state were differentiated on the basis of clinical presentation.

Determination of genotype:-
Genotype was determined using manufactures protocol by Hb variant BIORAD HPLC

Complete Blood Count (CBC), Serum Electrolyte (Na^+ and K^+):-
Heparinized blood was subjected to Agape BC 3000 for CBC, according to manufacturer protocol.
Serum Electrolyte was estimated by Roche iLyte using manufacturer protocol.

Serum Ferritin:-
Serum ferritin was determined using kit based ELISA (BIORAD)method Monobind.Inc. Lake forest, CA 92630,USA as per the manufactures protocol.

Statistical analysis:-
Statistical analysis was done using IBM software SPSS 21.

Result:-
Assessment of Hematological parameters in the homozygous SCD group compare to trait and control groups:-
In present study we collected blood samples from subjects attending sickle cell mobile screening camp organized in different regions of Chhattisgarh. Following confirmation of the genotype as homozygous SCD (HbSS), trait(HbAS) and control (HbAA) by hemoglobin electrophoresis and HPLC, 100 samples were selected randomly for each group (HbSS, HbAS and HbAA) as per inclusion and exclusion criteria, described in material methods. Male: female ratio in each group was 1:1 (**Table 1**). Average age distribution in HbSS, HbAS and HbAA were 14.66±7.7, 27.78±13 and 23.28±11.65 respectively (**Table 1**). Out of 100 homozygous SCD patients 84 were in stable and 16 were in crisis state. Hematological parameters were evaluated using Agappe BC 3000 CBC counter. Interestingly we found, there is significant decrease in MCV and MCHC in homozygous SCD in comparison with trait and normal (**Table2**). 27% (27/100) of SCD patients shows MCV of below 76.8fl, (<95% confidence interval of HbAA).Among SCD patients 46% shows Microcytic RBC (MCV<80fl) [**9**] compared to 23% and 13% in trait and control respectively. Hypochromic RBC (MCHC<32g/dl) [2**0**] was observed in 24% of SCD patients compared to 4% and 13% in trait and control respectively (**Table 3**). Interestingly 88% (14/16 SCD) patients presented in crisis state shows MCHC more than 32 gm/dL. Only two cases of crisis show MCHC in hypochromic range (< 32 gm/dL). Overall only 2 SCD patients out of 100 (2%) shows MCHC more than normal range for the control population (37.7% gm/dL, upper limit of 95% confidence interval of HbAA). **Figure 1** explains distribution of MCHC and MCV and incidence of crisis state in SCD group with reference to control (HbAA) subjects as base line values.

However as reported from other geographical regions in this study population also, increase mean leukocyte count was observed in homozygous SCD (HbSS: 10.45±4.1x10^9/l**) in comparison with trait (HbAS:6.5±1.8x10^9/L) and control (HbAA:6.7±2.4x10^9/L). Leukocytosis was observed in 25% (25/100) 0f SCD patients (>11.5x10^9/l, 95% confidence interval of HbAA) (**Table 2**).

Among the SCD patients mean HbF level was 20±6.5 %. There was 80% (20/25) of SCD patients with leukocytosis (>11.5x10^9/l) having HBF level <20% whereas only 20% of the patients shows HbF level>20%.
Among the SCD patients 16% (16/100) were affected by clinical crisis. Interestingly 12 out of 16 patients in crisis (75%) shows leukocyte count more than 9.8x10^9/L (median value in SCD patients) as wells as HbF level less than 21.1% (median value in SCD patients). However in 3 cases of crisis (18%) leukocyte count was less than 9.8x10^9/L, as well HbF level was more than 21.1% (median value of SCD) (**Table 4**).So accordingly when the cut-off values for correlating leukocyte count and HbF level were set at the respective median values, 68% of the SCD patients with leukocyte count greater than 9.8x10^9/l, were observed to have HbF level less than 21.1% whereas rest32 % of the patients shows HbF level more than or equal to 21.1% (**Table 4**).(**Figure 2** shows the negative correlation of HbF with WBC total count in SCD patients and distribution of crisis state).

Assessment of serum Sodium level in the homozygous SCD group compare to trait and control groups:-
To analyze if any alteration of Plasma osmolality may contribute to RBC hydration and maintenance of MCHC in normal or below normal range in SCD patient group, we measured serum electrolyte of the study groups (HbSS, HbAS vs. HbAA). As expected we found that there was significantly overall decrease in mean serum sodium concentration in HbSS (125.68±3.5) compared to HbAA (137.7±3.25)and HbAS (134.0±5)($p<0.0001$ HbSS vs. HbAA/HbAS) (**Table5)**. However there was no significant difference was found between HbAS and HbAA. Serum sodium level of 92% (92/100) of SCD patients shows below 131.2 mEq/L (<95% confidence interval of HbAA). Mean value of serum sodium in stable and crisis condition in SCD patients were not differ significantly (125.5±3.4 vs. 125.5±3.5 mEq/L respectively).**Figure 3** shows the distribution of serum Na+ and MCHC in SCD patients along with crisis state with reference to control (HbAA) subjects as base line values.

Assessment of serum Potassium level in the homozygous SCD group compare to trait and control groups:-
Unlike reported elsewhere in this study we observed significant reduction of mean serum potassium level in homozygous SCD (HbSS: 3.2±0.86) as compared to trait (HbAS:3.7±0.84)and normal (HbAA: 4±0.37);($p<0.05$HbSSvs.HbAA) (**Table6**).Mean values of serum potassium in stable and crisis condition were not differ significantly (3.2 ± 0.58 vs. 3.2 ± 0.91 mEq/L respectively). 68% (68/100) of SCD patients shows serum potassium level of below3.26mEq/L (<95% confidence interval of HbAA). Potassium level below 3.26mEq/L was found in 69% (11/16) of SCD patients in crisis state. Out of 100, only 6 SCD patients (stable state) show potassium level more than 5 mEq/L.

Assessment of serum Ferritin level in the homozygous SCD group compare to trait and control groups:-
To verify if co-existence of iron deficiency anaemia may cause RBC microcytosis (low MCV), as a pilot study we examined average serum ferritin level in the limited number of SCD patients, trait and background control population and interestingly we found significantly low serum ferritin in SCD patients relative to trait and background population (**Table 7**). 60% of SCD patients show serum ferritin level below 15µg/L compared to 30% of control group.

Table 1: Age, sex distribution of the study population.

Genotype	**Male**	**Female**	**Age (mean±SD)**
AA	50	50	23.28±11.65
AS	50	50	27.78±13
SS	50	50	14.66±7.7

Table 2: Distribution of haematological indices in SCD, trait and control group.

Group	RBC Total count ($x10^{12}$/L)	WBC Total count ($x10^{9}$/L)	MCHC (g/dL)	MCV (fL)
AA	4.24±0.65	6.7±2.41	34.13±1.8	84.2±3.7
AS	4.3±3	6.5±1.84	34.7±1.5	83.4±3.7
SS	2.7±0.77**	10.45±4.1**	31.9±6.4*	79.65±9.1*

*significance<0.001
**significance<0.00001

Table 3: Distribution of RBC combined morphology in SCD, trait and control group.

Genotype	% RBC Microcytic (MCV<80)	% RBC Hyperchromia (MCH<32)
HbAA	13	13
HbAS	23	04
HbSS	46	23

Table 4: Profile of SCD patients using median values of HbF% and total WBC count as cut off (Number of patients in crisis state indicated within bracket).

HbF%	**WBC (TC)** >$9.8x10^{9}$/L	**WBC (TC)** <$9.8x10^{9}$/L	**Total**
>21.1	16 (**1**)	34 (3)	50 (**4**)
<**21.1**	34 (**12**)	16 (0)	50 (**12**)
Total	50 (**13**)	50 (3)	100 (**16**)

Table 5: Distribution of Serum Sodium and Potassium level in SCD, trait and control group.

Serum Parameters	AA (Mean ±SD)	AS (Mean±SD)	SS(Mean±SD)		p-value SS vs AA/AS
			Stable	Crisis	
Sodium (mEq/l)	137.7±3.25	134.0±5	125.5±3.4	125.5±3.5	<0.0001*
Potassium (mEq/l)	4±0.37	3.7±0.84	3.2±0.9	3.3±0.58	<0.0001*

Table 6: Serum electrolyte distribution in Hypochromic microcytic or normocytic SCD patients.

Parameter	Hypochromic (MCHC<32g/d), Microcytic (MCV<80fL)	Hypochromic (MCHC<32g/dL, Normocytic (MCV>80fL)	p- value
Sodium (mEq/L)	126±3.5	123±2.67	<0.05*
Potassium (mEq/L)	3.1±0.67	2.9±0.41	=0.32

Table 7: Serum ferritin level in the study group.

Genotype	Ferritin (µG/L)
HbAA	42.76±37
HbAS	27.2±10.9
HbSS	16.4±12*

(P* < 0.05)

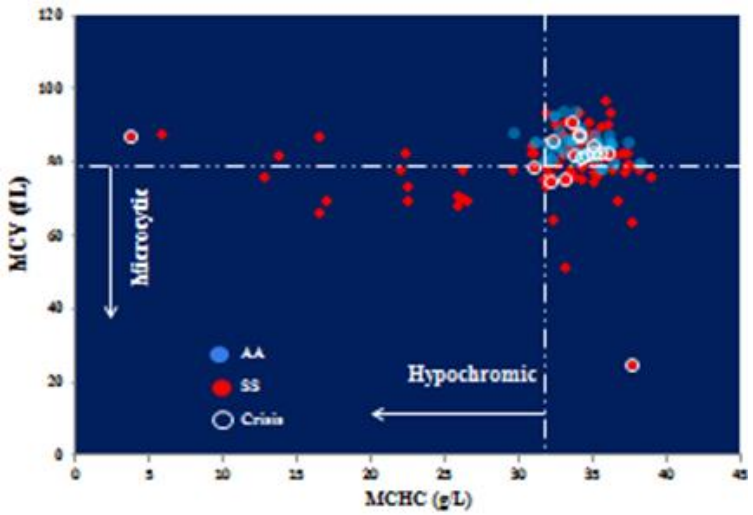

Figure 1: The distribution of serum MCV and MCHC in SCD patients (SS) along with crisis state with reference to control (AA) subjects as base line values. Light blue dots: AA; Red dots:SS; White circle: crisis state.

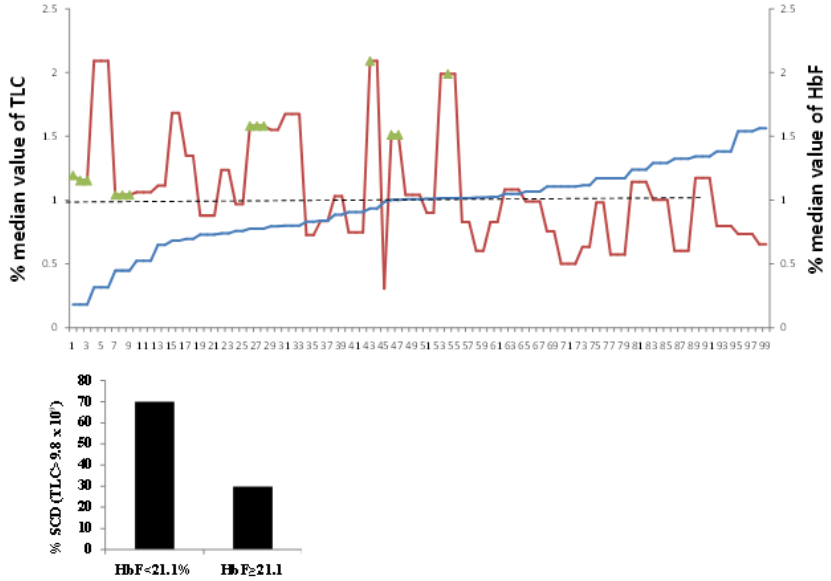

Figure 2: A. Relation of total leukocyte count (TLC) with HbF% in SCD patients. HbF% and TLC values were expressed as % of respective medians. (Median value of HbF%: 21.1; Median value of TLC: 9.8 x10^9/L. Values of HbF % are plotted chronologically, indicated by blue line; corresponding TLC values are indicated by brown line. Green triangles represents crisis state B. Relative frequency (percentage) of SCD patients (TLC >9.8x10^9/L) having HbF< 21% or > 21% as indicated.

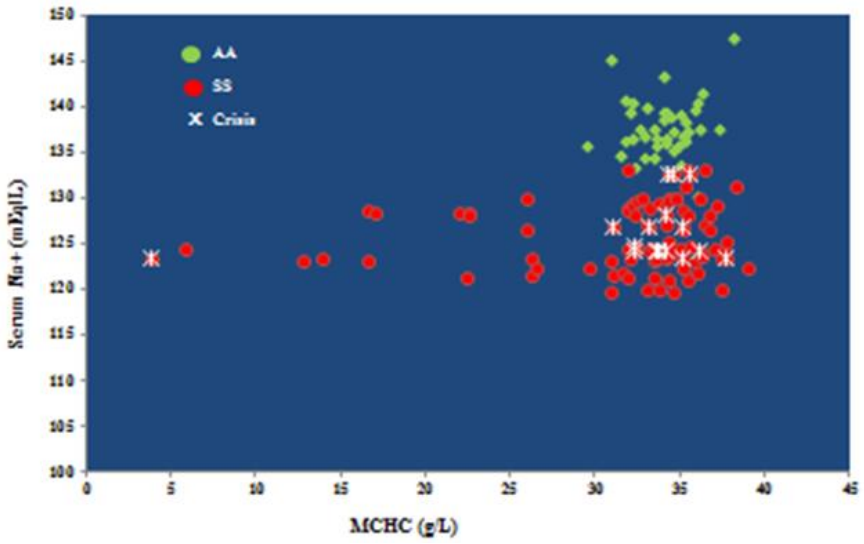

Figure 3: The distribution of serum Na^+ and MCHC in SCD patients (SS) along with crisis state with reference to control (AA) subjects as base line values. Green dots: AA; Red dots: SS; White cross: crisis state.

Discussion:-

In this study we evaluated the hematological parameters in HbSS patients and compared with HbAS and HbAA. HbS polymerization in SCD patients is facilitated by increase hemoglobin concentration following RBC dehydration and shrinkage. Low MCV and high MCHC in SCD have been observed by different investigator previously [**2,45**]. However, interestingly, in this study we observed significant reduction of MCHC compared to trait and normal. The maintenance of low MCHC in the HbSS patients in this study group might protect, HbS from polymerization and frequent attack of vaso-occlusive crisis (VOC) in turn this might affect the clinical outcome of the disease in our study group as evident by lesser incidence of crisis (12.5%) when MCHC is observed at hypochromic range.

Corpuscular hemoglobin concentration is regulated in SCD by various factors intrinsic to RBC or present in plasma. Among the intrinsic factors, some nutritional deficiency or co-existing thalassemia may causes lower hemoglobin synthesis and consequently low MCHC. Changes in plasma osmolality could also regulate MCHC by changing hydrodynamic balance in RBC, particularly in SCD. Perillie and Epstein have shown that sickled RBC shrink in hyperosmolar solution behaving like osmometer (cannot regulate its own volume) resulting increase MCHC [**30**].It is reported that hyper-osmolality in the renal medulla could also induce RBC sickling and vasoocclusive crisis in kidney. So it is expected that plasma hypo-osmolality may rehydrate and swell sickled RBC maintaining normal or low MCHC. The infusion of hypotonic solution is already in clinical practice for acute sickle cell crisis [**11, 38**]. There was some report on limited patients that chronic induced hyponatremia could rehydrate and maintain sickled RBC volume[**39**] So the observed hyponatremia in the SCD group even in steady state may contribute to maintain MCHC in low or normal range and consequently to characteristic milder clinical presentation in this region. Interestingly the mean serum sodium level (125.68 mEq/L), observed in our SCD study group, also was well aligned with the therapeutic target of induced hyponatremia (125-120 mEq/L) achieved previously for chronic or acute management of sickle cell crisis (25, 26). There was a estimation that when serum sodium decrease to 120-125 mmol/L, there is 30-100 fold increase of time require for polymerization of HbS, allowing RBC to pass through tiny capillary without sickling. Thus hyponatremia in this SCD patient group may help to reduce SCD related morbidity [1**9**]. Very recently, though a report from Nigeria claimed hyponatremia in SCD patients [**1**]. However in that study average serum sodium concentration in steady state SCD group was not differed significantly to corresponding control group (132.13±1.51 mEq/l vs. 134±2mEq/l respectively). Such a minute change in serum sodium

concentration in SCD patients (within 2SD of control) is unlikely to impact on RBC rehydration to maintain stable state. More over in that study sodium concentration was not correlated with MCHC or any other blood parameter. There was no other study reported particularly from this geographical region, suggesting normal or low MCHC could be maintained by natural hyponatremia in SCD particularly in stable state. The physiological mechanistic connection between RBC sickling events and hyponatremia is yet to be elucidated.

However in contrary to expectation we observed simultaneous low MCV in spite of swelling of rehydrated RBC. It could be due to co-existence of microcytic anaemia of varied origin most commonly due to iron deficiency or thalassemia in the SCD patients. However we already excluded the co-existing thalassemia in our SCD patient pool (exclusion criteria). Usually in hereditary hemolytic anaemias like thalassemia, sickle cell anemia iron deficiency is not expected [**17,28,41,13**]. However subsequent studies from various parts of the world including India, report existence of iron deficiency anemia in transfused SCD patients [**44,29,14,36**]. We also observed the similar iron deficiency in SCD patients relative to trait and control. However it was a pilot study on a small representative fraction of the study population. Interestingly in this study we observed two distinct SCD population based on RBC morphology. SCD patients having low MCHC and low MCV (hypochromic microcytic) shows little lesser hyponatremia compared to patients having low MCHC and normal MCV (126±3.5 mEqvs 123±2.66 respectively; $p<0.05$) (**Table 6**). This might suggest that in presence of co-existing microcytic anemia of other origin (e.g. iron deficiency anaemia) little severe hyponatremia might be needed to compensate MCV to normal state.

Previously it was reported by Agoreyo that there is increase K level in stable state which further increase in crisis state in SCD patients indicating potassium leakage from de-oxygenated and dehydrated RBC into plasma and or hemolysis [**1**]. However in our study group, overall reduced level of potassium is observed in SCD patients in stable as well as in crisis state. At this point it is not clear the undercurrent mechanism and significant of low potassium in SCD patients. One possible mechanism could be that hyponatremia mediated rehydration of RBC may inhibit repeated HbS polymerization depolymerization cycles within the RBC resulting less sickling, less potassium leakage and reduced hemolysis. Jaitly et al reported an isolated case of SCD, presented in crisis condition with hypokalaemia which was thought to be due to intermittent mineralocorticoid excess but in that case also serum sodium was in normal range [**21**]. Patients with severe hyponatremia (<115mEq/L) frequently present with neurological manifestation and needs active intervention [**3**]. In our study, no SCD patients had serum sodium level below 119mEq/L as well as any clinical manifestation of hyponatremia. However simultaneous hyponatremia and hypokalemia is a unusual finding in SCD patients particularly in stable asymptomatic state.

Expression of HbF is another independent prognostic factor which inhibits HbS polymerization by making relative dilution of HbS in the RBC and low HbF level in SCD shows more disease manifestation. In our study mean HbF% (20±6.6) was observed much higher than in other Hb haplotypes found in other part of the world[**25,34**]. Very recently Duyen Ngo et al, also have similar observation on indo-Arabian haplotypes [**27**].On the other hand, increased mean Leukocyte count which is another precipitating factor for VOC in SCD patients, is also observed to be comparable to the reports from other part of the world. Expectedly together both the parameter correlated negatively (-0.434). Interestingly we observed 75% (12/16) of the crisis patients in this study have HbF level<21.1% (Median value of HbF %) and leukocyte count $>9.8x10^9$/L (Median value of WBC count). These combined criteria could be used to determine the high risk SCD patients for prophylactic measure in this region. However this is the pilot study and bigger study group from this region is needed to authenticate the cut off values. It would be interesting to look if the cut off for disease manifestation in Indian population is set at higher level. However in the current study, 76% (38/50) of the SCD patients having HbF level <21.1%, presented as stable status without any complication suggesting there must be other additional contributing factor/s which might protecting against VOC. Most likely a protective background of uniform low osmolality and hypochromic microcytic anaemia due to nutritional deficiency in SCD group may contribute to keep the patients in stable state while VOC precipitation is still determined by the two key haematological parameters, HbF level and leukocytosis.

Taken together we report here maintaining reduced mean MCHC in SCD patients having simultaneous natural hyponatremia and hypokalemia. Hyponatremia may contribute to rehydrate sickled RBC. Prevalent microcytic anaemia in this tribal region of India due to co-existing nutritional anaemia in the poor socioeconomic group of SCD patients might interplay with hyponatremia on the background of high HbF response, protecting against HbS polymerization and episode of VOC. A cut off values of low HbF and high leukocyte count could be used in combination to screen high risk SCD patients. Our result suggests that even only leukocyte count could be effective to asses HbF response in a poorly equipped set up.

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