Homocysteine ,Folic acid ,Vitamin B_{12} and Pyridoxine : Effects on Vaso-Occlusive Crisis in Sickle Cell Anemia and Sickle –Thalassemia

Sawsan Sati' Abbas*, Najat Abul –Razak**, Noor Mustafa***, Razeqa Abd Ali****

ABSTRACT:

BACKGROUND:
Hemoglubiopathies include sickle cell anemia and the Thalassemia . S/ β0- Thalassemia have a clinical course similar to HbSS. Elevated concentration of Homocysteine contribute to thrombosis , a frequent event in sickle cell anemia . Vitamin B_{12}, Pyridoxine , and folic acid deficiencies lead to dangerous increase in plasma Homocysteine.

OBJECTIVE:
1- To test whether children with sickle cell anemia and Sickle cell – Thalassemia have elevated concentration of serum Homocysteine with diminished level of folate, B_6, B_{12}.
2- To determine whether hyperomocysteinaemia has a correlation with the frequency of Vaso-occlusive crisis.

PATIENTS AND METHOD:
A case- control study was carried over a period of one year from Jan.- Dec. 2010 inclusive, 30 patients were collected from the Thalassemia centre in Ibn –AL-Baldy Hospital together with healthy 30 cases, age and sex matched ,were taken from AL- Kadhimiyia Teaching Hospital. Venous blood sample were aspirated from both groups to estimate serum Homocysteine, Folic acid, B_{12} and B_6 level. Statistical analysis was done, using the student T-test (P. value < 0.05 is considered as statistically significant) . Pearson correlation analysis was performed.

RESULTS :
The age of the patients range between (5-29) years, the majority of the patients were between (10 - 19) years , 10 cases (38 .46 %). More than one half were male ,16 cases (61.54 %) . Sickle cell – Thalassemia constitute 20 cases (76.93%). Vaso-occlusive crisis was mainly involving the large joints, 15 cases (57.69 %). Mild attacks constitute more than half of the patients, 16 cases (61.54 %).Homocysteine level was higher in the patients group compared with control group with a mean and standard deviation of (44.52 ± 23.008) and (18.65 ± 4.56)µmol/L respectively . Folic acid level was lower, B_{12} level was higher, B_6 level was lower in the patients group compared with control group with a mean and standard deviation of (11.32± 3.23) and (14.71 ± 3.39)ng /ml, (172.57± 61.34) and (103.45 ± 30.45)pg /ml, (4.43± 3.93) and (10.23 ± 2.30) ng/ml respectively, the results were statistically not significant, P. value > 0.05. Significant inverse correlation was found between Homocysteine level and B_6 level. A strong positive correlation between Homocysteine level and the frequency of Vaso-occlusive crisis was found.

CONCLUSION:
Patients with sickle cell disease have high serum level of Homocysteine with low level of folic acid and pyridoxine. This Hyperomocysteinaemia is significantly inversely correlated with pyridoxine deficiency , but positively correlated with the frequency of Vaso-occlusive crisis.

KEY WORDS: Sickle cell anemia ,Sickle –thal , children ,Homocysteine ,Folic acid ,Vitamin B_{12} ,pyridoxine (B_6) . Vaso-occlusive crisis.

INTRODUCTION:
Hemoglubiopathies are diseases caused by genetic mutations that result in abnormal, dysfunctional hemoglobin molecules or lower level of normal hemoglobin molecules [1]. The most common hemoglubiopathies are sickle cell anemia and Thalassemia[1]. S/ β0- Thalassemia have a clinical course similar to HbSS[2]. Elevated concentration

*Department of Pediatric /College of Medicine /AL-Nahrain University.
**Department of Biochemistry /College of Medicine /AL-Nahrain University.
***Medical Research Unite /College of Medicine /AL-Nahrain University.
****ThalassemiaCentre/ Iben AL-Balady Hospital.

of Homocysteine contributes to thrombosis, a frequent event in sickle cell anemia[3]. More recently degrees of folate inadequacy, not severe enough to produce anemia, have been found to be associated with high levels of amino acid Homocysteine[4]. Vitamin B_{12}, Pyridoxine (B_6) and folic acid deficiencies lead to dangerous increase in plasma Homocysteine[5].

AIM OF THE STUDY:

1- To test whether children with sickle cell anemia and Sickle cell – Thalassemia have elevated concentration of serum Homocysteine with diminished levels of folate, B_6, B_{12}.

2- To determine whether hyperomocysteinaemia has a correlation with Vaso-occlusive crisis.

PATIENTS AND METHOD:

A case-control study was carried over a period of one year from first of Jan. 2010 to the end of Dec. 2010 , 30 cases of patients (sickle cell anemia and Sickle cell – Thalassemia) were collected from the Thalassemia centre in Ibn –AL-Baldy Hospital together with 30 healthy cases, age and sex matched ,were taken from AL- Kadhimiyia Teaching Hospital (relative of patients admitted for other reasons , after taking their consent) .

Data obtained from the patients concerning name , age , gender, residence ,frequency of occurrence of Vaso-occlusive crisis , severity of pain and site , whether they took folic acid , received desferal and mode of it's administration . The severity of Vaso-occlusive crisis was determined according to the pain scale (1-10) plus whether the patient use hospital, emergency or unscheduled ambulatory care for pain in the previous day [6, 7]. Physical examination was performed.

A 3cc of venous blood sample were aspirated from both groups and centrifuged ,after centrifugation the serum were taken to the Medical Research Unit, in the College of Medicine / AL-Nahrain University , Baghdad, to estimate serum Homocysteine ,Folic acid ,B_{12} and B_6 level , 4 samples of the patients and 5 of the control were neglected because the serum is not enough ,so the tests were performed on 26 cases of the patients and 25 cases of the control group.

Using High Performance Liquid Chromatography (HPLC), Shimadzu (Kyoto, Japan) which consisted of a system controller model SCL-10 AVP, a degasser model DGU-12A, two liquid delivery pumps model LC-8AVP, UV-Visible detector model SPD-10AVP, and injector model SIL-10A, equipped with 20 µl sample loop. The HPLC system has been interfaced with computer via a Shimadzu class-VP5 chromatography data system program supplied by the manufacturer; Epson LQ-300 printer model P852A (Japan)

Standard preparation

A stock solution of 100 µmol/L of standard Homocysteine (sigma) was prepared by dissolving 10mg of Homocysteine methanol and diluted to 100ml. The same procedure for folate, vitamin B_6 and vitamin B_{12} were followed in the preparation of their stock solutions. Other standard solutions were prepared by subsequent dilution of these stocks solutions.

Serum sample preparation

Samples prepared by adding 50µl of 15% 5-sulphosalsic to 400µl of serum, then mixed and centrifuge at 5000 rpm for 10 min. The supernated was taken and diluted ten folds with distilled water and filtrated using minipore filter paper.

Analysis of folate, vitamin B_6 and vitamin B_{12}

All samples and standard solutions of folate, vitamin B_6 and vitamin B_{12} chromatographically analyzed with C-18 column using mobile phase acetonitrile (A) and 50mM NaH_2PO_4 pH 2.5 (B), gradient 3min 100% (B), 6min 80% (B), 15min 60% (B), flow rate 1ml/min and UV-VIS detection at wavelength 230 nm in order to estimate vitamins. Normal value for serum folate is (5-20 ng / ml) , a level < 3 ng is considered as deficiency [8] , B_{12} (140- 700 pg / ml) [9] , B_6 (3.6 -18 ng / ml) [10] .

Analysis of Homocysteine

All samples and standard solutions of Homocysteine chromatographically analyzed with C-18 column using mobile phase linear gradient from 0.1M acetate buffer pH 4 containing 2% methanol to 0.1M phosphate buffer containing 6% methanol over 15min (0-100%), flow rate 0.5ml/min and UV-VIS detection at wavelength 245 nm . Normal Homocysteine level is (5- 15 µmol /L) [11].

Statistical analysis

Was done using Microsoft Excel program to estimate the mean and the standard deviation as well as to draw tables and figures , student T-test was performed between the patients group and the control group using the (SPSS) program (version 16) , P. value < 0.05 indicates statistical significance. Pearson correlation analysis was done between serum Homocysteine level and Folic acid, B_{12} and B_6 level as well as between Homocysteine level and the frequency of Vaso-occlusive crises, r. value range from -1.0 to 1.0 inclusive and reflects the extent of a linear relationship between two data sets. P.value was estimated, a value < 0.05 indicates statistical significance.

RESULTS:

In this study a total number of 26 cases of patients and 25 cases of the control were studied. The age of the patients range between (5-29) years with a mean and standard deviation of (14 .36 ± 7.62), while the age of the control range between (5-30) years with a mean and standard deviation of (14 .40 ± 7.69) , the majority of the patients were between (10 -19) years , constituting 10 cases (38 .46 %) as it is shown in (Table-1) . Applying the t-test, no statistical difference was found between the age of the two groups (t-test = 0.493260) , p . value = 0.311 845 i.e. > 0.05

Of the patients group 10 (38 .46 %) cases were female and 16 (61.54 %) cases were male, male: female ratio equal to 1.6:1. In the control group, 12 cases (48%) were male and 13 cases (52%) were female. All of the patients were from Baghdad (100%).

The patients group includes 6 cases (23.07%) with Sickle cell anemia and 20 cases (76.93%) with Sickle- Thalassemia.

All of them received folic acid tablet (5 mg) regularly (100%), 11 cases (42.31 %) only on regular desferal which is given by infusion pump in all of them (100%).

Vaso-occlusive crisis (painful crisis) was mainly involving the large joints in 15cases (57.69%) followed by the small joints in 10 cases (38 .46 %) ,6 cases (23.07%) with back pain , 1 case (3.84 %) for each of the abdominal crisis and acute chest syndrome , no stroke , multiple sites encountered in 10 cases (38 .46 %) ,as it is shown in (Table-2).

The attacks were mild in more than half of the patients 16 cases(61.54 %) followed by moderate in 6 cases (23.08%) and severe in 4 cases (15.39%) as it is shown in (Table-3 -).

Homocysteine level was higher in the patients group compared with control group with a range of (17.59-91.91) µmol/L, the mean and standard deviation was ((44.52 ± 23.008) for the patients group while in the control group the range was (9.44 – 25.93) µmol/L with a mean and standard deviation of (18.65 ± 4.56) as it is shown in (Table- 4), applying the student T-test the result is statistically not significant (t- value =1.2872E -06) with a P.value > 0.05 .

Folic acid level was lower in the patients group compared with control group with a range of (5.20 -17.81) ng/ml , the mean and standard deviation was (11.32± 3.23) while in the control group the range was(7.87 -22.12) ng/ml with a mean and standard deviation of (14.71 ± 3.39) as it is shown in (Table -4-) , applying the student T-test the result is statistically not significant (t- value = 0.000644) with a P.value > 0.05 .

B_{12} level was higher in the patients group compared with control group with a range of (83.70 -313.27) pg/ml , the mean and standard deviation was (172.57± 61.34) while in the control group the range was(56.90 – 163.78) pg/ml with a mean and standard deviation of (103.45 ± 30.45) as it is shown in (Table-4),. Applying the student T-test the result is statistically not significant (t – value = 6.21365E -06) with a P.value, > 0.05.

B_6 level was lower in the patients group compared with control group with a range of (1 - 10. 46)ng/ml , the mean and standard deviation was (4.43± 3.93) while in the control group the range was(4.06 -15.71) ng/ml with a mean and standard deviation of (10.23 ± 2.30) as it is shown in (Table-4-) , applying the student T-test the result is statistically not significant (t- value = 5.80683E-08) with a P.value > 0.05 .

Pearson correlation shows no significant correlation between Homocysteine level and Folic acid level (r. value = 0.1, p. value > 0.05) as it is shown in (Fig-1-). No significant correlation was found between Homocysteine level and Vitamin B_{12} level (r. value = 0.231, p. value > 0.05) as it is shown in (Fig -2-) .A significant inverse correlation was found between Homocysteine level and B_6 level (r. value = - 0.393 ,p. value < 0.05) as it is shown in (Fig -3-) .For the patients group, a positive strong correlation was found between Homocysteine level and the frequency of Vaso-occlusive crises (r.value = 0.9 , p. value < 0.05) as it is shown in (Fig-4-).

Table 1:Shows Age Distribution

Age / year	No.	%
1- 9	8	30.77
10-19	10	38.46
20 -29	8	30.77

Table 2: Distribution of cases according to the site of Vaso- occlusive crisis (Painful crisis)

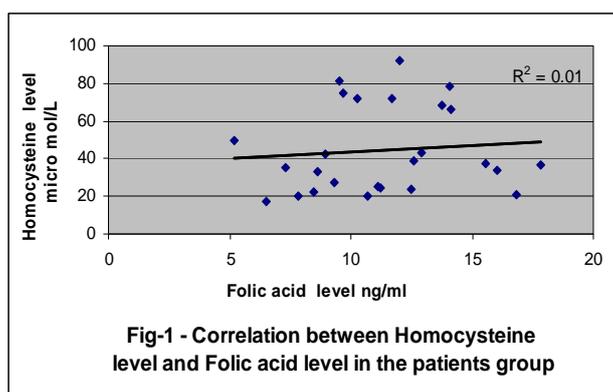| Site	No.	%
Large joints	15	57.69
Small joints	10	38.46
Back pain	6	23.07
Abdominal crisis	1	3.84
Acute chest syndrome	1	3.84
Central nervous system	/	/
Multiple sites	10	38.46

Table 3: Shows The Severity of The Vaso- occlusive crisis (Painful crisis)

Severity	Sickle –cell anemia		Sickle- Thalassemia		Total	
	No.	%	No.	%	No.	%
Mild	3	50	13	65	16	61.53
Moderate	1	16.66	5	25	6	23.08
Severe	2	33.34	2	10	4	15.39

Table 4: Shows the mean and standard deviation of the serum level of Homocysteine ,Folic acid B_{12} ,B_6 for the patients and the control group
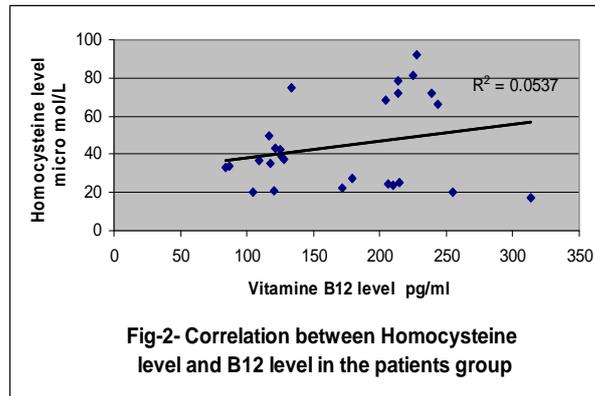
Serum level	patient	control	p. value*
Homocysteine level (µmol/L)	44.52 ± 23.008	18.65 ± 4.56	0.999999
Folic level (ng/ml)	11.32 ± 3.23	14.71±3.39	0.999248
B_{12} level pg/ml)	172.57±61.34	103. 45± 30.45	0.999995
B_6 level (ng/ml)	4.43 ± 3.93	10.23± 2.30	1

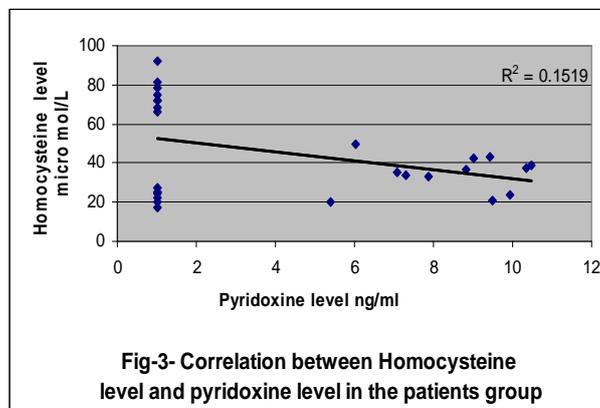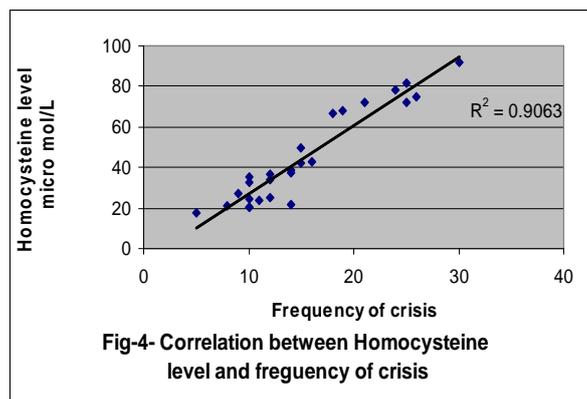- P. value > 0.05, i.e. not significant

Fig-1 - Correlation between Homocysteine level and Folic acid level in the patients group

r = 0.100
P .value = 0.627 i.e. > 0.05 (not significant)

Fig-2- Correlation between Homocysteine level and B12 level in the patients group

r = 0.231
p. Value = 0.255 i.e. > 0.05 (not significant)

Fig-3- Correlation between Homocysteine level and pyridoxine level in the patients group

r = - 0.393
P. value = 0.049 i.e. < 0.05 (statistically significant)

Fig-4- Correlation between Homocysteine level and freguency of crisis

r. value = 0.951
p. value = 0.000 i.e. < 0.05 (highly significant)

DISCUSSION:

The mean age of the patients is(14 .36 ± 7.62) ,10 (38 .46 %) cases were female and 16 (61.54 %) cases were male ,in comparison with a study done in Lebanon , the median age was (11.1 year) ,33 (64.70 %) were male and 18 (35.30%) cases were female [12] . In this study, 6

cases (23.07%) were Sickle cell anemia and 20 cases (76.93%) were Sickle- Thalassemia, in a study done in Lebanon, 48 cases (94%) were Sickle cell anemia and 3 cases (6 %) were Sickle- β Thalassemia[12,13]. This difference can be attributed to the fact that all cases were from Baghdad where Thalassemia is more common while sickle cell anemia is more encountered in Basra and other southern governorates.

The attacks were mild in more than half of the patients (61.54 %) followed by moderate (23.08%), then severe (15.39%), in comparison with a study done in Nigeria (20.81 %) were mild, (62.5%) were moderate and (16.7%) were severe. [6] In this study the large joints were involved in (57.69%) followed by the small joints in comparison with studies done elsewhere ,frequent pain crisis occur in (39.2%) followed by acute chest syndrome (31.4%) ,(Lebanon) [12,13] . In Brazil frequent painful crisis occur in (61.8%) [14]. In a Guadeloupe, painful crisis and acute chest syndrome were noticed in (65.4%) and (58.8%) respectively [15].

In this study multiple sites encountered in (38.4%) compared with Nigerian study multiple sites were involved in (45.8%) [6].

In this study , Homocysteine level was higher in the patients than the control , while Folic acid level is lower , B_{12} level is higher , B_6 level is lower in the patients than the control , there is no correlation between Homocysteine level and folic acid level as well as between Homocysteine level and B_{12} level , while a significant inverse correlation was found between Homocysteine level and B_6 level . In comparison with studies done elsewhere , Homocysteine level was also higher in the patients than the control, Ohio[16] ,Cincinnati[17] , Birmingham[18] , folate is significantly higher in patients than the control [16,17, 18] .Concerning B_{12} , a study done in Netherlands showed that the patients and the control had a similar level of B_{12} [19], no significant difference in B_{12} level between the patients and the control is seen in Birmingham study [18] while B_{12} is elevated in the patients group in Cincinnati study [17] . Studies done elsewhere showed that B_6 is significantly lower in the patients than the control group [16, 17]. Concerning correlation, Homocysteine is inversely correlated to folate [20]. An inverse correlation is seen between Homocysteine and B_{12} [3, 21]. Pyridoxine has a negative correlation with Homocysteine in a study done in Cincinnati [17] .

In this study significant positive correlation was found between Homocysteine level and the frequency of crisis which indicate that Hyperomocysteinaemia contributes for initiation of Vaso –occlusive crisis through occlusion of small blood vessels. This hyperomocysteinaemia may be attributed to pyridoxine deficiency , as it is known that Homocysteine is an intermediate compound of methionine degradation ,is normally remethylated to methionine , This methionine-sparing reaction is catalyzed by the enzyme methionine synthase, which requires a metabolite of folic acid (5-methyltetrahydrofolate) as a methyl donor and a metabolite of vitamin B_{12} (methylcobalamin) as a cofactor[22], in addition Homocysteine is trans –sulferated to cystathionine ,this pathway require Vitamin B_6[23],deficiencies of this vitamin will contribute to high serum Homocysteine level through disturbance in the metabolic pathway of Homocysteine[22] .

Although patients with sickle cell anemia and sickle- Thalassemia were given daily folic acid of 5 mg , still their folic acid is low and the supplementation need to be increased to 15 mg /day , in addition they need a daily supplementation of pyridoxine (100 mg/ day) which may be effective in alleviating the risk(s) conferred by hyperomocysteinaemia in sickle cell anemia and Sickle cell – Thalassemia.

CONCLUSION:

Patients with sickle cell anemia and Sickle-Thalassemia have high serum level of Homocysteine with low level of folic acid and pyridoxine compared with the control group. This Hyperomocysteinaemia is significantly inversely correlated with pyridoxine deficiency. There is strong positive correlation between Homocysteine level and the frequency of Vaso-occlusive crisis.

REFERENCES:

1. Vij R, Machado RF, Pulmonary complications of hemoglubiopathies , Chest , 2010;138: 973-83.
2. Micheal R. Debaun and Elliott Vichinsky ,Disease of the blood : In Behrman and Kliegman , Nelson Textbook of Pediatrics ,18ed , Philadelphia ,WB Saunders Company , 2008 ,Ch. 462.1 ,2038
3. Segal JB ,Miller ER ,Brereton NH , Rear LM , Concentration of B vitamins and Homocysteine in children with sickle cell anemia ,South Med. J. ,2004;97:149-55.

4. Malouf M ,Grimley EJ ,Areosa SA ,Folic acid with or without B_{12} for cognition and dementia , Cochrane Data base Sys Rev ,2003;4.

5. Cattaneo M , Hyperhomocystenemia ,atherosclerosis and thrombosis .Thrombo Haemost , 1999 ;81:165- 76.

6. Taiwo Kotila ,Sunday Ocheni ,Acute painful crisis in Sickle cell disease : severity assessment , the Internal journal of Tropical Medicine ,2006; 3 :44.

7. Wally R. Smith , Lynne T. Peberthy . Victor E. Bovbjerg , Daily assessment of pain in adults with Sickle cell disease ,Ann. Intren. Med. journal , Jan ,15, 2008;148 : 94 -101.

8. Micheal R. Debaun and Elliott Vichinsky , Disease of the blood : In Kliegman , Behrman, Nelson Textbook of Pediatrics ,18ed , Philadelphia ,WB Saunders Company , 2008;454.1 :2012 -13.

9. C. Charlton Mabry , Reference range for laboratory test : In Kliegman , Behrman, Nelson Textbook of Pediatrics ,13ed , Philadelphia ,WB Saunders Company , 1987;29 :1558.

10. Burak Turgut , Murat Kaya,Sermal Arslan et al , level of circulating Homocysteine and vitamin B_6 ,vitamin B_{12} ,and folate in different of types of open angle glaucoma : Dove press journal: Clinical intervention in aging , April, 2010 :133- 39.

11. Ueland PM , Refsum H, Stabler SP , et al ,Total Homocysteine in plasma or serum : Methods and clinical applications , Clin Chem ,1993;39:1764 -79.

12. Adlette K.Inati ,Mode N. Ojaimi ,Fouade M. Ziade et al , Risk factors for adverse outcome in patients with sickle cell disease in north Lebanon , proceeding of the 42 annual meeting of the American Society of Hematology, 2000.

13. Adlette K.Inati ,Mode N. Ojaimi ,Fouade M. Ziade et al, Clinical feature of sickle cell disease in north Lebanon , proceeding of the 42 annual meeting of the American Society of Hematology ,2000.

14. Angela Maria D Zanette, Ana Cristina C. Strapasson , Isa L. Queiroz et al Clinical and hematologic features in sickle cell syndrome (SS and Sc) in Salvador ,Bahia ,Brazil , proceeding of the 42 annual meeting of the American Society of Hematology , 2000 .

15. Tarer V., Etienne – Julan M. ,Diara JP et al ,Sickle cell anemia in Guadeloupean children : Pattern of acute clinical events ,Eur. J. Haematol ,2006 ; 76: 193-99.

16. Balassa V V , Kalinyak KA , Bean JA , et al , hyperhomocysteinemia is associated with low plasma pyridoxine level in children with sickle cell disease ,J. Pediatric Hematol Oncol ,2002;24 :374 -79.

17. VV Balassa ,K. Kalinyak ,Homocysteine and vitamin co- factor levels in patients with sickle cell disease, proceeding of 43 annual meeting of the American Society of Hematology , 2002 .

18. Lowenthal EA ,Mayo MS , Cornwell PE , Thornley – Browen D , Homocysteine elevation in sickle cell disease ,J. Am. Coll .Nutr . 2000;19 : 608 -12.

19. Vander Dijs FP ,Schnog JJ ,Brouwer DA , et al , Elevated Homocysteine indicate suboptimal folate status in pediatric sickle cell patients, Am. J. Hematol ,1998; 59:192- 8.

20. Houston PE , Rana S ,Sekhsaria S. ,Perlin E. , Kim KS Castro OL ,Homocysteine in sickle cell disease : relationship to stroke ,Am .J.Med ,1997 ; 103:192-96.

21. ER Miller , LMS Resar ,Vitamins and Homocysteine in children with sickle cell anemia , proceeding of the 43 annual meeting of the American Society of Hematology , 2002.

22. Iraj Rezvani and David S. Rosenblatt , Metabolic disease: In Kliegman , Behrman, Nelson Textbook of Pediatrics ,18ed , Philadelphia ,WB Saunders Company , 2008; 85:536.

23. Guilland JC ,Favier A ,Potier de Courcy G, et al , hyperhomocysteinemia : an independent risk factor or a simple marker of vascular disease ? 1. Basic data ,J. Pathol Biol (paris) ,2003;51:101-10.