

## Homocysteine ,Folic acid ,Vitamin B<sub>12</sub> and Pyridoxine : Effects on Vaso-Occlusive Crisis in Sickle Cell Anemia and Sickle –Thalassemia

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### ABSTRACT:

#### BACKGROUND:

Hemoglubiopathies include sickle cell anemia and the Thalassemia . S/  $\beta$ 0- Thalassemia have a clinical course similar to HbSS. Elevated concentration of Homocysteine contribute to thrombosis , a frequent event in sickle cell anemia . Vitamin B<sub>12</sub>, 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<sub>6</sub>, B<sub>12</sub>.
- 2- To determine whether hyperhomocysteinaemia 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- Kadhimiya Teaching Hospital. Venous blood sample were aspirated from both groups to estimate serum Homocysteine, Folic acid, B<sub>12</sub> and B<sub>6</sub> 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  $\pm$  23.008) and (18.65  $\pm$  4.56) $\mu$ mol/L respectively . Folic acid level was lower, B<sub>12</sub> level was higher, B<sub>6</sub> level was lower in the patients group compared with control group with a mean and standard deviation of (11.32 $\pm$  3.23) and (14.71  $\pm$  3.39)ng /ml, (172.57 $\pm$  61.34) and (103.45  $\pm$  30.45)pg /ml, (4.43 $\pm$  3.93) and (10.23  $\pm$  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<sub>6</sub> 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 Hyperhomocysteinaemia 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<sub>12</sub> ,pyridoxine (B<sub>6</sub>) . Vaso-occlusive crisis.

### INTRODUCTION:

Hemoglubiopathies are diseases caused by genetic

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mutations that result in abnormal, dysfunctional hemoglobin molecules or lower level of normal hemoglobin molecules <sup>(1)</sup>. The most common hemoglubiopathies are sickle cell anemia and Thalassemia<sup>(1)</sup>. S/  $\beta$ 0- Thalassemia have a clinical course similar to HbSS<sup>(2)</sup>. Elevated concentration

of Homocysteine contributes to thrombosis, a frequent event in sickle cell anemia<sup>(3)</sup>. 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<sup>(4)</sup>. Vitamin B<sub>12</sub>, Pyridoxine (B<sub>6</sub>) and folic acid deficiencies lead to dangerous increase in plasma Homocysteine<sup>(5)</sup>.

### 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<sub>6</sub>, B<sub>12</sub>.

2- To determine whether hyperhomocysteinaemia 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- Kadhimiya 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 its 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<sup>(6, 7)</sup>. 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<sub>12</sub> and B<sub>6</sub> 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<sub>6</sub> and vitamin B<sub>12</sub> 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-sulphosalicylic acid to 400µl of serum, then mixed and centrifuge at 5000 rpm for 10 min. The supernatant was taken and diluted ten folds with distilled water and filtrated using minipore filter paper.

### Analysis of folate, vitamin B<sub>6</sub> and vitamin B<sub>12</sub>

All samples and standard solutions of folate, vitamin B<sub>6</sub> and vitamin B<sub>12</sub> chromatographically analyzed with C-18 column using mobile phase acetonitrile (A) and 50mM NaH<sub>2</sub>PO<sub>4</sub> 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<sup>(8)</sup>, B<sub>12</sub> ( 140- 700 pg / ml )<sup>(9)</sup>, B<sub>6</sub> ( 3.6 -18 ng / ml )<sup>(10)</sup>.

### 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)<sup>(11)</sup>.

### 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<sub>12</sub> and B<sub>6</sub> 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<sub>12</sub> 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<sub>6</sub> 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<sub>12</sub> 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<sub>6</sub> 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 |

## SICKLE CELL ANEMIA AND SICKLE –THALASSEMIA

**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 |

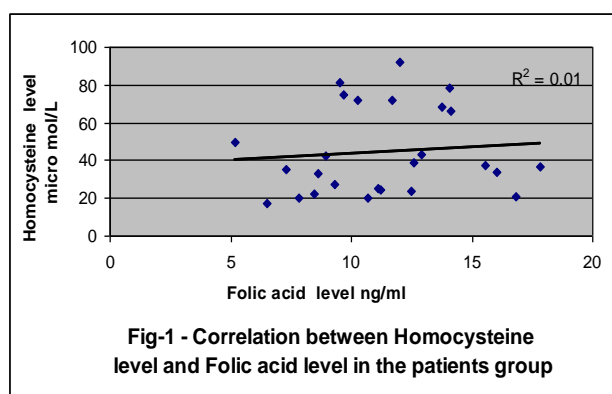
**Table 3: Shows The Severity of The Vaso- occlusive crisis ( Painful crisis)**

| 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<sub>12</sub> ,B<sub>6</sub> for the patients and the control group**

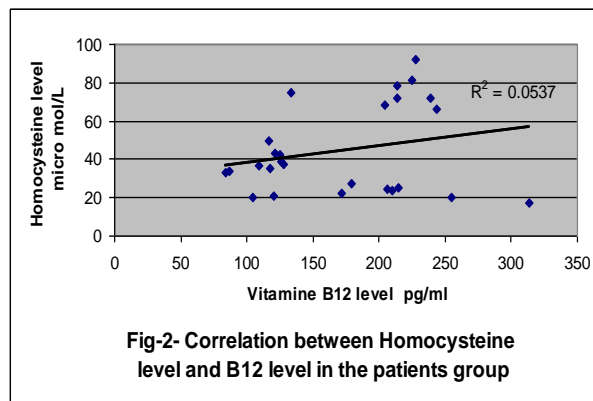
| Serum level                  | patient        | control        | p. value* |
|------------------------------|----------------|----------------|-----------|
| Homocysteine level (µmol/L)  | 44.52 ± 23.008 | 18.65 ± 4.56   | 0.999999  |
| Folic level (ng/ml)          | 11.32 ± 3.23   | 14.71±3.39     | 0.999248  |
| B <sub>12</sub> level pg/ml) | 172.57±61.34   | 103. 45± 30.45 | 0.999995  |
| B <sub>6</sub> level (ng/ml) | 4.43 ± 3.93    | 10.23± 2.30    | 1         |

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

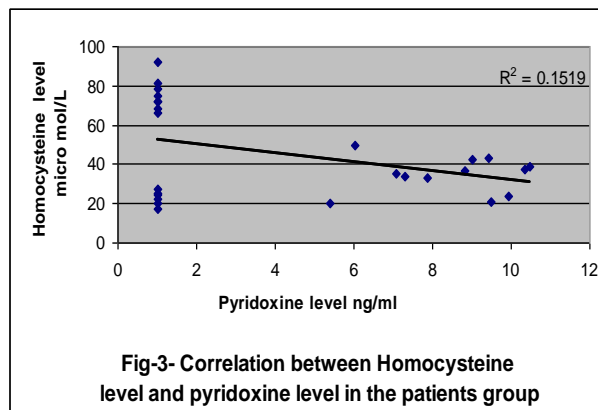


$r = 0.100$

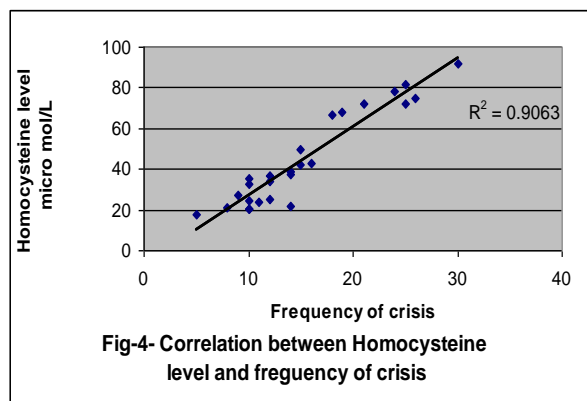
P .value = 0.627 i.e. > 0.05 (not significant)



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



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



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 <sup>(12)</sup> . 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-  $\beta$  Thalassemia<sup>(12,13)</sup>. 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.<sup>(6)</sup> 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)<sup>(12,13)</sup> . In Brazil frequent painful crisis occur in (61.8%)<sup>(14)</sup>. In a Guadeloupe, painful crisis and acute chest syndrome were noticed in (65.4%) and (58.8%) respectively<sup>(15)</sup>.

In this study multiple sites encountered in (38.4%) compared with Nigerian study multiple sites were involved in (45.8%)<sup>(6)</sup>.

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<sup>(16)</sup> ,Cincinnati<sup>(17)</sup> , Birmingham<sup>(18)</sup> , folate is significantly higher in patients than the control<sup>(16,17, 18)</sup> .Concerning  $B_{12}$  , a study done in Netherlands showed that the patients and the control had a similar level of  $B_{12}$ <sup>(19)</sup> , no significant difference in  $B_{12}$  level between the patients and the control is seen in Birmingham study<sup>(18)</sup> while  $B_{12}$  is elevated in the patients group in Cincinnati study<sup>(17)</sup> . Studies done elsewhere showed that  $B_6$  is significantly lower in the patients than the control group<sup>(16, 17)</sup> . Concerning correlation, Homocysteine is inversely correlated to folate<sup>(20)</sup>. An inverse correlation is seen between Homocysteine and  $B_{12}$ <sup>(3, 21)</sup>. Pyridoxine has a negative correlation with Homocysteine in a study done in Cincinnati<sup>(17)</sup> .

In this study significant positive correlation was found between Homocysteine level and the frequency of crisis which indicate that Hyperhomocysteinaemia contributes for initiation of Vaso –occlusive crisis through occlusion of small blood vessels. This hyperhomocysteinaemia 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<sup>(22)</sup> , in addition Homocysteine is trans –sulferated to cystathionine ,this pathway require Vitamin  $B_6$ <sup>(23)</sup>,deficiencies of this vitamin will contribute to high serum Homocysteine level through disturbance in the metabolic pathway of Homocysteine<sup>(22)</sup> .

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 hyperhomocysteinaemia 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 Hyperhomocysteinaemia is significantly inversely correlated with pyridoxine deficiency. There is strong positive correlation between Homocysteine level and the frequency of Vaso-occlusive crisis.

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