Asian Hematology Research Journal

1(2): 67-76, 2018; Article no.AHRJ.41741

Non-Fasting Total Serum Homocysteine (tHcy) Levels in Folate-Supplemented Omani Arabs with Sickle Cell Disease in the Steady State and in Crisis

Huxley Knox-Macaulay[1], David Gravell[2], Gavin Ross[3], Fehmida Zia[1], Anil Pathare[1] and Salam Al Kindi[1*]

[1]Department of Haematology, Sultan Qaboos University, Oman.
[2]Sultan Qaboos University Hospital, Oman.
[3]Department of Statistics, Rothamsted Research (Formerly Rothamsted Experimental Station), Harpenden, Herts AL5 2JQ, UK.

Authors' contributions

This work was carried out in collaboration with all authors. Author HKM designed the study and wrote the first draft of the manuscript. Author DG carried out the bench work. Authors GR and AP did the statistical analysis. Author FZ performed the literature searches. Author SAK supervised the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AHRJ/2018/41741
Editor(s):
(1) Shubhangi Agale, Associate Professor, Department of Pathology, Grant Medical College, Mumbai, India.
Reviewers:
(1) Godfrey Mutashambara Rwegerera, University of Botswana, Botswana.
(2) Sergio Parco, University of Trieste, Italy.
(3) Nina Filip, Grigore T. Popa University of Medicine and Pharmacy, Romania.
(4) Kalima Nzanzu Adelard, Catholic University of Graben, Democratic Republic of the Congo.
Complete Peer review History: http://www.sciencedomain.org/review-history/24897

Original Research Article

Received 14th March 2018
Accepted 25th May 2018
Published 31st May 2018

ABSTRACT

The aim of this investigation is to determine tHcy levels in SCD Omani Arabs in (i) the steady state (ii) crisis (iii) relative to non-SCD subjects as such values might help clarify the role of tHcy in SCD. Serum tHcy concentrations and other laboratory analyses including serum folate and cobalamin measurements were performed on non-fasting blood samples in 287 non-smoking predominantly adult subjects comprising 133 SCD (SCDstst and SCDcr) patients, 23 HbAS individuals and 131 volunteers acting as controls. Serum tHcy concentration was measured by the Abbott IMx Analyzer using a competitive fluorescence polarization immunoassay (FPIA) while serum folate was determined using the Abbott IMx folate reagent kit based on ion capture technology and serum

**Corresponding author: E-mail: sskindi@yahoo.com;*

cobalamin by the Abbott IMx B_{12} microparticle enzyme intrinsic factor immunoassay (MEIA). Other investigations performed included detailed blood cell counts and variant hemoglobin analyses. Blood counts of the whole cohort whose ages ranged from 8-50 years were essentially normal; nevertheless, hypochromic microcytic red cells were found in about 80% of cases suggestive of α thalassemia trait. Serum folate and cobalamin values post-fortification were normal. Serum tHcy levels were lower in SCD patients than in HbAS and normal volunteers who acted as controls while SCDstst tHcy concentration was higher than that of SCDcr. Our results in Omani Arabs showed the tHcy levels in SCD patients during steady state are higher than those in crisis and are at variance with those obtained in African-Americans and Nigerians in whom tHcy values in the steady state are lower than in a crisis. However, differences in assay methods and varying proportions in SCD haplotypes (50% Benin, 25% Bantu and 25% Arab Indian) amongst the Omani SCD patients are likely to explain this variance. Folate and cobalamin supplementation facilitated vaso-occlusion. The nature of the β^S haplotype and the prevalence of α thalassemia appear to militate against the development of stroke in our SCD Omani patients.

Keywords: Omani Arab; Serum Total Homocysteine (tHcy); SCD; folic acid; cobalamin.

1. INTRODUCTION

Homocysteine is a non-dietary sulphur-containing amino acid whose in vivo synthesis is at the crossroads of two methionine metabolic pathways: (i) the cobalamin (vitamin B_{12}) – dependent and non-dependent remethylation pathway and (ii) the transulphuration pathway [1]. It is found in serum or plasma in several chemical forms including uncomplexed 'simple' homocysteine (Hcy), and Hcy complexed with (a) inorganic disulphides (Hcy-S-S-Hcy; homocystine) (b) mixed disulphide (Hcy-S-S-cysteine) and (c) protein, in particular, albumin (Hcy-S-S-albumin). Thus, estimation of 'homocysteine' levels in serum or plasma is that of the 'total homocysteine' (tHcy) species with estimated values being usually higher in serum. Functionally, homocysteine as a metabolite accumulates in the cells and is processed into other substances, such as S-Adenosyl methionine (SAM). SAM plays a key role in the initial stages of synthesis for many metabolic pathways, but folic acid and vitamin B12 are necessary for homocysteine conversion. If these vitamins are deficient, the metabolic step cannot take place leading hyperhomocysteinemia.

Serum or plasma tHcy levels are increased frequently in venous thromboembolism [1,2] and in acquired atherothrombosis of various blood vessels such as peripheral, coronary, cerebral (including strokes) [3,4] and retinal arteries [5-7] which may lead to ischemia with or without infarction of the relevant organs. It is therefore not surprising that hyperhomocysteinemia is being reported increasingly in sickle cell disease (SCD), an inherited vaso-occlusive disorder with hemolytic and vaso-occlusive manifestations. Several intrinsic factors which facilitate vaso-occlusion in SCD include a low arterial pH, high red cell 2,3 DPG concentration and a reduced HbF. Fever, infection, dehydration, trauma and non-infectious illnesses are some of the extrinsic factors which often precipitate in vivo sickling with resultant vaso-occlusion.

Hyperhomocysteinemia has been demonstrated in diverse ethnic groups of SCD patients [8,9]. There are a number of published investigations on the concentrations of serum or plasma tHcy of various nationalities and races. However, information on the circulating tHcy status of SCD Arabs and its possible relevance to vaso-occlusive episodes in this racial group is singularly lacking. We, therefore, embarked on determinations of serum tHcy levels in fully folate-supplemented SCD Omani Arabs in the steady state (*SCDstst*) and in crisis (*SCDcr*); we then compared the values in these two groups to each other as well as to those of their healthy compatriots. SCD patients received a 5 mg folic acid tablet perorally daily. Possible correlations between concentrations of circulating tHcy and other variables of the SCD participants were also determined. A complimentary evaluation of serum tHcy levels of non anemic HbAS (sickle cell trait) Omani Arabs and Omani blood donor control volunteers (BDCVs) were included in the study. Folic acid supplements were not administered to HbAS individuals and BDCVs.

2. SUBJECTS, MATERIALS AND METHODS

2.1 Study Subjects

The cohort of male and female patients was recruited primarily and randomly from the adult

SCD clinic of the Hematology Department of Sultan Qaboos University Hospital (SQUH). Four children aged ≤ 10 years were included also in the analysis. All the recruited participants had been compliant in taking their medications. The following were excluded from the study: (i) pregnant females (ii) patients who had received a blood transfusion within three months preceding this investigation (iii) patients with various significant comorbidities including diabetes, hypertension, renal dysfunction, neurovascular and cardiac disorders and (iv) patients on hydroxyurea therapy. The SCD participants were regarded as being in a '*steady state'* (*SCDstst*) provided they had remained essentially asymptomatic, pain-free and clinically stable during four weeks prior to the start of investigations and throughout the study period. Acutely ill patients who presented with features indicative of a vaso-occlusive event were admitted directly into hospital as they were diagnosed as being in *crisis*.

Clinical history was elicited from the whole cohort of SCD *(SCDstst & SCDcr*) patients, HbAS individuals and BDCVs. *SCDstst* patients were examined in the outpatients SCD clinic while *SCDcr* patients were admitted directly to the hospital wards for evaluation and management. Specific detailed instructions about eating patterns on the day of collection of non-fasting blood samples were usually given initially on recruitment to *SCDstst* patients, HbAS individuals and BDCVs followed by a reminder on the morning of the actual day of blood sample collection. The emphasis on the management of crisis patients was in ensuring adequate fluid intake and replacement. Initial and follow-up clinical assessments of *SCDstst* and *SCDcr* patients were carried out by the same medical personnel.

2.2 Materials and Methods

2.2.1 Laboratory investigations

Non-fasting blood samples were collected in the supine position from asymptomatic *SCDstst* patients in the SCD clinic in the mornings; similar samples were obtained from HbAS individuals and BDCVs. Acutely ill *SCDcr* patients provided blood samples immediately on admission into the hospital.

Laboratory analyses were carried out on 287 non-smoking subjects comprising 133 SCD (*SCDstst* and *SCDcr*) patients, 23 HbAS individuals and 131 healthy BDCVs. Total blood cell and differential counts were determined by automated methods using the Abbott CELL-DYN 4000 (Abbott Diagnostics, Abbott Park, IL) [10,11]. Constituent normal and variant hemoglobins were separated and measured by high performance liquid chromatography (HPLC) using the Bio-Rad Variant II^{TM} instrument (Bio-Rad Laboratories, Hercules, CA, USA) [12]. Three BDCVs were excluded from the study because 18% of an unidentifiable variant haemoglobin were found in one volunteer while two other volunteers seemed to have inherited the HbC trait (HbAC).

Sera for determination of folic acid and cobalamin concentrations were separated from clotted blood samples within one hour of collection. Relevant vitamin assays were carried out immediately following blood extraction or were performed on samples stored at -20°C. Duplicated levels of serum folic acid were measured on batches of thawed samples within six weeks of freezing the sera. Duplicate folate assays of hemolysates of either fresh or thawed EDTA red cell samples used the Abbott IMx folate reagent kit based on ion capture technology (Abbott Laboratories, Diagnostics Division, Abbott Park, Illinois, USA). Serum cobalamin assays were carried out in duplicate with the Abbott IMx B_{12} microparticle enzyme intrinsic factor immunoassay (MEIA) (Abbott Laboratories, Diagnostics Division, Abbott Park, Illinois, USA). Appropriate cyanocobalamin controls yielding recommended serum cobalamin concentrations were included in the batches' assays.

Serum tHcy concentrations were measured on separated fresh or thawed sera stored at -20°C for two to six weeks. The tHcy values of the serum samples were estimated quantitatively on the Abbott IMx Analyzer using a competitive fluorescence polarization immunoassay (FPIA) [13]. Hcy controls comprised L-Hcy in non-reactive processed human serum at three concentration ranges: 5.25-8.75 µmol/L, 10.0-15.0 µmol/L and 20.0-30.0 µmol/L. Appropriate quality control procedures were performed for the Abbott Imx tHcy calibration and controls.

2.2.2 Statistical analysis

After exclusion of the three BDCVs from the original group of 287 participating subjects, the data of the remaining 284 subjects were then analyzed. Thus, the final cohort (Table 1)

Table 1. Age-ranges and gender distribution, mean hemoglobin values and other red cell indices of participants in the Omani Total Homocysteine (tHcy) study

Categories	No.	Mean age (Y)	Hb g/l	Retics (x 10^9/l)	HbS (%)	MCHbs (pg)
SCDstst; M+F	69	18.8 ± 5.4	102.5± 15.0	214.6±82.2	84.6±8.0	20.01±3.2
SCDstst; M	36		105.8±15.6	206.6±11.4	83.4±10.1	19.52±3.5
SCDstst; F	33		98.8±12.6	224. ±94.84	86.0±4.4	20.54±2.6
SCDcr; M+F	64	23.2±6.1	90.6±16.0	197.9±85.0	83.1±10.9	20.36±3.8
SCDcr; M	24		95.0±15.0	207.6±71.0	81.2±12.1	19.54±3.5
SCDcr; F	40		88.0±16.0	192.1±93.0	84.3±10.1	20.85±4.0
HbAS; M+F	23	27.3±14.5	133.1±14.9	85.8±45.8	28.5±4.5	6.84±1.7
HbAS; M	15		139.1. ±13.61	86.2±53.4	28.3±4.3	6.90±1.7
HbAS;F	8		121.2±9.9	85.2±	28.9±5.2	6.73±1.8
BDCVs;M+F	128	25.9±7.0	140.9±11.7	79.22±26.2	Nil	Nil
BDCVs;M	109		143.5±10.2	79.94±26.6	Nil	Nil
BDCVs;F	19		126.0±7.6	75.09±24.3	Nil	Nil

No.: number of subjects; Hb: haemoglobin; Retics: absolute reticulocyte count; HbS%: percentage concentration of sickle haemoglobin; MCHbS: mean cell sickle haemoglobin (pg); SCDstst: sickle cell disease in steady state; SCDcr: sickle cell disease in crisis; HbAS: sickle cell trait; BDCVs: blood donor control volunteers; M: males; F: females; M+F: combined total of males and females

comprised the following: (i) 69 *SCDsts*t patients (ii) 64 *SCDcr* patients (iii) 23 HbAS individuals and (iv) 128 BDCVs. They were analyzed using a "Maximum Likelihood Program (MLP)" statistical package comprising a number of standard statistical tests including Student's't' test and correlation coefficients [14]. Apart from serum *tHcy* concentration, other evaluated variables were age, red blood cell count, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width, absolute reticulocyte count, white blood cell count, platelet count, serum cobalamin (vitamin B_{12}), serum folate, red cell folate, HbA_2, HbF, HbS%, mean cell HbS (MCHbSpg). The mean ± standard deviation of each variable was calculated in each clinical category for (a) male and female subjects separately (b) and the combined total of male and female subjects (Table 1). A logarithmic scale was considered appropriate in cases where the distribution was skewed.

Using the standard Student's 't' test with derived probability (p) values, we compared the means of the serum tHcy concentrations of the following pairs of age and gender-dependent subgroups: total male ('TM') versus (vs) total female ('TF'), 'young' male ('YM') vs 'young' female ('YF'), 'old' male ('OM') vs 'old' female ('OF'). Also, similar comparisons were made between the means of the non-serum tHcy variables of three or more gender- and age-dependent subgroups by a one-way analysis of variance standard F test. Differences between selected pairs of subgroups were appropriately evaluated only in cases in which F was significant. Pearson correlation coefficients (r) with their related probability (p) values were determined between serum tHcy concentration and the other variables.

3. RESULTS AND DISCUSSION

3.1 Results

The age-ranges and gender-distribution, mean hemoglobin values and other red cell indices of participants in the Omani tHcy study of the whole cohort are outlined in Table 1. At one of the more recent censuses (2014), the population of the Sultanate was just over 3 million (3,219,775) [15] with immigrants making up just over 30% of the total population. The age structure of the Omani population is relatively 'young' with 50% < 25 years and 93% < 55 years. Excluding an isolated 80-year-old HbAS 'outlier', a 50-year-old BDCV was the oldest individual in the whole cohort. The mean ages as shown in Table 1 of the subjects in the various clinical categories are:

SCDstst (69 patients): 18.8± 5.4 years;
SCDcr (64 patients): 23.2 ± 6.1 years;
HbAS (23 subjects): 27.3 ± 14.5 years;
BDCVs (128 healthy volunteers): 25.9±7.0 years.

Relevant hematological data as summarized in Table 1 shows a significantly higher mean Hb in males (105.8 ± 15.6 g/L) than females (98.8 ± 12.6 g/L; p = 0.046) among *SCDstst* patients. However, no significant gender differences in

mean values of (i) absolute reticulocyte counts (p = 0.35) (ii) HbS% concentration (p = 0.17) and (iii) MCHbSpg (p = 0. 21) were noted. Mean Hb concentrations of *SCDcr* males and females were not significantly different from each other (p = 0.084) nor were the means of the absolute reticulocyte counts (p=0.49), HbS% concentration (p = 0.27) and MCHbSpg (p = 0.19). The mean Hb concentration of the small group of 23 HbAS individuals was significantly higher among males than females (p = 0.0047) but the mean values of the absolute reticulocyte counts (p = 0.98), HbS% concentration (p = 0.77) and MCHbSpg (p = 0.83) were essentially similar in both sexes. Male members of the healthy BDCVs group had a higher mean Hb than female members (p< 0.01) but the mean absolute reticulocyte count was not significantly different between the sexes (p = 0.46). The gross inequality in the numbers of BDCV males (109) and females (19) may invalidate gender comparisons within this group.

Further evaluation of group differences using the F test analysis of variance revealed the following: (i) the mean Hb of the total number of male and female *SCDcr* patients was less than that of the total number of male and female *SCDstst* patients which was also less than the mean Hb of HbAS individuals and BDCVs (p ≤ 0.01) (ii) the mean absolute reticulocyte counts of HbAS individuals and BDCVs were less than the mean absolute reticulocyte counts of *SCDstst* and *SCDcr* patients (p ≤ 0.01) and (iii) the mean HbS% of HbAS subjects was less than the mean values of *SCDstst* and *SCDcr* patients (p≤ 0.01) (iv) the mean MCHbS pg of HbAS subjects was less than that of *SCDstst* and *SCDcr* patients (p ≤ 0.01).

Mean serum and red cell folate concentrations of *SCDstst* and *SCDcr* patients were normal and consistent with their daily intake of folic acid, while the mean serum and red cell folate levels of HbAS subjects and BDCVs were within their respective SQUH laboratory reference ranges. Mean serum cobalamin values of the various clinical categories were unremarkable. The mean serum tHcy concentrations of *SCDstst* and *SCDcr* patients were within the BDCVs' control range though the mean tHcy values of the *SCDstst* and *SCDcr* patients were respectively lower than those of the HbAS subjects (Table 2).

Table 3 demonstrates clearly a significant difference between the mean serum tHcy concentration of (i) *SCDstst* and *SCDcr* patients (p≤ 0.01) (ii) *SCDstst* patients and BDCVs (p≤ 0.01) (iii) *SCDcr* patients and BDCVs (p≤ 0.01). However, no significant difference was noted between the mean serum tHcy concentrations of the two normal healthy groups - HbAS subjects and BDCVs. Comparative analyses of the mean serum tHcy levels of HbAS males and females (not shown in Table 3) and of the mean serum tHcy concentrations of male and female *SCDcr* and *SCDstst* patients revealed no significant gender differences. Moreover, further evaluation

Table 2. Mean serum Cobalamin, Folate and Total Homocysteine (tHcy) levels of (i) Omani Arabs with Sickle Cell Disease (SCD) and Sickle Cell Trait (HbAS) and (ii) Omani Arab Blood Donors Control Volunteers (BDCVs)

Subjects (No)	Hb g/l	Cobl pg/ml	Sfol ng/ml	RBCfol ng/ml	tHcy µmol/l
SCDstst; M+F(69)	102.5± 15.0	436.2±40.1	34.4±5.0	367.2±	8.8±2.2
SCDstst; M(36)	105.8±15.6	428.1±38.4	29.1±6.3	357.2±35.5	9.1±5.5
SCDstst; F(33)	98.8±12.6	445.0±38.4	40.1±7.9	378.2±36.3	8.6±1.8
SCDcr; M+F(64)	90.6±16.0	380.4±32.5	59.9±7.3	398.8±27.8	7.3±2.6
SCDcr; M(24)	95.0±15.0	373.4±35.4	49.7±10.5	368.5±35.1	7.4±2.8
SCDcr; F(40)	88.0±16.0	384.8±48.5	66.3±10	416.9±39.2	7.2±2.4
HbAS; M+F(23)	133.1±14.9	430.8±39.8	11.8±4.4	224.7±33.7	11.9±7.3
HbAS; M(15)	139.1±13.61	440.5±48.1	9.5±2.5	140.3±8.3	12.9±8.5
HbAS;F(8)	121.2±9.9	410.0±80.6	16.7±3.6	383.0±67.0	9.6±3.2
BDCVs;M+F(128)	140.9±11.7	440.4±18.7	10.4±3.1	142.2±64.0	11.2±4.5
BDCVs;M(109)	143.5±10.2	448.4±20.8	10.4±3.0	141.0±64.0	11.2±4.8
BDCVs;F(19)	126.0±7.6	394.8±38.6	10.6±3.2	148.9±71.0	10.8±2.9

SCDstst: sickle cell disease in steady state; SCDcr: sickle cell disease in crisis; HbAS: sickle cell trait; BDCVs: blood donor control volunteers; M: Male; F: Female; M+F: combined total of males and females; Hb: Hemoglobin; Cobl: serum cobalamin concentration; SQUH serum reference range for cobalamin: 148-943 pg/ml; Sfol: serum folate concentration; SQUH serum folate reference range:2.6-10.2 ng/ml; RBC fol: red blood cell folate concentration; SQUH rbc folate reference range : 170-500 ng/ml; tHcy: total serum homocysteine concentration

Table 3. The Total Homocysteine (tHcy) Investigation in Omanis: comparison of mean serum tHcy concentration of Sickle Cell Disease (SCDstst and SCDcr) Patients with HbAS (sickle cell trait) subjects and Blood Donor Control Volunteers(BDCVs)

Mean tHcy conc. (µmol/l)	t	P
SCDstst: 8.8±2.2 vs SCDcr: 7.3±2.6	3.38	<0.01
SCDstst: 8.8±2.2 vs BDCVs: 11.16±4.5	-3.44	<0.01
SCDcr: 7.3±2.6 vs BDCVs: 11.16±4.5	-5.61	<0.01
HbAS: 11.9±7.3 vs BDCVs: 11.16±4.5	0.44	0.66

Conc.: concentration; SCDstst: sickle cell disease in steady state; vs: versus; SCDcr: sickle cell disease in crisis; HbAS: sickle cell trait; BDCVs: blood donor control volunteers; t: significance test; p: probability value

showed no significant differences between the mean serum tHcy values of the age-gender dependent subgroups - 'OM' vs 'YM', 'OF' vs 'YF' - of each clinical category except those of BDCVs in which the mean serum tHcy of 'YM' aged 8-20 years was 11.63 ± 0.71µmol/L and of 'OM' aged 21 – 34 years was 10.11 ± 1.52µmol/L ($p = 0.04$).

Fifty percent of the 133 SCD cases in this study were SS/αthalassemia with $S\beta^0$ thalassemia accounting for almost 25%. The diagnosis of concomitant α and β thalassemia was non-molecular and was based on red cell count, MCV, MCH, hypochromic microcytic red cells as well as HbA_2 values. The rarer HbSD and HbSE diseases accounted for only five percent of the SCD cohort (Table 4). Evaluation of the inter-relationships of (a) serum tHcy concentration with each of the other listed variables and (b) the numerous non-tHcy variables with each other showed that the only significant correlations were between serum tHcy levels and (i) red cell folate concentration in *SCDstst* patients ($r = 0.401$, $p = 0.006$) (ii) red cell folate concentration in *SCDcr* patients ($r = - 0.327$, $p = 0. 008$) (iii) platelet count in *SCDstst* patients ($r = - 0.258$, $p = 0.03$) (iv) serum cobalamin levels in BDCVs ($r = - 0. 274$, $p = 0.002$) and (v) serum folate levels in BDCVs ($r = - 0.204$, $p = 0.02$).

3.2 Discussion

Since McCully's observation in 1969 on the relationship between premature atherosclerosis and homocystinuria [16], numerous retrospective and prospective studies have confirmed an independent association between raised circulating tHcy concentration and vascular disease affecting primarily ocular[5,6,7], coronary, peripheral, and cerebral vessels [1-4]. A rising serum or plasma tHcy level tends also to accompany increasing age while greater tHcy values are observed more frequently in healthy males than females [17].

After an 11-year follow-up of 7048 men and women from two provinces in eastern Finland, Alfthan et al found that mean serum tHcy values were similar among those with myocardial infarction and strokes compared to matched controls [18]. Their findings therefore indicated the absence of a positive relationship between circulating tHcy and vascular disease, an association which up till then had been assumed to be universal. Nevertheless, in a review of the subject of hyperhomocysteinemia and vascular disease, doubts were expressed by Townend and colleagues about the validity of the tHcy assay in this Finnish study [4].

Dietary folate deficiency may lead to deleterious changes in small blood vessels associated with increased circulating tHcy concentration [3]. Evidence of the interaction of tHcy with medium-sized and small blood vessels followed by subsequent vascular abnormmalities has been accumulated over the years in SCD and non SCD individuals. In a meta-analysis of the tHcy-vascular relationship, Boushey and colleagues (1995) [3] noted a positive association between rising serum or plasma tHcy levels and atherosclerosis of the carotid and coronary arteries in 11 of 27 relevant studies; in 9 case control studies of cerebrovascular disease, a similar positive relationship was observed. However, in two prospective studies, no association was observed between tHcy concentration and cerebrovascular disease. Also, in a meta-analysis of peripheral vascular disease, Boushey et al. [3] provided supporting evidence for the association of

Table 4. Haemoglobin phenotypes of Sickle cell disease (SCD) Patients, Sickle Cell Trait (HbAS) subjects and Blood Donor Control Volunteers (BDCVs) in Oman

Hb phenotype	SCDstst	SCDcr	Hb AS	BDCVs	133SCD patients (100%)
SS/α thal	35	32	-	-	67(50.4%)
SS	13	19	-	-	32(24.1%)
$S\beta^0$ thal	18	9	-	-	27(20.3%)
SE/α thal	2	0	-	-	2(1.5%)
SD/ α thal	1	4	-	-	5(3.7%)
AS/ α thal	-	-	18	-	18(NA)
AS	-	-	5	-	5(NA)
AA	-	-	-	55	55(NA)
AA/ α thal	-	-	-	67	67(NA)
α β thal	-	-	-	6	6(NA)
Total	69	64	23	128	284

Hb: haemoglobin; SCDstst: sickle cell disease in steady state; SCDcr: sickle cell disease in crisis; HbAS: sickle cell trait; BDCVs: blood donor control volunteers; SCD:133(100%) sickle cell disease (SCDstst+SCDcr) includes listed Hb phenotypes; SS/αthal: homozygous sickle cell disease (sickle cell anemia) with α thalassaemia trait; SS: homozygous sickle cell disease (sickle cell anemia); $S\beta^0$ thal: sickle-β^0 thalassaemia disease; SE/αthal: sickle cell-hemoglobin E disease with α thalassaemia trait; SD/αthal: Sickle cell-hemoglobin D disease plusα thalassaemia trait; AS/αthal: sickle cell trait plus α thalassaemia trait; AS:sickle cell trait; AA: normal haemoglobin homozygote; AA/αthal: normal haemoglobin homozygote withα thalassaemia trait; α βthal: the diagnosis of α thalassaemia was based on hematological and not molecular criteria; βthal: βthalassaemia trait; NA: not known

increased tHcy levels and peripheral vascular damage in nine case control studies (seven non-population – and two population – based). It should be noted that the variation in (a) methodologies of the plan of different studies (b) experimental assay techniques (c) statistical analysis form the major drawback of comparing data and conclusions from studies in meta-analyses.

Mild hyperhomocysteinemia, independent of patients' folate and cobalamin status has been reported from the New York Methodist Hospital (NYMH) by Dhar and colleagues [19] who showed that the mean and median plasma tHcy concentrations of 90 SCD adults were significantly greater than the respective values of 76 adult controls ($p = 0.03$). Moreover, in this NYMH study, increased plasma tHcy concentration uninfluenced by the intake of nutritional factors (particularly folate, cobalamin and pyridoxine) was more often a feature of 20% of SCD patients compared to 3% of non SCD controls ($p = 0.005$).

In our investigation, the mean serum tHcy levels of *SCDcr* or *SCDstst* were not greater than either those of HbAS subjects or BDCVs irrespective of their folate or cobalamin reserves (Table 3). Based on our observation, the mean serum tHcy level was surprisingly lower in the *SCDcr* than in the *SCDstst* Omani patients (Table 2). It is therefore unlikely that serum tHcy has a consistently independent primary role in the pathogenesis of vaso-occlusive crisis in this particular SCD population of Omanis. Nevertheless, a secondary etiopathogenetic role cannot be excluded entirely. In contrast to our observations in Omani patients, Olaniyi and colleagues found a greater plasma tHcy concentration in Nigerian patients in crisis than in the steady state [20]. These results in Nigerian patients which are patently dissimilar from those in Omanis may reflect simply a difference between these two populations (African and Arab) in the speed and severity of the intra- and inter-erythocytic sickling processes which culminate in the genesis of sickling polymers at the capillary level. Variation in assay methodologies could possibly account to some extent for the differences between the Omani and Nigerian results.

One of the more devastating consequences of SCD is the development of a stroke. However, none of our 133 Omani SCD patients presented with a stroke. Therefore, we could not draw any conclusions regarding the role of tHcy in the genesis of strokes in this particular group of SCD Omanis. Nevertheless, it should be recalled that in a US study in 1997, Houston & colleagues [8] showed that median and mean serum tHcy

values of 100 SCD (SS and $S\beta^{o}$thal) African-American adults (≥ 18 years) and children (< 18 years), were significantly higher in 16 patients with a stroke than in those without a stroke ($p = 0.02$) [8]. Also, these authors found that circulating tHcy levels were independently directly correlated with a stroke ($p<0.026$) but inversely with serum folic acid concentration ($r = - 0.41$, $p< 0.00005$). Moreover, these African-American SCD patients were 3.5 times as likely to have experienced a stroke if their serum tHcy levels were above the median value of 10.1µmol/L of the group. Houston et al. [8] found also that serum tHcy levels were independently correlated directly with disease-severity scores ($p<0.02$, $r = 0.24$). Nevertheless, after adjustment for age, the concentration of tHcy in serum had no significant relationship with disease severity [8]. Similarly, in 2000, Lowenthal and coauthors showed that SCD adults, on a daily dose of 1mg folic acid, had a median plasma tHcy level of ≈ 1.5 times greater than that of 16 healthy blacks ($p = 0. 0008$) [21]. Serum cobalamin levels in the SCD and control groups were not significantly different from each other in this investigation.

In 2014, Domingos and colleagues reported an incidence of ischemic stroke of 9.5% among 261 unrelated Brazilian sickle cell anemia (SCA) patients in whom the homozygous CAR/CAR (Bantu) β^{s} globin haplotype was predominant in 56.3% of the subjects [22]. This β^{s} globin haplotype which is independently associated with an almost threefold risk of stroke is comparatively infrequent in Oman where the Benin β^{s} globin haplotype predominates [23].

The coinheritance of α-thalassemia ($\alpha^{-3.7kb}$) appeared also to contribute towards reducing the risk of stroke in these Brazilian cases [22]. Table 4 demonstrates the widespread presence of the α-thalassemia gene in Omanis including its occurrence in steady state and crisis SCD patients as well as in non-SCD participants (HbAS subjects and BDCVs). Based on Hb Barts (γ^{4}) sampling in Omani neonates, Alkindi and coworkers found an overall incidence of α^{+}thalassemia of 48.5%, with homozygosity for the α^{+}thalassemia gene ($\alpha^{-3.7kb}$) being demonstrated in more than 60% of the neonatal samples tested [24].

In summary, the following factors possibly limit the incidence of stroke in our group of Omani SCD patients: (a) comparative infrequency of the CAR/CAR β^{s} haplotype [23] (b) predominance of the Benin β^{s} globin haplotype [23] (c) widespread distribution of the α-thalassemia gene [24] and (d) the relatively small population of SCD patients in our study.

4. CONCLUSION

The role of serum tHcy in the mechanisms underlying the reversible vasculopathy of SCD including the 'switch' from the steady state to the crisis and vice versa is unclear. Nevertheless, possible potential mechanisms include (i) direct tHcy toxicity on capillary vessel walls (ii) increase DNA synthesis in vascular smooth muscle cells and induction of proliferation of vascular smooth cells (iii) decrease cell surface expression of thrombomodulin and inhibition of protein C activation which encourages thromboses.

Based on the results of our investigation of Omani Arabs, serum tHcy does not appear to be a major pathogenetic factor in the evolution of vaso-occlusive events such as the sickling *crisis-steady-state* reversible interconversion.

Our group of Omani SCD patients is probably heterogeneous and may have included some individuals with genetic polymorphisms such as 5,10-methylene tetrahydrofolate reductase (MTHFR). Varying reactions of small blood vessels to tHcy in different ethnic groups may also account for differences in vaso-occlusion among various nationalities. The overall 'young' age-structure of our Omani population is possibly also an additional factor which distinguishes our Omani patients from other groups.

CONSENT

Informed consent was obtained from each individual participant.

ETHICAL APPROVAL

This project was reviewed and approved by the Research and Ethics Committees of Sultan Qaboos University (SQU).

ACKNOWLEDGEMENT

We sincerely thank the Medical Research and Ethics Committee, Sultan Qaboos University and the Sultan Qaboos University Hospital administration for allowing the use of the clinical material.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. D'Angelo A, Selhub J. Homocysteine and thrombotic disease. Blood. 1997;90:1-11.
2. Haynes WG. Homocysteine and atherosclerosis: Potential mechanisms and clinical implications. Proceedings of the Royal College of Physician of Edinburgh. 2000;30:114-122.
3. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA. 1995;274(13):1049-57.
4. Townend J, O'Sullivan J, Wilde JT. Hyperhomocysteinaemia and vascular disease. Blood Rev. 1998;12(1):23-34.
5. Wenzler EM, Rademakers AJ, Boers GH, Cruysberg JR, Webers CA, Deutman AF. Hyperhomocysteinemia in retinal artery and retinal vein occlusion. Am J Ophthalmol. 1993;115(2):162-7.
6. Ducloux D, Bresson-Vautrin C, Chalopin JM. Ocular venous occlusion and hyperhomocysteinemia. Ann Intern Med. 1999;131(7):548-9.
7. De Bruijne EL, Keulen-de Vos GH, Ouwendijk RJ. Ocular venous occlusion and hyperhomocysteinemia. Ann Intern Med. 1999;130(1):78.
8. 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(3):192-6.
9. Pandey S, Pandey HR, Mishra RM, Pandey S, Saxena R. Increased homocysteine level in Indian sickle cell anemia patients. Indian J Clin Biochem. 2012;27(1):103-4.
10. Briggs C, Bain B. Basic haematological techniques in Dacie & Lewis practical haematology. 2014;Chapter 3:23-56.
11. Bain B, Lewis S. Preparation and staining methods for blood and bone marrow films in Dacie & Lewis practical haematology. 2014;Chapter 4:57-68.
12. Wild B, Bain B. Investigations for abnormal hemoglobin's and thalassemia in Dacie & Lewis practical haematology. 2014; Chapter 14:301-332.
13. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. Clin Chem. 1995;41(7):991-4.
14. Ross GJS. A program for fitting nonlinear models, MLP. In: Ross GJS Nonlinear Estimation, New York, Springer Series in Statistics, Springer-Verlag. 1990;143-172.
15. Oman Demographic Profile. CIA World Textbook and Times, New Series; 2014.
16. McCully KS. Vascular pathology of homocysteinemia: Implications for the pathogenesis of arteriosclerosis. Am J Pathol. 1969;56(1):111-28.
17. Selhub J, Jacques PF, Rosenberg IH, Rogers G, Bowman BA, Gunter EW, Wright JD, Johnson CL. Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991-1994): Population reference ranges and contribution of vitamin status to high serum concentrations. Ann Intern Med. 1999;131(5):331-9.
18. Alfthan G, Pekkanen J, Jauhiainen M, Pitkäniemi J, Karvonen M, Tuomilehto J, Salonen JT, Ehnholm C. Relation of serum homocysteine and lipoprotein(a) concentrations to atherosclerotic disease in a prospective Finnish population-based study. Atherosclerosis. 1994;106(1):9-19.
19. Dhar M, Bellevue R, Brar S, Carmel R. Mild hyperhomocysteinemia in adult patients with sickle cell disease: A common finding unrelated to folate and cobalamin status. Am J Hematol. 2004;76(2):114-20.
20. Olaniyi JA, AKinlade KS, Atere AD, Arinola OG. Plasma homocysteine, methyl-malonic acid, vitamin B12 and folate levels in adult Nigerian sickle cell anaemia patients. British Journal of Medicine & Medical Research. 2014;4(6):1327-1334.
21. Lowenthal EA, Mayo MS, Cornwell PE, Thornley-Brown D. Homocysteine elevation in sickle cell disease. J Am Coll Nutr. 2000;19(5):608-12.
22. Domingos IF, Falcão DA, Hatzlhofer BL, Cunha AF, et al. Influence of the βs haplotype and α-thalassemia on stroke development in a Brazilian population with sickle cell anaemia. Ann Hematol. 2014;93(7):1123-9.

23. Daar SF. β cluster haplotypes linked to βS in Oman- in haemoglobinopathies in the sultanate of Oman: Study of clinically significant β globin gene mutation. PhD Thesis. 2000;184. Table 4.

24. Alkindi S, Al Zadjali S, Al Madhani A, Daar S, et al. Forecasting hemoglobinopathy burden through neonatal screening in Omani neonates. Hemoglobin. 2010;34(2): 135-44.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/24897