

# Determinants and Associations of Homocysteine and Prothrombotic Risk Factors in Kuwaiti Patients with Cerebrovascular Accident

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## Key Words

Cerebrovascular accident · Homocysteine · Prothrombotic risk factors

## Abstract

**Objective:** The objective of this study was to evaluate the determinants and associations of some prothrombotic risk factors in patients with cerebrovascular accidents (CVAs). **Subjects and Methods:** In this case-control study, plasma total homocysteine (tHcy), lupus anticoagulant, protein C, protein S, activated protein C resistance (APC-R) and antithrombin were measured in 102 patients (60 males and 42 females) and 167 controls (87 males, 80 females). Serum vitamin B<sub>12</sub>, folate, red cell folate, creatinine, lipid profile and glucose were also determined. Glomerular filtration rate (GFR) was calculated. **Results:** 13 (22%) of the 60 male patients, and 16 (39%) of the 42 female patients had hyperhomocysteinemia. Median (interquartile range) tHcy was higher in male patients [11.22 μmol/l (9.60–15.40)] than female patients [10.05 μmol/l (8.72–17.54)]. On binary logistic regression analysis, the significant ( $p < 0.05$ ) determinants of tHcy were urea, creatinine and GFR. Comparing patients with control subjects showed that tHcy, age, fasting glucose, urea, serum cre-

atinine, white blood cell count, protein S, APC-R and factor VIII were significantly higher, while protein C, factor II, total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were significantly lower in patients. Lupus anticoagulant was not associated with tHcy and not detected in patients and controls. Low concentrations of vitamins B<sub>12</sub> and folate were not associated with tHcy. Logistic regression analysis showed a significant association of tHcy with CVA (OR = 9.55;  $p = 0.047$ ) in males in the presence of other traditional CVA risk factors but tHcy is not independently associated with CVA in females. **Conclusion:** Hyperhomocysteinemia is common in Kuwaiti patients with CVA and tHcy probably interacts with prothrombotic factors (protein C, APC-R and factor VIII) to increase CVA risk. The main determinants, age and GFR markers, should be kept in mind when determining the risk associated with tHcy.

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## Introduction

Thrombotic disorders are the commonest causes of death worldwide. Fatal consequences of thrombosis include mainly ischemic heart disease, stroke or cerebro-

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vascular accidents (CVAs). The striking difference in incidence in different populations and at different age groups has led to an intensive search for risk factors. Factors that have been identified as risk factors for arterial thrombosis include endothelial injury, platelet activation, hyperlipidemia, stasis, elevated levels of activated coagulation factors, reduced levels of inhibitors of coagulation such as protein C (PC), protein S (PS), antithrombin, activated protein C resistance (APC-R), factor V Leiden, and others [1–3].

The battery of tests performed on patients with premature thrombotic events has been extending with discovery of more factors that are implicated as risk factors. Increased plasma total homocysteine (tHcy) is one of the newly recognized factors that increase the risk for vascular diseases [4], but the mechanism is not known. Mild hyperhomocysteinemia is seen in patients with heterozygous genetic defects for cystathionine  $\beta$ -synthase or methylenetetrahydrofolate reductase gene defects [5]. However, there are several preventable causes for hyperhomocysteinemia that include deficiencies of folic acid, vitamin B<sub>12</sub> and vitamin B<sub>6</sub>, renal failure, excessive protein intake or the administration of certain drugs such as nitrous oxide, methotrexate and antiepileptics [6].

The aims of this study were to evaluate the determinants and associations of prothrombotic risk factors such as PC, PS, antithrombin, APC-R and the presence of lupus anticoagulant in relation to tHcy in Kuwaiti patients presenting with CVA.

## Subjects and Methods

### Subjects

One hundred and two Kuwaiti patients [60 males (age range 26–60 years) and 42 females (age range 20–60 years)] admitted to Mubarak Hospital were studied. Inclusion criteria were patients less than or equal to 60 years of age with thrombotic CVA that is confirmed by computerized tomography (CT). All patients were given full neurological examination and CT scan of the brain. Cerebral infarction was confirmed by brain CT indicating the presence of infarction and absence of hemorrhage or tumors. The control subjects [n = 167; 87 males (age range 19–58 years) and 80 females (age range 30–60 years)] were recruited from healthy volunteer blood donors and subjects referred for health screen. All patients or first-degree family members and control subjects gave informed voluntary consent to participate in the study according to the protocol approved by the Ethics Committee and in accordance with the ethical standards laid down in the Helsinki declaration. The following data were acquired from patients and controls: age, sex, weight, blood pressure and/or history of hypertension, diabetes mellitus, smoking status, previous thrombotic episodes, family history of CVA and diabetes mellitus. Drug history was also obtained.

### Methods

Venous blood samples were collected without tourniquet. Samples for homocysteine estimation were collected in EDTA tubes within 12 h of stroke symptoms in the patients. For coagulation studies, blood samples were collected in trisodium citrate and platelet-poor plasma was obtained and kept frozen at  $-70^{\circ}\text{C}$  until analyzed [7].

### Assays

Estimation of tHcy was carried out with the IMx analyzer (Abbott Laboratories, Abbott Park, Ill., USA). The method measures the total concentration of homocysteine. As tHcy is known to be significantly higher in males, hyperhomocysteinemia is defined as fasting tHcy  $>15.0\ \mu\text{mol/l}$  for males and  $>11.4\ \mu\text{mol/l}$  for females, as previously suggested [8, 9].

### Vitamin Status

Radioassay (dual count) was used to estimate vitamin B<sub>12</sub> and folate (Diagnostic Product Company, Los Angeles, Calif., USA). The red blood cell folate level was calculated from folate and hematocrit according to the kit manufacturer's instructions (Diagnostic Product Company).

### Hematological Parameters

Complete blood count was determined by Gen S™ Coulter® analyzer (Beckman Coulter Inc., Fullerton, Calif., USA). PC, PS, and factors II and VIII were measured with functional assays (Tectron International Laboratory Co., Milan, Italy). Antithrombin was measured by a chromogenic assay; APC-R was tested by an APTT-based method (both by Tectron International Laboratory Co.). Lupus anticoagulant was detected using two methods – dilute Russell's viper venom time (American Diagnostica Inc., Conn., USA) followed by an automated confirmatory test on the ACL 9000 (Tectron International Laboratory Co., IL Test LA Screen and IL Test LA Confirm tests). Coagulation studies were performed on the automated coagulation analyzer ACL 9000 (Tectron International Laboratory Co.).

### Other Assays

Serum creatinine was determined on the Beckman LX20 automated analyzer (Beckman Corporation, Brea, Calif., USA) and creatinine clearance estimated glomerular filtration rate (eGFR) was calculated with the modified Modification of Diet in Renal Disease (MDRD) formula [10] to assess renal function in patients and controls. Fasting glucose and full lipid profile [total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol] were determined for each subject and low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula [11].

### Statistical Methods

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS® for Windows®, Chicago, Ill., USA) version 12.0. Distributions of continuous variables were tested for normality with the Kolmogorov-Smirnov test and the natural log transformations of tHcy and vitamin B<sub>12</sub> (the only skewed variables) were used to improve normality to be able to use parametric methods. Data are presented as mean and standard deviation (SD) except for the non-normally distributed variables which are presented as median and interquartile range (25–75th percen-

**Table 1.** Summary data of the variables in male patients and control subjects

	Patients	Control subjects	p (Student's t test)
Age, years	49.4 (7.4)	40.9 (8.1)	<0.0001
White blood cells, $\times 10^9/l$	9.8 (3.9)	7.0 (2.1)	<0.0001
PC, %	105.4 (38.5)	115.7 (20.4)	0.030
PS, %	89.3 (29.8)	85.2 (19.1)	NS
Antithrombin, %	103.3 (16.9)	103.3 (14.7)	NS
APC-R	2.4 (0.3)	2.3 (0.3)	0.006
Factor II, %	82.6 (12.6)	87.5 (19.4)	NS
Factor VIII, %	71.9 (29.2)	57.0 (24.8)	0.005
Vitamin B <sub>12</sub> <sup>a</sup> , pmol/l	301 (104–301)	220 (121–330)	0.002 <sup>b</sup>
Serum folate, nmol/l	24.8 (12.4)	24.3 (10.5)	NS
Red cell folate, nmol/l	1,349.7 (634.8)	1,120.1 (363.5)	0.010
Fasting glucose, mmol/l	8.2 (3.3)	6.4 (2.9)	0.002
Urea, mmol/l	7.3 (3.1)	4.9 (1.5)	<0.0001
Creatinine, $\mu\text{mol/l}$	116 (122)	86 (15)	0.024
GFR by MDRD formula, ml/min/1.73 m <sup>2</sup>	89 (29)	94 (19)	NS
Total cholesterol, mmol/l	4.73 (0.99)	5.10 (1.14)	0.050
HDL cholesterol, mmol/l	0.89 (0.26)	1.01 (0.23)	0.010
LDL cholesterol, mmol/l	3.01 (0.90)	3.37 (1.1)	NS
Triglycerides, mmol/l	1.63 (0.86)	1.63 (1.4)	NS
Homocysteine <sup>a</sup> , $\mu\text{mol/l}$	11.22 (9.60–15.40)	8.5 (6.99–9.75)	<0.0001 <sup>b</sup>
Hypertension	35 (58%)	0	
Diabetes mellitus	27 (45%)	0	
Nonsmoker	26 (44%)	59 (68%)	
Current smoker	31 (52%)	26 (30%)	

Results are means with SD in parentheses, unless indicated otherwise.

<sup>a</sup> Results are median (25–75th) percentiles. <sup>b</sup> log-transformed values used.

tiles). The Pearson correlation coefficient was used to assess the association between log-transformed values of tHcy and other variables. In view of the well-known decline of the glomerular filtration rate (GFR) with age and since there was a significant gender difference in tHcy, partial correlation was used to assess the association between tHcy and other variables after controlling for age and sex. Comparison between groups was done by Student's t test. To assess the effect of variables on the risk of CVA, expressed as odds ratio (OR), logistic regression analysis was used to ascertain the association between CVA (which was recoded as a binary outcome – control = 0; CVA = 1) and the variables. Logistic regression analysis was also used to ascertain the association between other variables and hyperhomocysteinemia as defined previously. Values of  $p < 0.05$  were considered to be statistically significant.

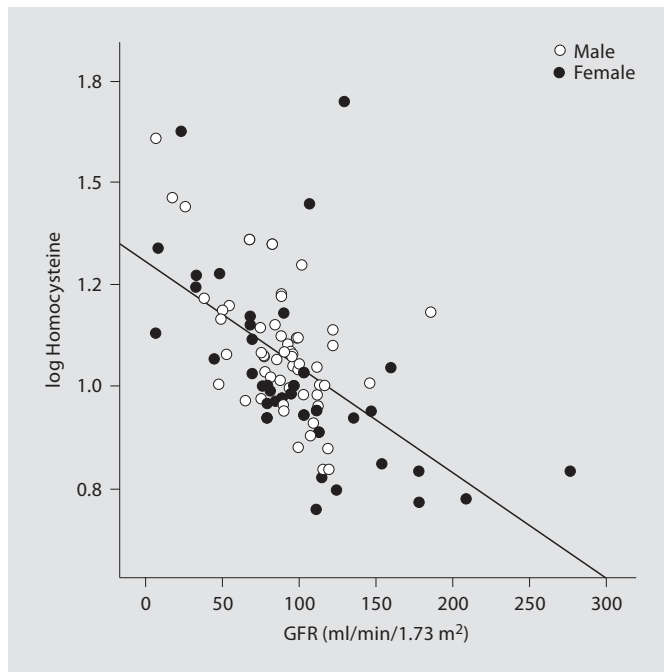
## Results

Of the 60 male patients, 13 (22.0%) had tHcy  $>15.0 \mu\text{mol/l}$  while of the 42 female patients, 16 (38.5%) had tHcy  $>11.4 \mu\text{mol/l}$ . All the variables in patient and con-

trol groups are summarized according to gender (table 1) showing that patients with CVA have higher homocysteine levels than controls among both males and females.

### Correlations of Homocysteine

In patients, tHcy (log transformed) showed significant correlations with urea ( $r = -0.440$ ,  $p < 0.0001$ ), serum creatinine ( $r = 0.625$ ,  $p < 0.0001$ ), GFR ( $r = -0.577$ ,  $p < 0.0001$ ), total cholesterol ( $r = -0.230$ ,  $p = 0.024$ ), PS ( $r = 0.212$ ,  $p = 0.038$ ) and log vitamin B<sub>12</sub> ( $r = -0.269$ ,  $p = 0.008$ ). The correlation between log tHcy and GFR in the patients is shown in figure 1. Partial correlation analysis after correcting for age and sex confirmed significant associations between tHcy and hemoglobin ( $r = -0.379$ ,  $p < 0.0001$ ), urea ( $r = 0.313$ ,  $p = 0.002$ ), serum creatinine ( $r = 0.468$ ,  $p < 0.0001$ ), GFR ( $r = -0.398$ ,  $p < 0.0001$ ), total cholesterol ( $r = -0.307$ ,  $p = 0.003$ ) and LDL cholesterol ( $r = -0.280$ ,  $p = 0.017$ ).



**Fig. 1.** Scatter plot showing the relationship between GFR calculated by the MDRD formula and log tHcy in the patients grouped by gender. The solid line indicates the regression-based fit line.

#### *Determinants of Hyperhomocysteinemia*

Binary logistic regression analysis of the determinants of hyperhomocysteinemia in the patients showed some gender differences. In male patients, the most significant determinants were markers of GFR (urea: OR = 6.10, 95% CI = 1.51–24.73,  $p = 0.011$ ; creatinine: OR = 16.91, 95% CI = 2.18–131.36,  $p = 0.007$ ; eGFR: OR = 0.97, 95% CI = 0.94–0.99,  $p = 0.011$ ) and serum vitamin B<sub>12</sub> (OR = 0.28, 95% CI = 0.10–0.75,  $p = 0.011$ ). However, in female patients, markers of GFR were the main determinants of hyperhomocysteinemia (urea: OR = 3.81, 95% CI = 1.13–12.82,  $p = 0.031$ ; creatinine: OR = 46.60, 95% CI = 2.62–828.70,  $p = 0.009$ ; eGFR: OR = 0.97, 95% CI = 0.94–0.99,  $p = 0.007$ ).

#### *Homocysteine as a Risk Factor for CVA*

Binary logistic regression of the association between tHcy and CVA in males shows that tHcy is significantly associated (OR = 9.55, 95% CI = 1.03–88.4,  $p = 0.047$ ) in the presence of potential confounding risk factors such as age, weight, smoking, cholesterol, HDL cholesterol, LDL cholesterol and triglycerides. However, in females, tHcy is not independently associated with CVA (OR = 3.67, 95% CI = 0.74–18.12,  $p = 0.110$ ).

#### *Hematological Parameters*

The hematological and other parameters in patients and controls are also summarized in tables 1 and 2 for male and female patients, respectively. White blood cell count was higher in patients than controls with no significant differences in hemoglobin and platelet count (data not shown). PC was significantly lower in patients than controls. APC-R and factor VIII levels were significantly higher in patients than controls. There was no significant difference of antithrombin between patients and controls. Factor II was not found to be significantly higher in patients than controls. No patient was positive for lupus anticoagulant. Interestingly, PS was found to be significantly higher in female patients compared to female controls.

#### **Discussion**

The major finding of the present study was that elevated tHcy is common and is the main prothrombotic factor associated with CVA. The most significant determinants of tHcy are related to age and markers of GFR. These findings are similar to those of previous studies [12, 13]. Our data show that tHcy is an independent risk factor for CVA, especially in the male population, as shown by the relatively high OR even when other risk factors and markers of GFR were included as potential confounders. This is in agreement with reports from other populations [14–16]. Furthermore, the magnitude of the association between tHcy and risk of CVA is consistent with the results from previous studies in Caucasian population [17, 18]. The underlying mechanisms of how hyperhomocysteinemia increases the risk of CVA are not clear but several possibilities have been proposed. Hyperhomocysteinemia causes increased arterial blood pressure [19] thereby increasing the risk of CVA. Elevated plasma tHcy has also been shown to induce oxidative injury to vascular endothelial cells and cause impairment of the endothelial production of nitric oxide, a strong vascular relaxing factor [20]. Other proposed mechanisms include enhancement of platelet adhesion to endothelial cells [21], promotion of the growth of vascular smooth muscle cells [22] and association of increased tHcy with higher levels of prothrombotic factors such as  $\beta$ -thromboglobulin, tissue plasminogen activator, and factor VIIc [23].

Although the risks associated with increased tHcy are now well known, the determinants of tHcy have not been extensively studied in the Gulf Arab population. In the present study, the prevalence of hyperhomocysteinemia

**Table 2.** Summary data of the variables in female patients and control subjects

	Patients	Control subjects	p (Student's t test)
Age, years	48.7 (10.8)	41 (8.0)	<0.0001
White blood cells, $\times 10^9/l$	9.2 (2.0)	6.7 (1.6)	<0.0001
PC, %	113.8 (37.2)	118.7 (25.8)	NS
PS, %	87.7 (21.8)	71.4 (23.4)	0.001
Antithrombin, %	105.1 (20.6)	99.8 (15.5)	NS
APC-R	2.5 (0.28)	2.3 (0.20)	<0.0001
Factor II, %	84.6 (17.9)	92.6 (11.6)	0.004
Factor VIII, %	87.4 (32.3)	62.5 (20.3)	<0.0001
Vitamin B <sub>12</sub> <sup>a</sup> , pmol/l	406 (122–406)	173 (104–243)	<0.0001 <sup>b</sup>
Serum folate, nmol/l	29.0 (11.9)	25.2 (11.7)	NS
Red cell folate, nmol/l	1,391.6 (355.1)	1,161.2 (421.8)	0.014
Fasting glucose, mmol/l	7.5 (3.1)	5.9 (1.7)	0.001
Urea, mmol/l	7.2 (3.3)	4.1 (1.2)	<0.0001
Creatinine, $\mu\text{mol/l}$	93.6 (37.3)	64.9 (9.7)	0.028
GFR by MDRD formula, ml/min/1.73 m <sup>2</sup>	101 (50.7)	96 (17)	NS
Total cholesterol, mmol/l	4.86 (1.1)	5.17 (0.9)	NS
HDL cholesterol, mmol/l	1.14 (0.44)	1.25 (0.28)	NS
LDL cholesterol, mmol/l	3.14 (0.91)	3.41 (0.74)	NS
Triglycerides, mmol/l	1.63 (0.88)	1.16 (0.64)	0.021
Homocysteine <sup>a</sup> , $\mu\text{mol/l}$	10.05 (8.72–17.54)	7.25 (5.57–8.62)	0.004 <sup>b</sup>
Hypertension	26 (62%)	0.0	
Diabetes mellitus	25 (59%)	0.0	
Nonsmoker	40 (94%)	79 (99%)	
Current smoker	3 (6%)	1 (1%)	

Results are means with SD in parentheses, unless indicated otherwise.

<sup>a</sup> Results are median (25–75th) percentiles. <sup>b</sup> log-transformed values used.

in all patients was 27.4%, being more common in females than in males. This is comparable to what was reported in other populations [16, 24]. In agreement with previous reports, we have found strong correlations between tHcy and endogenous markers of GFR [9, 25] confirming that this is a significant determinant of tHcy. The kidney plays a significant role in tHcy metabolism. Studies in an animal model showed that catabolism of filtered homocysteine in renal tubular cells is a major determinant of homocysteine clearance [26]. Therefore, in addition to changes in glomerular clearance, reduced metabolism in renal tissue in our patients could be an important underlying determinant of tHcy.

In our population, we found an inverse correlation between tHcy and vitamin B<sub>12</sub> and logistic regression analysis also confirmed a strong association between hyperhomocysteinemia and vitamin B<sub>12</sub> in male patients. However, we have found that patients with CVA had higher homocysteine levels than controls and none of the patients had vitamin B<sub>12</sub> deficiency. This is in contrast with the reports from other populations [27, 28]. A possible

explanation for this discordant finding is the fact that the Kuwaiti society is an affluent society where nutritional vitamin deficiencies are extremely rare.

Although many studies showed a significant relation between hyperhomocysteinemia and folate deficiency [29], we did not find such an association in agreement with other studies [30, 31]. We cannot exclude a potential contribution from genetic defects (such as mutations in methylenetetrahydrofolate reductase and cystathionine  $\beta$ -synthase genes) as we did not assess these. The value of vitamin supplementation, however, has been documented in many studies to lower tHcy level [32, 33].

A number of prothrombotic factors were found to be significantly different when patients were compared with controls. Our study suggests that the factors that are potentially contributory to development of CVA are PC, APC-R and factor VIII. However, these factors do not appear to be independent risk factors for CVA as there were no significant associations when the confounding effects of other risk factors were included in multivariate logistic regression analysis. Risk factors are known to act syner-

gistically and the finding of significant associations between tHcy and several prothrombotic factors may further help to explain the association between tHcy and risk of CVA. There are conflicting reports on the associations between these prothrombotic factors and CVA [34, 35], but these studies did not evaluate the potential interaction of these factors with tHcy. It is noteworthy that none of the patients in this study was positive for the presence of lupus anticoagulant. This is in agreement with other studies [36].

A limitation of the present study is the absence of assessment of vitamin B<sub>6</sub>, which would have provided important information about the contribution of vitamin B<sub>6</sub> to increased tHcy in our population. However, additional measurements of vitamin B<sub>6</sub> would not have altered the main conclusions of our study with regard to the association between tHcy and CVA. Another limitation is the fact that we cannot exclude the possibility that the timing of sampling in relation to the onset of CVA may have contributed to some of the variability in tHcy levels although a publication has shown insignificant variability after CVA [37]. Furthermore, collection of samples from the patients was standardized and limited to within the first 12 h of the onset of symptoms. Finally, as tHcy is known to increase with age, the age difference between patients and controls (tables 1 and 2) may be viewed as a limitation. However, published studies show that the same tHcy

reference range could be used for adults less than 65 years of age [38]. Furthermore, GFR and vitamin status, two of the main factors that contribute to age-related increases in tHcy, were not significantly different between patients and controls (tables 1 and 2). Nevertheless, age may be the main factor in the significantly higher PS level found in female patients compared to female controls because PS increases with age [39].

## Conclusion

The results of our study show that elevated tHcy is common and may be one of the important risk factors for CVA. tHcy probably interacts with the prothrombotic factors (PC, APC-R and factor VIII) found to be significantly different when patients were compared with controls. The other determinants of tHcy in patients with CVA are age and markers of GFR and these determinants should be kept in mind when using tHcy as a risk factor for CVA.

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