

Association of Low Red Blood Cell Folate Concentrations with Coronary Artery Disease in Iranians: A Matched Case-Control Study

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Key Words

Coronary artery disease · Homocysteine · 5-Methyltetrahydrofolate · Red blood cells · Remethylation pathway

Abstract

Background: It is not fully established whether the increasing risk of coronary artery disease (CAD) is associated with high plasma homocysteine levels or components of the homocysteine remethylation pathway, e.g. vitamin B₁₂ or 5-methyltetrahydrofolate (5-MTHF) in plasma and red blood cells (RBC). In this study, we tested the hypothesis that 5-MTHF in RBC, which represents the long-term folate status of individuals, may be a more reliable marker of homocysteine remethylation pathway disturbances, and its deficiency may be associated with CAD in Iranians. **Methods:** Plasma total homocysteine (tHcy), vitamin B₁₂, and plasma and RBC 5-MTHF were measured in 200 angiographically documented patients and 200 controls matched for sex and age. **Results:** In the plasma, tHcy levels were significantly higher in cases compared to controls (geometric mean 12.9 ± 6.5 vs. $10.6 \pm 5.6 \mu\text{mol/l}$, $p = 0.04$). However, RBC 5-MTHF (527.2 ± 185.9 vs. $461.3 \pm 117.9 \text{ nmol/l}$, $p = 0.007$) and vitamin B₁₂ (254.2 ± 132.8 vs. $182.2 \pm 110.4 \text{ pmol/l}$, $p = 0.04$) were significantly higher in controls than patients. RBC 5-MTHF was a strong and independent predictor of plasma tHcy ($\beta = -0.01$, $p = 0.003$, $r^2 = 0.19$). Subjects in the

lowest quartile of red-cell 5-MTHF had a 2.5-fold increased prevalence of CAD compared to subjects in the highest quartile. The association of CAD in the first quartile with red-cell 5-MTHF remained significant when adjusted for plasma tHcy, vitamin B₁₂, hypertension and hypercholesterolemia (odds ratio, OR 2.3, confidence interval: 1.1–3.9, $p = 0.01$). However, the association between CAD in the highest quartile and plasma tHcy decreased and became insignificant when adjusted for red-cell 5-MTHF, vitamin B₁₂, hypertension and hypercholesterolemia (OR 1.27, confidence interval: 0.96–1.69, $p = 0.11$). **Conclusion:** In this study, the association between CAD and low RBC 5-MTHF was stronger than with plasma 5-MTHF and plasma tHcy levels, indicating that RBC 5-MTHF may be a more stable parameter to study disturbances in the homocysteine remethylation pathway in Iranians.

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Introduction

Homocysteine is a sulfur-containing amino acid, and its plasma concentrations are determined by genetic factors and nutritional deficiencies in vitamin B₆, B₁₂ and folic acid [1]. High plasma total homocysteine (tHcy) causes oxidative stress and vascular damage and is increasingly recognized as an independent risk factor for vascular disease in different populations [2–5]. tHcy con-

centrations above the 80th percentile of normal have been reported in almost 40% of patients with vascular disease, including coronary artery disease (CAD) [4]. In a meta-analysis of 20 prospective studies involving 3,820 participants, lowering of current tHcy concentrations by 3 $\mu\text{mol/l}$ (achievable by increasing folic acid intake) would reduce the risk of ischemic heart disease by 16% (11–20%), deep vein thrombosis by 25% (8–38%) and stroke by 24% (15–33%) [5]. However, recent intervention studies in patients with stable CAD [6] and in patients with stroke [7] do not support the data from this meta-analysis.

Differences in the prevalence of vascular disease and in the effect of hyperhomocysteinemia on vascular disease among countries and races have been reported [8, 9]. However, there are no documented data regarding the association of plasma tHcy and the folate status with CAD in Iranians.

Remethylation of homocysteine to methionine required 5-methyltetrahydrofolate (5-MTHF) as a methyl donor and vitamin B₁₂ as a cofactor, and hyperhomocysteinemia can be associated with low 5-MTHF and vitamin B₁₂. A deficiency in any of these components could have an impact on the pathogenesis of CAD.

Homocysteine concentrations and its metabolites can be varied between body compartments, and it has been suggested that its levels in plasma might not be an indication of intracellular processes and hence may not indicate the risk of CAD [10]. Most previous studies have focused on the association of plasma tHcy and plasma folate with CAD and other components of the homocysteine remethylation pathway. Particularly red blood cell (RBC) folate, a possible risk factor for CAD, has not been fully investigated. RBC folate reflects the folate status during the past 120 days and thus represents the long-term folate status of the individuals.

In this case-control study in an Iranian population, we tested whether low RBC 5-MTHF may be a more reliable marker of CAD than other components of the homocysteine remethylation pathway.

Patients and Methods

Participants

At the time of subject enrolment, relevant data on past medical history, current smoking habits and drug therapy were obtained from all study participants. All subjects were of Iranian ancestry and were not first- or second-degree relatives. This study was approved by the local ethics committee, and all individuals gave written, informed consent. Exclusion criteria for both patients and con-

trols included age >70 years, cardiomyopathy, diabetes mellitus, pregnancy, liver dysfunction, a history of anemia and renal dysfunction. Subjects on drug therapy known to interfere with homocysteine metabolism (such as anti-epileptic and antifolate drugs) and on vitamin supplements, and women receiving hormone replacement therapy were excluded from this study. Using the first three digits of the telephone directory, representing an area in the Shiraz region, 635 subjects (aged <70 years) were contacted by phone for interview and sampling, 174 were not interested and 115 were excluded because 19 had a history of cardiovascular disease (myocardial infarction and unstable angina), 5 were diabetic, 2 were pregnant, 26 had a history of renal dysfunction, 23 had a history of anemia, 18 were on drug therapy known to interfere with homocysteine, 10 were on vitamin supplements and 12 women were on hormone replacement therapy. Out of the 346 subjects, 200 were matched for age, sex and area (lived in the same area of Shiraz) with 200 control cases. Patients (n = 489; age <70 years) diagnosed with stable CAD in the last 4 months, which was documented angiographically in cardiology outpatient departments or coronary care units at two major university hospitals in Shiraz (Faghihi and Namazi), were phoned to participate in this study. The criterion for angiographically proven CAD was >50% stenosis in one or more major epicardial vessel in multiple projections. Out of the 489 patients, 388 agreed to participate and completed the questionnaire; 90 subjects were excluded because 14 had renal dysfunction, 8 were diabetic, 3 had anemia, 1 was pregnant, 49 were on vitamin supplements, 4 had cardiomyopathy, 3 were on anti-epileptic drugs and 8 women were on hormone replacement therapy. Therefore, out of the remaining 298 subjects, 200 were matched for sex, age and area with the controls. Our laboratory is located on the campus of the Shiraz University of Medical Sciences in the center of Shiraz, and the participants had good access to our laboratory from all over the city. Controls were included within 3 months of patient enrolment.

Analytical Methods

Samples from both cases and controls were obtained within 3 months. Samples for plasma tHcy were taken in the fasting state (overnight), placed on ice and centrifuged within 1 h, and the separated plasma was stored at -70°C until assayed. Additional fasting samples were collected for serum creatinine, cholesterol, plasma 5-MTHF, RBC 5-MTHF and vitamin B₁₂. Plasma tHcy were analyzed by high-performance liquid chromatography after the reduction in plasma disulfides with tris(2-carboxyethyl)phosphine, precipitation of proteins with trichloroacetic acid, derivatization with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate and fluorescent detection [11]. The inter-assay coefficient of variation for the determination of tHcy in plasma was less than 7.2%.

Plasma and RBC 5-MTHF and vitamin B₁₂ were measured using commercially available radioassay kits (SimulTRAC-SNB; ICN Pharmaceuticals, Costa Mesa, Calif., USA). The intra- and inter-assay coefficients of variation for the determination of plasma 5-MTHF were less than 5.8 and 8.9%, respectively, being less than 6.1 and 9.2% for RBC 5-MTHF and less than 6.2 and 7.5% for plasma vitamin B₁₂, respectively.

The use of any tobacco was defined as current smoking. Hypertension was defined as a systolic blood pressure ≥ 140 mm Hg or a diastolic pressure of ≥ 90 mm Hg, or treatment with angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists, β -blockers, vasodilators and calcium channel blockers. Hypercho-

lesterolemia was considered present if subjects were taking lipid-lowering drugs such as statins, clofibrate, gemfibrozil and resins or had a serum cholesterol ≥ 6.5 mmol/l. Serum creatinine and cholesterol were determined with enzymatic photometry.

Statistical Analysis

Student's t test for continuous variables and χ^2 analysis for frequency measures were used to determine statistically significant differences in cardiovascular risk factor levels between cases and controls. The normality of distribution of any variable was assessed using the Kolmogorov-Smirnov test and where skewed (tHcy, plasma 5-MTHF was positively skewed) logarithmically transformed to reduce kurtosis, and then geometric means were calculated. The logarithmic transformations were also used in further statistical analysis.

Pearson's correlation coefficients were used to examine the correlation between plasma tHcy and RBC 5-MTHF with other cardiovascular risk factors in controls. Partial correlation was used to examine the correlation of plasma 5-MTHF and vitamin B₁₂ with tHcy controlling for RBC 5-MTHF and also between RBC 5-MTHF with plasma tHcy controlling for plasma 5-MTHF and vitamin B₁₂. Stepwise multiple regression analysis was used to determine predictors of plasma tHcy and RBC 5-MTHF in controls.

Quartiles (25%) of tHcy, RBC/plasma 5-MTHF and vitamin B₁₂ concentrations were determined in controls. Logistic regression was used in both univariate and multivariate analysis to determine the crude odd ratios (OR) of RBC and plasma 5-MTHF, plasma tHcy and vitamin B₁₂ and adjusted OR for RBC 5-MTHF and plasma tHcy for CAD and their 95% confidence intervals. Confounding variables such as hypertension, hypercholesterolemia, plasma vitamin B₁₂ and plasma tHcy were selected a priori and adjusted in the multivariate model for RBC 5-MTHF. In addition, the OR of plasma tHcy were also adjusted for hypertension, hypercholesterolemia, plasma vitamin B₁₂ and RBC 5-MTHF. The statistical analyses were performed using SPSS software. All reported p values are two tailed.

Results

Baseline Characteristics

The characteristics of the cases and the controls are shown in table 1. Student's paired t test and χ^2 analysis showed that the presence of hypertension and hypercholesterolemia was significantly different between cases and controls. In addition, plasma tHcy, a component of the remethylation pathway, was significantly higher in cases than controls whereas RBC 5-MTHF and vitamin B₁₂ were significantly higher in controls than cases. The concentrations of plasma 5-MTHF were not statistically significant between the cases and the controls.

Correlates of Plasma tHcy and RBC 5-MTHF

Results from the Pearson correlation coefficient showed that among all the variables listed in table 1, plasma tHcy was significantly and negatively correlated with

Table 1. Characteristics of the cases and the controls

Characteristics	Cases (n = 200)	Controls (n = 200)	p value
Age, years	50.2 ± 10.3	49.6 ± 11.2	0.71
Males, %	54	52	0.86
Body mass index	25.6 ± 3.7	26.5 ± 3.3	0.68
Current cigarette smokers, %	38	29	0.17
Hypertension, %	43	17	<0.001
Hypercholesterolemia, %	38	19	0.003
Creatinine, $\mu\text{mol/l}$	74.3 ± 10.6	71.6 ± 22.1	0.39
RBC 5-MTHF, nmol/l	461.3 ± 117.9	527.2 ± 185.9	0.007
Plasma 5-MTHF, nmol/l ^a	9.7 ± 4.6	10.3 ± 4.7	0.11
Plasma tHcy, $\mu\text{mol/l}$ ^a	12.9 ± 6.5	10.6 ± 5.6	0.04
Plasma vitamin B ₁₂ , pmol/l	182.2 ± 110.4	254.2 ± 132.8	0.04

Results are expressed as means \pm SD.

^a Results are expressed as geometric means \pm SD.

plasma 5-MTHF ($r = -0.35$, $p = 0.003$), RBC 5-MTHF ($r = -0.42$, $p \leq 0.001$) and plasma vitamin B₁₂ ($r = -0.35$, $p = 0.002$). The significant negative correlation of plasma 5-MTHF and vitamin B₁₂ with plasma tHcy became insignificant when this correlation was controlled for RBC 5-MTHF, yielding r values of -0.08 ($p = 0.45$) and -0.10 ($p = 0.36$) for plasma 5-MTHF and vitamin B₁₂, respectively. Among all the variables, RBC 5-MTHF was significantly correlated with plasma 5-MTHF ($r = 0.68$, $p \leq 0.001$), vitamin B₁₂ ($r = 0.64$, $p \leq 0.001$) and plasma tHcy ($r = -0.42$, $p \leq 0.001$). The negative correlation of RBC 5-MTHF with plasma tHcy remained significant ($r = -0.25$, $p = 0.035$) when this correlation was controlled for plasma 5-MTHF and vitamin B₁₂.

Predictors of Plasma tHcy and RBC 5-MTHF

Stepwise multiple regression analysis showed that the independent predictor of plasma tHcy was RBC 5-MTHF ($\beta = -0.01$, $p = 0.003$, $r^2 = 0.19$) when all the variables listed in table 1 were used in the model. The results from stepwise multiple regression analysis to determine the predictors of RBC 5-MTHF showed that the main independent predictors of RBC 5-MTHF were plasma 5-MTHF ($\beta = 0.16$, $p = 0.01$) and vitamin B₁₂ ($\beta = 0.50$, $p = 0.02$).

Prevalence of CAD

Table 2 shows the OR for CAD comparing each quartile of RBC and plasma 5-MTHF, plasma vitamin B₁₂ and plasma tHcy concentrations with those of the first

Table 2. OR of CAD for quartiles of plasma tHcy, RBC and plasma 5-MTHF and plasma vitamin B₁₂ determined in controls

	Cases (n = 200)	Controls (n = 200)	OR (95% CI)	p value
RBC 5-MTHF				
<384	68	42	2.8 (1.5–4.9)	<0.001
			2.3 (1.1–3.9) ^a	0.01
385–454	42	40	1.8 (0.91–3.3)	0.06
			1.6 (0.5–4.1) ^a	0.11
455–646	58	63	1.5 (0.87–2.6)	0.12
			1.3 (0.77–3.4) ^a	0.22
>646	32	55	1.0	
Plasma 5-MTHF				
<8.2	70	42	1.8 (1.0–3.2)	0.03
8.3–9.7	36	46	0.89 (0.49–1.6)	0.69
9.8–12.9	48	62	0.87 (0.50–1.5)	0.62
>12.9	46	50	1.0	
Vitamin B₁₂				
<174	76	70	1.0 (0.6–1.8)	0.89
175–218	48	48	0.95 (0.53–1.7)	0.87
219–304	32	40	0.76 (0.4–1.4)	0.39
>304.5	44	42	1.0	
tHcy				
<8.4	90	108	1.0	
8.5–11.5	34	36	1.1 (0.63–2.0)	0.65
			0.97 (0.59–1.3) ^b	0.89
11.6–15.7	26	20	1.56 (0.78–3.1)	0.17
			1.02 (0.77–1.35) ^b	0.93
>15.7	50	36	1.67 (0.97–2.8)	0.04
			1.27 (0.96–1.69) ^b	0.11

CI = Confidence interval.

^a Adjusted for plasma tHcy, vitamin B₁₂, hypertension and hypercholesterolemia.

^b Adjusted for RBC 5-MTHF, vitamin B₁₂, hypertension and hypercholesterolemia.

quartile. In the first quartiles of RBC 5-MTHF and plasma 5-MTHF, the prevalence of CAD was more than 2.5- and 1.5-fold increased compared with the highest quartiles. In subjects in the fourth quartile of tHcy, the prevalence of CAD was more than 1.5-fold increased compared with the subjects in the lowest quartile. The association between CAD and RBC 5-MTHF in the first quartile remained significant when adjusted for plasma tHcy, vitamin B₁₂, hypertension and hypercholesterolemia. However, the association between CAD and plasma tHcy in the highest quartile tHcy became insignificant when adjusted for RBC 5-MTHF, vitamin B₁₂, hypertension and hypercholesterolemia.

Discussion

The most important result from this study is that the association of low 5-MTHF concentrations in RBC with CAD is stronger than any other major component of the homocysteine remethylation pathway.

There was a general disequilibrium of the homocysteine remethylation cycle in patients with CAD compared with controls. This was characterized by higher tHcy concentrations, lower concentrations of 5-MTHF and B₁₂ in plasma, and lower 5-MTHF concentrations in RBC in patients compared with controls (table 1). The difference in the RBC 5-MTHF concentrations between controls and cases was more pronounced and significant than in other components of the homocysteine remethylation pathway examined in this study.

Univariate and multivariate analyses of plasma total tHcy with other components of the remethylation pathway showed that there was a stronger and independent correlation of plasma tHcy with RBC 5-MTHF than plasma 5-MTHF and plasma vitamin B₁₂. Furthermore, stepwise multiple regression analysis showed that RBC 5-MTHF is the main and independent predictor of plasma tHcy in controls when adjusted for other variables such as plasma 5-MTHF, vitamin B₁₂, hypertension and hypercholesterolemia. In addition, results from logistic regression analysis showed that the association of CAD with decreasing RBC 5-MTHF was stronger than those with decreasing plasma 5-MTHF, plasma vitamin B₁₂ and increasing plasma tHcy concentrations (table 2). The correlation between CAD and tHcy was also weaker and became insignificant at the highest quartile of plasma tHcy when adjusted for RBC 5-MTHF, vitamin B₁₂, hypertension and hypercholesterolemia. Therefore, results from this study suggest that folate in the RBC is a more stable parameter to study the association of components of the homocysteine remethylation pathway with CAD in Iranians.

In most studies, the folate status has so far been measured in plasma and has been mostly regarded as a determinant of tHcy concentration, but its association with CAD has been inconsistent: some found an association [12, 13] in contrast to others [14–17]. This is because plasma folate may not be a true indicator of the folate status as it can be impaired by exogenous factors such as drugs and diet, which could mask the association between folate and homocysteine status and the risk of CAD. However, results from this study are consistent with a few reports that found a positive association of RBC 5-MTHF with CAD [12, 17, 18]. Folate concentrations in RBC are

regarded as indicators of the long-term folate status and are not affected by exogenous factors. While the commonly determined folate concentrations in plasma or serum represent the circulating folate, determining 5-MTHF in RBC assesses the intracellular folate status and may reflect the folate status during the past 120 days [19] and thus the amount of folate available as a cofactor in the metabolism of homocysteine.

In this study, the intra-erythrocyte 5-MTHF content was nearly 50-fold higher than that of plasma 5-MTHF and it has been established that the transport and incorporation of plasma folate into erythroblasts is facilitated by folate receptors during erythropoiesis [20]. Progressive deficiency of plasma folate will therefore first induce a decrease in the RBC 5-MTHF concentrations. Folic acid fortification has been reported to increase RBC 5-MTHF concentrations, and it has been used as the main indicator of the folate nutritional status and folate insufficiency to establish the new dietary reference intakes [21]. Consistent with this study, a cross-sectional study from the Framingham Heart Study in the US [22] and the COMAC multicenter case-control study in Europe [18] demonstrated that low plasma folate, low folate intake (Framingham Heart Study) and low RBC folate (European COMAC study) were associated with CAD and vascular disease, respectively. In addition, recently RBC 5-MTHF has also been reported as a better marker to study the association between tHcy and the remethylation pathway and venous thromboembolism compared to plasma 5-MTHF and homocysteine [23].

The beneficial effect of folate on endothelial function has been reported and was initially thought to be a consequence of the reduction in plasma tHcy concentrations. However, recent studies suggest that other mechanisms beside the lowering effect of plasma tHcy by folic acid may be involved in the beneficial effects of folate on vas-

cular endothelial function. Folic acid therapy has been reported to improve endothelial function in patients with CAD with normal fasting plasma tHcy [24, 25]. In vitro studies suggest that folate itself is an effective free radical scavenger and inhibitor of microsomal lipid peroxidation [26]. In addition, folic acid supplementation may have beneficial effects by improving the bioavailability of tetrahydrobiopterin, a cofactor required for nitric oxide synthase activity [1] or by directly acting on endothelial nitric oxide via pteridine binding at the active site of nitric oxide synthase [27].

In this study, to eliminate the effects of confounding factors, cases and controls were matched for age and sex, and individuals who took vitamins were excluded. Furthermore, sampling in cases and controls was carried out at the same time of the year.

However, although this case-control study proves an association, it remains debatable whether RBC 5-MTHF concentrations are causally related to CAD. To prove this association, further prospective randomized studies including patients receiving folate supplementation to reduce CAD are required.

In conclusion, this study firstly described the association of plasma tHcy and folate status with CAD in Iranians. In addition, results from this study suggest that folate measurements in RBC seem to be the most reliable marker indicating 5-MTHF deficiency and disturbances in the homocysteine remethylation pathway in association with CAD in Iranians.

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