High Homocysteine Levels are Closely Associated with the Categories of Coronary Artery Diseases as well as Low Status of Folic Acid and Vitamin B₁₂

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Introduction

Coronary artery disease (CAD) is seriously to harm people’s healthy disease in both developed and developing countries, which was predominantly caused by atherosclerosis with endothelial dysfunction [1,2]. Despite best efforts, available therapies protect only 30-40% of individuals at risk, and no therapeutic cure is anticipated for those who currently suffer from the disease [3]. The endothelium is a single layer of cells lining all blood vessels. It plays an important role in many physiological functions, including the control of blood cell trafficking, vasomotor tone, vessel permeability, and hemostatic balance. Endothelial cells produce a wide variety of substances in response to various physical and chemical stimuli, including vasodilator substances, and vasoconstrictor substances [4]. Researches have confirmed that endothelial dysfunction plays an important role in all categories of CAD from stable angina pectoris (SAP) to unstable angina pectoris (UAP), and to acute myocardial infarction (AMI) [5]. Early warning and immediate risk stratification of patients with different categories of CAD is frequently a challenging task in the current.

A large number of studies have confirmed that elevated homocysteine (Hcy) has been associated with endothelial dysfunction of atherosclerosis owing to oxidative stress [6], endoplasmic reticulum stress [7], increased level of asymmetric dimethylarginine (ADMA) [8], involved inflammation [9,10]. Genetic or nutritional deficiencies in Hcy metabolism lead to hyperhomocysteinemia (HHcy) and cause endothelial dysfunction, a hallmark of atherosclerosis [11]. Folic acid and vitamin B₁₂ play an important role in regulating the metabolic process of Hcy [12]. Current studies have shown that folic acid supplementation can significantly improve endothelial dysfunction in patients with CAD [13]. Vitamin B₁₂ deficiency and HHcy are related to cardiovascular risk factors in patients with coronary artery disease [14]. In present study, we evaluate the correlations between Hcy and folic acid as well as vitamin B₁₂, and explore the associations of Hcy with the categories of CAD as well as the levels of folic acid and vitamin B₁₂ in patients with atherosclerotic CAD.

Materials and Methods

This study included 292 CAD patients (103 SAP, 116 UAP, 73 AMI) and 100 chest pain non-CAD patients confirmed by coronary angiography from the Sichuan Academy of Medical Sciences & Sichuan Provincial People’s Hospital. Patients with the following diseases were excluded from this study: cancer, liver diseases, renal insufficiency, blood diseases, hyperthyroidism, thyroid dysfunction, systemic lupus erythematosus, malnutrition, pregnant woman, and supplemented folic acid and vitamin B₁₂. Participating subjects were explained their participation rights and written informed consent was
obtained, and were asked about excessive drinking (yes or no, it was defined as yes at least once a week and drinking over 45 degrees of alcohol more than 200 mL) and smoking habits (yes or no, nonsmokers including never smoking and stop smoking more than one year).

Hypercholesterolemia and hypertriglyceridemia were defined as TC ≥ 6.22 mmol/L and TG ≥ 2.26 mmol/L, respectively. According to 2007 China Adult Dyslipidemia Prevention Guide. Diabetes mellitus was diagnosed when patients’ fasting plasma glucose (FPG) ≥ 7.0 mmol/L. Hypertension was diagnosed when patients' systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg. Overweight and obesity were defined as BMI (24.0-27.9) kg/m², and ≥ 28 kg/m², respectively, according to 2006 Guidelines for Prevention and Control of Overweight and Obesity in Chinese Adults.

The concentrations of Hcy, TC, TG, HDL-C, LDL-C and FPG were measured, respectively, by Hitachi 7600 Automatic Biochemistry Analyzer (Hitachi High-Tech Instruments Co., Ltd., Japan). The levels of folic acid and vitamin B₁₂ were quantified by ACCESS 2 Immunoassay System (Beckman Coulter, Inc., USA). The references intervals of Hcy, folic acid and vitamin B₁₂ were (5-15) µmol/L, ≥ 6.8 nmol/L and (133-675) pmol/L, respectively.

Statistical Analysis
The data were analyzed by using the statistical package for social science SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Normal distribution variables were expressed as mean ± SD, which of more than two samples were compared with the One-Way ANOVA, and the differences between two groups were compared by using the SNK-q test when p < 0.05. Categorical variables were expressed as percentage and compared by χ²-test. The correlation study between Hcy and folic acid as well as vitamin B₁₂ were performed on the measured data by using Pearson correlation a coefficient. A p-value < 0.05 was considered as significant.

Results

Comparison of principal characteristics between high Hcy and normal-low Hcy levels in CAD patients

Compared to normal-low Hcy group, High Hcy group were characterized by smoking, Diabetes mellitus, hypercholesterolemia and hypertriglyceridemia. There were no significant differences in the ratio of elder age, male, excessive drinking, hypertension, BMI>24 kg/m² between high Hcy and normal-low Hcy groups. The comparison of principal characteristics between high Hcy and normal-low Hcy levels in CAD patients (Table 1).

| Groups            | n   | Hcy (µmol/L) | Vitamin B₁₂ (pmol/L) |
|-------------------|-----|--------------|----------------------|
| Controls          | 100 | 10.81 ± 4.62 | 222.34 ± 62.58      |
| Stable angina pectoris | 103 | 16.63 ± 6.73  | 167.52 ± 56.25 b     |
| Unstable angina pectoris | 116 | 22.62 ± 6.37 ac | 148.65 ± 62.51 bd   |
| Acute myocardial infarction | 73  | 23.44 ± 5.78 ac | 144.57 ± 52.24 bd   |

Table 2: Comparison of Hcy, folic acid and vitamin B₁₂ in CAD patients and controls.

Prevalence of high Hcy, and low folic acid and vitamin B₁₂ in CAD patients

Approximately four-fifths of CAD patients (79.11%) had a prevalence of high Hcy. However, the levels of folic acid and vitamin B₁₂ in CAD patients were reduced, the prevalence of low folic acid and vitamin B₁₂ were 41.78% for folic acid, and 34.93% for vitamin B₁₂, respectively. The prevalence of high Hcy, and low folic acid and vitamin B₁₂ in CAD patients (Figure 1).

Figure 1: Prevalence of high Hcy, and low folic acid and vitamin B₁₂ in 292 CAD patients aged 36-85 years.
There was a significant trend toward an increase in the prevalence of high Hcy from SAP to AMI ($\chi^2=19.93$, p < 0.01). The prevalence of high Hcy progressively increased from 66.02% in SAP group to 81.90% in UAP group, and to 93.15% in AMI group. Similarly, low folic acid and vitamin B12 also had significant trend toward rise in the prevalence from SAP to AMI ($\chi^2=13.03$ and $\chi^2=13.13$, respectively, both p<0.01). The prevalence of low folic acid and vitamin B12 progressively increased from 32.04% and 24.27% in SAP group to 39.66% and 34.48% in UAP group, and to 58.90% and 50.68% in AMI group, respectively.

**Comparison of prevalence of low folic acid and vitamin B12 between high Hcy group and normal-low Hcy group in CAD patient and the correlations between Hcy and folic acid and vitamin B12**

More than half of the CAD patients (51.08%) with high Hcy had low folic acid levels, 7 times higher than that (6.56%) in CAD patients with normal-low Hcy concentrations ($\chi^2=39.33$, p<0.001), and 41.99% CAD patients with high Hcy had low vitamin B12 levels, 5 times higher than that (8.20%) in CAD patients with normal-low Hcy concentrations ($\chi^2=24.25$, p<0.001). There were strongly moderate negative correlations between Hcy and folic acid ($r=-0.67$, p<0.001) and vitamin B12 ($r=-0.56$, p<0.001).

**Discussion**

Vascular endothelium has important regulatory functions in the cardiovascular system and a pivotal role in regulating blood flow, mediating vasodilation, coagulation reactions, platelet activation, leukocyte adhesion, and vascular muscle function [15]. Current knowledge suggests that endothelial dysfunction, as an impairment of endothelium-dependent relaxation of blood vessels, occurs as the initial event in the pathogenesis of atherosclerosis, which considered to be the initiating factor and the key point of cardiovascular disease [16,17]. A major mechanism of endothelial dysfunction appears to be related to decreased bioavailability of endothelium-derived nitric oxide (NO). NO as a most important mediator of endothelium-dependent relaxation, plays a key role in normal vascular physiology in preserving the vessel wall in a quiescent state by inhibition of inflammation, thrombosis, and cellular proliferation. This gas is a potent vasodilator to activate soluble guanylyl cyclase in vascular muscle, resulting in accumulation of cGMP and relaxation, which is produced from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of several cofactors [18].

During the last two decades, overwhelming clinical and epidemiological studies and animal experiments has been actively exploring the roles and mechanisms of Hcy in atherosclerosis with endothelial dysfunction [19,20]. HHcy may lead to increase of the level of ADMA which has been identified as a potential risk factor for atherosclerosis with endothelial dysfunction. Elevated ADMA can results in decreasing NO concentration and bioavailability [21]. Extensive experimental evidence, both in vitro and in vivo, indicates that Hcy is an independent risk factor for cardiovascular disease and elevated serum Hcy level is associated with CAD events [22-24]. Homocysteine Studies Collaboration research revealed that elevated approximately 3 µmol/L Hcy will increase about 10% risk of cardiovascular events [25]. Humphrey et al. [26] analyzed has also demonstrated that increased 5 µmol/L Hcy concentration will increase approximately 20% risk of CAD events. In present study, AMI patients had the highest level of Hcy with (23.44 ± 5.78) µmol/L, and UAP patients were a little lower than AMI but were the second highest with (22.62 ± 6.37) µmol/L, SAP patients had the third higher level of Hcy with (18.63 ± 6.73) µmol/L, which were significantly higher than that of controls with (10.81 ± 4.62) µmol/L (p < 0.01). Compared to SAP patients, patients with AMI and UAP had higher Hcy levels with approximately average elevated (4-5) µmol/L, while SAP patients were higher Hcy with approximately 8 µmol/L than controls. There was a significant trend toward an increase in the prevalence of HHcy from SAP to AMI (p<0.01). The prevalence of HHcy progressively increased from 66.02% in SAP group to 81.90% in UAP group, and to 93.15% in AMI group. The present study has provided the valuable evidence that high Hcy levels are closely associated with the categories of CAD. The more serious patients with CAD suffer, the more higher concentration their Hcy have.

Folic acid and vitamin B12 as two vital regulators play an important role in regulating the metabolic process of Hcy [27,28]. Folic acid and vitamin B12 deficiency would result in elevated level of Hcy [29,30]. In biological cells, Hcy is derived from methionine after its utilization as a methyl group donor in biological methylation reactions. However, approximately 50% Hcy is produced to remethylate back to methionine by the transmethylation of methionine, while 50% Hcy metabolize via transulfuration to cystathionine [31]. In this cycle, methionine is activated by condensation with ATP to give the methyl donor, S-adenosylmethionine (SAM). SAM is transformed into S-adenosylhomocysteine (SAH) by donating its methyl group to the substrates of methylation reactions. Subsequently, SAH gives rise to Hcy in a reversible reaction that favors SAH over Hcy production [32]. Methyl-tetra-hydrofolic acid (MTHF) which derive of folic acid provide methyl to remethylated of Hcy. Vitamin B12 is agon of methionine synthetase that catalyzed this reaction and participate transulfusion of methyl [19]. Folic acid deficiency will prevent remethylation of Hcy because of raw material deficiency. Moreover, Folic acid deficiency will also influence the production of MTHF through to affect activity of methylene tetrahydrofolate reductase (MTHFR) [33,34]. Vitamin B12 deficiency will also result in HHcy because methyl of MTHF can’t transfer, which influence production of methionine and regeneration of tetrahydro-folic acid (THF) [35].

Guo et al. [36] measured plasma levels of Hcy, folic acid and vitamin B12 levels in 152 patients with > or = 3 risk factors of CAD and 136 patients with 1-2 risk factors and 48 individuals with no risk factors, found that folic acid supplementation may be useful in reducing plasma Hcy level in high risk patients with hyperhomocysteinemia. Cheng et al. [37] investigated also demonstrated that supplement folic acid, vitamin B12 in patients with HHcy could reduce Hcy levels. Our study revealed that the levels of folic acid and vitamin B12 in AMI and in UAP patients were obviously lower compared to those in SAP and non-CAD patients (p<0.05). The prevalence of low folic acid and vitamin B12 progressively increased from 32.04% and 24.27% in SAP group to 39.66% and 34.48% in UAP group, and to 58.90% and 50.68% in AMI group, respectively. 51.08% CAD patients with HHcy had low vitamin B12 levels, 5 times higher than that (6.56%) in CAD patients with normal-low Hcy concentrations (p<0.001), and 41.99% CAD patients with HHcy had low vitamin B12 levels, 5 times higher than that (8.20%) in CAD patients with normal-low Hcy concentrations (p<0.001). There were strongly moderate negative correlations between Hcy and folic acid ($r=-0.67$, p<0.001) and vitamin B12 ($r=-0.56$, p<0.001). Our results confirmed that serum folic acid and vitamin B12 influence Hcy metabolism as cosubstrate and cofactor, respectively.
and low status of folic acid and vitamin B12 are closely associated with High Hcy levels.

Limitations
Since present study was just an investigation that the association between Hcy levels and the categories of CAD, and the correlation of Hcy with folic acid and vitamin B12. The larger sample number of multicenter study and longer prospective investigation are necessary to further observe serum Hcy changes and incidence of adverse cardiovascular events by supplementation of folic acid and vitamin B12 in CAD patients.

Conclusion
The present study confirmed that HHcy and traditional cardiovascular risk factors may be synergistically prompt the formation and development of atherosclerosis in CAD patients. High Hcy levels are closely associated with the categories of CAD as well as low status of folic acid and vitamin B12.

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References
1. Choi BJ, Matsuo Y, Aoki T, Kwon TG, Prasad A, et al. (2014) Coronary endothelial dysfunction is associated with inflammation and vascular von Willebrand factor in patients with early atherosclerosis. Arterioscler Thromb Vasc Biol 34: 2473-2477.
2. Ruggiero D, Paolillo S, Ratta GD, Mariniello A, Formisano T, et al. (2013) Endothelial function as a marker of pre-clinical atherosclerosis: assessment techniques and clinical implications. Monaldi Arch Chest Dis 80: 106-110.
3. Subbotin VM (2012) Neovascularization of coronary tunica intima (DIT) is the cause of coronary atherosclerosis. Lipoproteins invade coronary intima via neovascularization from adventitial vasa vasa, but not from the arterial lumen: a hypothesis. Theor Biol Med Model 9: 1-22.
4. Aird WC (2004) Endothelium as an organ system. Crit Care Med 32: S271-279.
5. Wen J, Wen Y, Zhihong L, Lingling C, Longxing C, et al. (2013) A decrease in the percentage of circulating mDC precursors in patients with coronary heart disease: a relation to the severity and extent of coronary artery lesions? Heart Vessels 28: 135-142.
6. Hoffman M (2011) Hypothesis: hyperhomocysteinemia is an indicator of oxidant stress. Med Hypotheses 77: 1088-1093.
7. Wang XC, Sun WT, Yu CM, Pun SH, Underwood MJ, et al. (2015) ER stress mediates homocysteine-induced endothelial dysfunction: Modulation of IKCa and SKCa channels. Atherosclerosis 242: 191-198.
8. Magne J, Huneau JF, Borderie D, Mathé V, Bos C, et al. (2015) Plasma asymmetric and symmetric dimethylarginine in a rat model of endothelial dysfunction induced by acute hyperhomocysteinemia. Amino Acids 47: 1975-1982.
9. Arzamasvet DD, Karpenko AA, Kostuchenko GI (2012) Inflammation of the vascular wall and hyperhomocysteinemia in patients with atherosclerosis obliterans of lower limb arteries. Angiol Sovus Khir 18: 27-30.
10. Antoniades C, Antonopoulos AS, Troussoulis D, Marinou K, Stefanadis C (2009) Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. Eur Heart J 30: 6-15.
11. Gurdka D, Handschuł L, Kotkowski W, Jakubowski H (2015) Homocysteine thiolactone and N-homocysteinylated protein induce pro-atherogenic changes in gene expression in human vascular endothelial cells. Amino Acids 47: 1319-1339.
12. Zeng R, Xu CH, Xu YN, Wang YL, Wang M (2015) The effect of folate fortification on folic acid-based homocysteine-lowering intervention and stroke risk: a meta-analysis. Public Health Nutr 18: 1514-1521.
13. Liu Y, Tian T, Zhang H, Gao L, Zhou X (2014) The effect of homocysteine-lowering therapy with folic acid on flow-mediated vasodilation in patients with coronary artery disease: a meta-analysis of randomized controlled trials. Atherosclerosis 235: 31-5.
14. Mahalle N, Culkarni MV, Garg MK, Naik SS (2013) Vitamin B12 deficiency and hyperhomocysteinemia as correlates of cardiovascular risk factors in Indian subjects with coronary artery disease. J Cardiol 61: 289-294.
15. Eren E, Ellidag HY, Aydin O, YA-Imaz N (2014) Homocysteine, Paraoxonase-1 and Vascular Endothelial Dysfunction: Omnibus viis Romam Perpervitur. J Clin Diagn Res 8: CE01-04.
16. Pushpakumar S, Kundu S, Sen U (2014) Endothelial dysfunction: the link between homocysteine and hydrogen sulfide. Curr Med Chem 21: 3662-3672.
17. Polovina MM, Potpara TS (2014) Endothelial dysfunction in metabolic and vascular disorders. Postgrad Med 126: 38-53.
18. Karbach S, Wenzel P, Waismann A, Munzel T, Daiber A (2014) eNOS uncoupling in cardiovascular diseases—the role of oxidative stress and inflammation. Curr Pharm Des 20: 3579-3594.
19. Lentz SR (2005) Mechanisms of homocysteine-induced atherothrombosis. J Thromb Haemost 3: 1646-1654.
20. Yang AN, Zhang HP, Sun Y, Yang XL, Wang N, et al. (2015) High-methionine diets accelerate atherosclerosis by HHcy-mediated FABP4 gene demethylation pathway via DNMT1 in ApoE(-/-) mice. FEBS Lett 589: 3998-4009.
21. Emekizki HC, Serdaroglu A, Biberoglu G, Gulbahar O, Arban E, et al. (2013) Assessment of atherosclerosis risk due to the homocysteine-asymmetric dimethylarginine-nitric oxide cascade in children taking antiepileptic drugs. Seizure 22: 124-7.
22. Akurek A, Akbal E, Gunes F (2014) Increase in the risk of ST elevation myocardial infarction is associated with homocysteine level. Arch Med Res 45: 501-506.
23. Wu Y, Huang Y, Hu Y, Zhong J, He Z, et al. (2013) Hyperhomocysteinemia is an independent risk factor in young patients with coronary artery disease in southern China. Herz 38: 779-784.
24. Alam N, Khan HI, Chowdhury AH, Haque MS, Ali MS, et al. (2012) Elevated serum homocysteine level has a positive correlation with serum cardiac troponin I in patients with acute myocardial infarction. Bangladesh Med Res Councl Bull 38: 9-13.
25. Homocysteine Studies Collaboration (2002) Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA 288: 2015-2022.
26. Humphrey LL, Fu R, Rogers K, Freeman M, Helfand M (2008) Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis. Mayo Clin Proc 83: 1203-1212.
27. Abdollahi Z, Elmadfa I, Djazayeri A, Sadeghian S, Freisling H, et al. (2013) Homocysteine-thiolactone and N-homocysteinylated protein induce pro-inflammatory response in children taking antiepileptic drugs. Eur J Clin Pharmacol 69: 1203-1212.
28. Chen KJ, Pan WH, Yang FL, Wei IL, Shaw NS, et al. (2005) Association of hyperhomocysteinemia with type 2 diabetes in Taiwan. Diabetes Res Clin Pract 69: 106-110.
30. Gonzalez-Gross M, Sola R, Albers U, Barrios L, Alder M, et al. (2007) B-vitamins and homocysteine in Spanish institutionalized elderly. Int J Vitam Nutr Res 77: 22-33.

31. Tchantchou F (2006) Homocysteine metabolism and various consequences of folate deficiency. J Alzheimers Dis 9: 421-427.

32. Troen AM, Lutgens E, Smith DE, Rosenberg IH, Selhub J (2003) The atherogenic effect of excess methionine intake. Proc Natl Acad Sci USA 100: 15089-15094.

33. Tavares EF, Vieira-Filho JP, Andriolo A, Perez AB, Vergani N, et al. (2004) Serum total homocysteine levels and the prevalence of folate acid deficiency and C677T mutation at the MTHFR gene in an indigenous population of Amazonia: the relationship of homocysteine with other cardiovascular risk factors. Ethn Dis 14: 49-56.

34. Bozok Cetinta V, Gunduz C (2014) Association between polymorphism of MTHFR c.677C>T and risk of cardiovascular disease in Turkish population: a meta-analysis for 2,780 cases and 3,022 controls. Mol Biol Rep 41: 397-409.

35. Green R, Miller JW (2005) Vitamin B12 deficiency is the dominant nutritional cause of hyperhomocystenemia in a folic acid-fortified population. Clin Chem Lab Med 43: 1048-1051.

36. Guo H, Lee JD, Ueda T, Cheng J, Shan J, et al. (2004) Hyperhomocysteinaemia & folic acid supplementation in patients with high risk of coronary artery disease. Indian J Med Res 119: 33-37.

37. Cheng D, Kong H, Pang W, Yang H, Lu H, et al. (2014) B vitamin supplementation improves cognitive function in the middle aged and elderly with hyperhomocysteinemia. Nutr Neurosci.