Original Article

Homocysteine and *MTHFR* and *VEGF* Gene Polymorphisms: Impact on Coronary Artery Disease

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Summary

Background: Polymorphisms in genes involved in the atherosclerosis development, angiogenesis, and homocysteine (Hcy) metabolism could be risk factors for coronary artery disease (CAD).

Objective: To evaluate the effect of the *VEGF* C-2578A and *MTHFR* C677T polymorphisms on CAD, and the association of these polymorphisms with the severity and extension of atherosclerotic lesions and Hcy concentrations.

Methods: Two hundred and forty-four subjects were evaluated by coronary angiography and included in the study (145 with CAD and 99 controls). The *VEGF* C-2578A and *MTHFR* C677T polymorphisms were investigated by the PCR-SSCP and PCR-RFLP techniques, respectively. Plasma Hcy was quantified by liquid chromatography/sequential mass spectrometry (LC-MS/MS).

Results: There was no significant difference in allele and genotype distribution between the groups, for both polymorphisms. The univariate analysis showed a higher frequency of the *VEGF* -2578AA genotype in the group with three-vessel disease (p=0.044). In addition, the *VEGF* -2578CA genotype was observed more frequently among individuals with <95% stenosis (p=0.010). After adjustment for other risk factors for CAD in a multivariate model, the *VEGF* C-2578A polymorphism was not found to be an independent correlate of CAD (p=0.688). The *MTHFR* polymorphism did not show any association with the extension and/or severity of the CAD. The *MTHFR* C677T polymorphism showed no direct association with hyperhomocysteinemia or increased mean plasma concentrations of Hcy.

Conclusion: Although there is an apparent association between *VEGF* C-2578A and the development of coronary atherosclerosis, this association is not independent of conventional cardiovascular risk factors. (Arq Bras Cardiol 2009;92(4):249-254)

Key words: Coronary artery disease; atherosclerosis; polymorphism, genetic; homocysteine.

Introduction

The gene encoding the vascular endothelial growth factor (*VEGF*) has been studied in the development of coronary diseases[1-3]. *VEGF*, present in the vessel walls, promotes the proliferation of vascular endothelial cells and angiogenesis, but its role in atherosclerosis is still obscure. There are studies pointing to *VEGF* as a protection factor in the atherosclerotic plaque development, by acting as a regulator of the coronary artery wall endothelial integrity[4,5]. On the other hand, there are studies showing that neovascularization, mediated by *VEGF*, influences the pathogenesis of arterial diseases[6].

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Alterations in the *VEGF* concentrations can be mediated by genetic polymorphisms[7,8]. Several single nucleotide polymorphisms (SNPs) have been described in the *VEGF* gene. Renner et al[7] described three polymorphisms of the *VEGF* gene (C702T, C936T and G1612A) and observed significantly lower *VEGF* plasma levels in 936T allele carriers compared to non-carriers. The C702T and G1612 polymorphisms showed no association with *VEGF* levels. Elevated levels were also associated with the G allele in the *VEGF* -1154 nucleotide[8]. In a study with patients undergoing coronary angiography, the C-2578A polymorphism was considered a risk factor for atherosclerosis development[1]. This genetic variation had already been associated with lower *VEGF* expression[8].

Hyperhomocysteinemia, i.e., increased blood homocysteine (Hcy) concentrations, is considered an independent risk factor for atherosclerosis and coronary artery disease (CAD)[9]. Increased Hcy concentrations are found in 40% of patients with coronary, cerebral or peripheral artery diseases, and only in 15% of healthy individuals[10]. The mechanism by which

hyperhomocysteinemia induces the development of vascular lesions is still obscure. Experimental evidence suggests that Hcy may be involved in atherogenesis and thrombogenesis, leading to muscle cell hyperplasia and fibrosis[10,11]. Abnormal Hcy concentrations can result from the interaction of genetic and dietary factors[12]. The enzyme methylenetetrahydrofolate reductase (*MTHFR*), which plays an important role in the Hcy metabolism, presents decreased activity as a result of the polymorphism in the position 677 of the *MTHFR* gene, and leads to increased Hcy concentrations[13]. The variant allele *MTHFR* 677T has been found at high frequencies in patients with vascular diseases[14-17].

In this study, we analyzed the frequency of the *VEGF* C–2578A and *MTHFR* C677T polymorphisms in patients with CAD and in a group without angiographic signs of the disease, and evaluated the association between these polymorphisms and the severity and extension of the atherosclerotic lesions, and the plasma Hcy concentrations.

Methods

Study subjects

This study was approved by the National Research Commission - CONEP, DF, Brazil. After signing a free informed consent form, 244 Caucasoid individuals were included in the research (145 with CAD and 99 without the disease). The diagnosis of CAD was confirmed or ruled out by coronary angiography, analyzed by two independent observers in a quantitative blind analysis, performed at the Cath Lab. Individuals who had undergone heart surgery and those with a coronary prosthesis were excluded from the study. Although miscegenation is widespread in Brazil, we considered as Caucasoid those individuals who had no other ethnic groups in the three generations that preceded them[18]. The extension of the CAD was determined according the number of involved coronary vessels (0 to 3), and the CAD severity by the degree of arterial obstruction (<50%, >50-75%, >75-95%, and >95% stenosis). Ejection fraction (EF) < 40% was considered as ventricular damage. Typical angina was considered as present or absent.

Classical risk factors for CAD were also investigated. The criteria used for defining diabetes were the use of oral hypoglycemic agents or insulin treatment, or glycemia levels higher than 126 mg/dl; for hypertension, the use of anti-hypertensive medication or blood pressure higher than 140/90 mmHg; for sedentary lifestyle, the absence of regular and controlled exercising; for alcoholism, the intake of alcohol with a frequency defined without a quantitative analysis; individuals who had smoked ≥100 cigarettes throughout their lifetime and currently smoked every day or on some days were considered smokers.

Biochemical analysis

Peripheral blood samples were collected from the patients during the coronary angiographic examination, after a 12-hour fasting. Plasma separation was performed within one hour after blood collection for Hcy quantitation, according to the liquid chromatography/sequential mass spectrometry (LC-MS/MS) method[19]. Hcy concentrations >15μmol/ml were considered as characterizing hyperhomocysteinemia[20].

The procedures for lipid profile determination were carried out at the Clinical Test Laboratory of the Base Hospital of the São José do Rio Preto Medical School. Serum triglycerides (TG), total cholesterol (TC), high-density lipoproteins (HDL_C), and low-density lipoproteins (LDL_C) were measured by the enzymatic colorimetric method.

Genotype determination

Genomic DNA was extracted from peripheral blood leukocytes[21]. The *VEGF* C-2578A polymorphism was investigated by the Polymerase Chain Reaction-Single-Strand Conformational Polymorphism (PCR-SSCP) method, according to Brogan et al[22]. Thirty amplification cycles (95°C for 30s, 62°C for 30s, and 72°C for 60s) were performed to amplify a 309 bp product. Then, 6 μl of the PCR product were denatured at 95°C for 5 min with 6 μl electrophoresis staining solution (80% v/v formamide; 10mM NaOH; 1 mM EDTA, pH 8.0; 0.1% w/v of bromophenol blue; and 1.1% xylenocyanol). The denatured products were analyzed by 9.6% polyacrylamide gel electrophoresis and visualized after silver nitrate staining.

Genotyping for the *MTHFR* C677T polymorphism was performed by PCR followed by enzyme digestion[16]. Thirty-five cycles (94°C for 60s, 58°C for 60s, and 72°C for 60s) were performed to amplify a 198 bp product. The PCR products were digested with the restriction enzyme *Hinf* I and the fragments were analyzed on a 9.6% polyacrylamide gel stained with silver nitrate. Allele C was not digested, whereas allele T was cut into two fragments of 175 bp and 23 bp, respectively.

Statistical analysis

Data are presented as mean ± standard deviation (SD) or number (percentages). Comparison between the groups was performed using the Chi-square, Student's *t*-test or Mann-Whitney's test, as appropriate. Dependence analysis was used to examine differences in genotype frequency between groups with one, two, or three affected arteries, and between groups with a different degree of stenosis The Hcy values were modeled based on a normal distribution on the logarithmic scale. Hcy concentration in relation to the *MTHFR* polymorphism was evaluated by analysis of variance (ANOVA). The independent relationship of the *VEGF* C-2578A and *MTHFR* C677T polymorphisms with risk factors in the presence of CAD was tested by multiple logistic regression analysis. A p-value ≤ 0.05 was used to establish statistical significance.

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Results

The characteristics of the studied population are shown in Table 1. The significantly different results obtained for patients with and without CAD are in agreement with previously established cardiovascular risk factors.

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Table 1 - Characteristics of the study population

	CAD (n = 145)	Controls (n = 99)	p-value
Age (years)	60.4±12.3	57.8±11.4	0.084
Gender			0.066
Males	93(64)	51(52)	
Females	52(36)	48(48)	
Hypertension	113(78)	66(67)	0.708
Diabetes	36(25)	13(13)	0.040
Sedentary lifestyle	63(43)	50(51)	0.339
Smoking	100(69)	52(53)	0.014
Angina	115(79)	68(69)	0.083
Ventricular damage	50(34)	14(14)	0.0007
Alcoholism	48(33)	25(25)	0.240
Total cholesterol (>200mg/dl)	60(41)	41(41)	0.996
HDLc (<40 mg/dl)	78(54)	31(31)	0.0008
LDLc (>130 mg/dl)	57(40)	36(36)	0.740
VLDLc (> 30 mg/dl)	58(40)	33(33)	0.356
Triglycerides (>150 mg/dl)	63(43)	36(36)	0.330

Values are presented as mean ± SD or number (%). CAD - Coronary artery disease; HDLc- high-density lipoprotein; LDLc - low-density lipoprotein; VLDLc - very-low-density lipoprotein.

Vascular endothelial growth factor (*VEGF*) gene polymorphism

There was no significant difference between the groups regarding allele (p=0.995) and genotype (p=0.254) frequencies of the *VEGF* C-2578A polymorphism. Calculations for the Hardy-Weinberg equilibrium (HWE) test showed that the genotype distribution observed was similar to the expected one, in both CAD and control groups (x^2_1=3.12; p=0.077 and x^2_1=0.46; p=0.496, respectively).

Genotype frequencies according to the number of involved vessels and degree of artery obstruction are shown in Tables 2 and 3, respectively. A significantly higher frequency of *VEGF* -2578AA genotype was found in individuals with three affected arteries (p=0.044). Regarding the degree of artery obstruction, the *VEGF* -2578CA genotype was observed more frequently in individuals with <95% stenosis (p=0.010).

Methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphism and homocysteine concentrations

There was no significant difference between the groups regarding allele (p=0.895) and genotype (p=0.884) frequencies of the *MTHFR* C677T polymorphism. The observed genotype distribution was similar to the expected one in the control group (x^2_1=2.23; p=0.135), but it showed departure from the HWE in the CAD group, with a significant difference between the observed and the expected frequencies (x^2_1=5.76; p=0.016).

Table 2 - Genotype frequencies in patients with one, two, and three affected arteries

Polymorphism		One affected artery N (%)	Two affected arteries N (%)	Three affected arteries N (%)	p-value
VEGF -2578	CC	15 (44)	14 (41)	5 (15)	0.044
	CA	32 (38)	28 (34)	23 (28)	
	AA	6 (21)	8 (29)	14 (50)	
MTHFR 677	CC	24 (45)	16 (30)	13 (25)	0.548
	CT	25 (31)	29 (36)	26 (33)	
	TT	4 (33)	5 (42)	3 (25)	

Table 3 - Genotype frequencies in patients with <50%, >50-75%, >75-95%, and >95% stenosis

Polymorphism		Degree of stenosis <50% N (%)	>50-75% N (%)	>75-95% N (%)	>95% N (%)	p-value
VEGF -2578	CC	2 (6)	6 (18)	16 (47)	10 (29)	0.010
	CA	9 (11)	17 (21)	45 (54)	12 (14)	
	AA	0 (0)	4 (14)	13 (47)	11 (39)	
MTHFR 677	CC	4 (7)	10 (19)	27 (51)	12 (23)	0.991
	CT	6 (8)	16 (20)	41 (51)	17 (21)	
	TT	1 (8)	1 (8)	6 (50)	4 (34)	

No association was observed between the *MTHFR* C677T polymorphism and the number of involved vessels and/or the degree of artery obstruction (Tables 2 and 3).

The mean Hcy concentration did not significantly differ between the groups (p=0.222), nor did the presence of hyperhomocysteinemia (p=0.266). The mean Hcy concentrations did not show any association with the number of involved vessels (p=0.793) or the degree of artery obstruction (p=0.700).

The *MTHFR* C677T polymorphism showed no direct association with hyperhomocysteinemia (p=0.860) or increased mean concentrations of Hcy (p=0.758).

Multivariate analysis

Multiple logistic regression was used to test independent correlates for the presence of CAD. The model included: smoking, hypertension, diabetes, HDL_c levels, and the *VEGF* C-2578A and *MTHFR* C677T polymorphisms. Smoking (p=0.006), diabetes (p=0.041), and HDL_c levels <40 mg/dl (p=0.0008) were independent correlates of the presence of CAD. Hypertension tended towards showing an association with CAD (p=0.061). The *VEGF* C-2578A (p=0.688) and *MTHFR* C677T (p=0.981) polymorphisms were not independent predictors of CAD.

Discussion

In our study, the allele and genotype frequencies of the *VEGF* C-2578A polymorphism did not differ between patients with and without CAD. However, the *VEGF* -2578AA genotype was observed at a higher frequency in individuals with three affected arteries. This result corroborates the findings of a recent study by Howell et al[1], in which the *VEGF* -2578AA genotype was considered to be a risk factor for atherosclerosis development, whereas the *VEGF* -2578CC genotype showed a protective effect. According to this study, the frequency of the *VEGF* -2578AA genotype gradually increased with the number of involved vessels, as compared to the wild-type *VEGF* -2578CC genotype, suggesting that the reduction in the VEGF expression resulting from the *VEGF* -2578AA genotype could promote the development of atherosclerosis. The higher frequency of the *VEGF* -2578CA genotype among individuals with <95% stenosis suggests an association between this genotype and the slower progression of the atherosclerotic lesion, possibly due to the presence of the wild-type allele. However, no association was found regarding the wild-type *VEGF* -2578CC genotype, possibly because of the small sample size.

Although our results regarding *VEGF* polymorphism and the severity and extension of CAD were significant, after adjustment for other risk factors in a multivariate model, the positive association between *VEGF* C-2578A polymorphism and severity or extension of CAD lost statistical significance. Since *VEGF* C-2578A polymorphism was not an independent risk factor for CAD, these data do not support a casual linkage between *VEGF* C-2578A polymorphism and coronary atherosclerosis.

The role of VEGF in atherosclerosis is a subject of debate in the literature. Some studies showed that the administration of recombinant human VEGF in animals enhances the progression of the atherosclerotic plaque[23], whereas others observed that it acts as an anti-atherosclerotic factor, promoting re-endothelization, reducing intimal thickening and preventing thrombus formation[5].

Correlations between genotypes and expression were not examined in our study. However, a study by Shahbazi et al[8], with renal transplant recipients, found an association between the wild-type allele *VEGF* -2578C and increased *VEGF* concentrations. Other polymorphisms of the *VEGF* gene have already been associated with variations in the expression of the protein[7,8]. According to Howell et al[1], the *VEGF* -2578AA genotype could be considered a risk factor for atherosclerosis due to the reduction in the protein expression. This result is consistent with the action of *VEGF* in the endogenous regulation of the endothelial integrity of the coronary artery wall[4]. On the other hand, atherosclerotic plaque neovascularization, mediated by *VEGF*, makes nutrients and components for the plaque available, increasing its volume. In artery walls with total occlusion by atherosclerotic plaques, as observed in a prospective study, microvessels named *vasa vasorum* were correlated, in their anatomy and quantity, to the degree of intimal plaque inflammation[24]. Moulton et al[25] observed a reduction in atherosclerosis progression secondary to the inhibition of *VEGF*-mediated neovascularization. Thus, further studies are necessary to clarify the true role of *VEGF* in CAD.

In this study, the allele and genotype frequencies of the *MTHFR* C677T polymorphism did not differentiate patients from controls. However, in the CAD group, the genotype distribution observed was different from the one expected according to the Hardy-Weinberg equilibrium (HWE). Case-control studies with SNP analysis have shown departure from HWE in patients, in controls or in both groups[26-28]. This disequilibrium can be expected for a number of genetic diseases, considering that there probably is a significant genetic contribution in complex diseases[29]. Although some studies found evidence of an association between the polymorphic *MTHFR* 677T allele and vascular diseases, carotid atherosclerosis, occlusive arterial disease and myocardial infarction[15-17,30-32], others did not confirm these hypotheses[11,33].

About 40% of patients with CAD present hyperhomocysteinemia[34]. In our study, 49.7% of the patients with CAD and 45.2% of the controls showed Hcy concentrations >15 μmol/L, and this difference was not significant. Another Brazilian study showed a significant difference in Hcy concentrations between the control group and a group with severe atheromatosis. These authors also observed a positive correlation between higher Hcy levels and CAD[35]. In our study, higher Hcy mean concentrations were found in the CAD group compared to the control group, but the difference was not significant (data not shown), which is in agreement with the findings of Yilmaz et al[36].

Clinical trials involving homocysteine-lowering treatment have demonstrated that folate supplementation did not reduce the risk of complications and death due to cardiovascular causes, despite a substantial reduction in plasma total Hcy levels[37,38]. Although observation studies have demonstrated that the plasma total Hcy level is a predictor of cardiovascular events[39], no causative role of Hcy has been substantiated by the results of intervention trials involving homocysteine-lowering treatment.

In the prospective study of Frederiksen et al[40], the Hcy mean concentration was higher in *MTHFR* 677TT genotype carriers compared to the carriers of other genotypes; however, the *MTHFR* C677T polymorphism was not associated with ischemic cardiovascular disease or thromboembolism. Similar observations were reported by Huh et al[41] and Yilmaz et al[36] in CAD studies. In the population studied by us, the *MTHFR* C677T polymorphism did not present a direct association with hyperhomocysteinemia or increased mean Hcy concentrations, either in the CAD or in the control group. A correlation between the presence of this mutation and hyperhomocysteinemia was also not observed by Lima et al[35], in another Brazilian study.

Sample size is an important factor in case-control studies, mainly in investigations of genetic polymorphisms with high frequency in the general population. Although the number of individuals evaluated in this study was sufficient to demonstrate a statistical difference in genotype distribution among the subgroups with different degrees of artery obstruction and number of involved vessels, no difference was observed between the CAD and control groups. Studies in the literature use larger samples, mainly to evaluate the association between polymorphisms and CAD or Hcy. Moreover, the investigation of alterations in other enzymes

involved in Hcy metabolism, plasma folate and vitamin B_6 and B_{12} concentrations, and vitamin intake, could provide more consistent data for the discussion about the effect of polymorphisms on Hcy concentrations.

In summary, there is an apparent association between the *VEGF* C-2578A polymorphism and atherosclerosis development; however, this association is not independent of conventional cardiovascular risk factors.

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Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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References

1. Howell WM, Ali S, Rose-Zerilli MJ, Ye S. VEGF polymorphisms and severity of atherosclerosis. J Med Genet. 2005; 42: 485-90.
2. Fleisch M, Billinger M, Eberli FR, Garachemani AR, Meier B, Seiler C. Physiologically assessed coronary collateral flow and intracoronary growth factor concentrations in patients with 1- to 3-vessel coronary artery disease. Circulation. 1999; 100: 1945-50.
3. Inoue M, Itoh H, Ueda M, Naruko T, Kojima A, Komatsu R, et al. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. Circulation. 1998; 98: 2108-16.
4. Tsurumi Y, Murohara T, Krasinski K, Chen D, Witzenbichler B, Kearney M, et al. Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nat Med. 1997; 3: 879-86.
5. Van Belle E, Tio FO, Chen D, Maillard L, Chen D, Kearney M, et al. Passivation of metallic stents after arterial gene transfer of phVEGF165 inhibits thrombus formation and intimal thickening. J Am Coll Cardiol. 1997; 29: 1371-9.
6. Bayer IM, Caniggia I, Adamson SL, Langille BL. Experimental angiogenesis of arterial vasa vasorum. Cell Tissue Res. 2002; 307: 303-13.
7. Renner W, Kotschan S, Hoffmann C, Obermayer-Pietsch B, Pilger E. A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. J Vasc Res. 2000; 37: 443-8.
8. Shahbazi M, Fryer AA, Pravica V, Brogan IJ, Ramsay HM, Hutchinson IV, et al. Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. J Am Soc Nephrol. 2002; 13: 260-4.
9. Stanger O, Herrmann W, Pietrzik K, Fowler B, Geisel J, Dierkes J, et al. Clinical use and rational management of homocysteine, folic acid, and B vitamins in cardiovascular and thrombotic diseases. Z Kardiol. 2004; 93: 439-53.
10. Welch GN, Upchurch GJR, Loscalzo J. Hyperhomocyst(e)inaemia and atherothrombosis. Ann NY Acad Sci. 1997; 811: 48-58.
11. Hankey GJ, Eikelboom JW. Homocysteine and vascular disease. Lancet. 1999; 354: 407-13.
12. Verhoef P, de Groot LC. Dietary determinants of plasma homocysteine concentrations. Semin Vasc Med. 2005; 5: 110-23.
13. Pisciotta L, Cortese C, Gnasso A, Liberatoscioli L, Pastore A, Mannucci L, et al. Serum homocysteine, methylenotetrahydrofolate reductase gene polymorphism and cardiovascular disease in heterozygous familial hypercholesterolemia. Atherosclerosis. 2005; 179: 333-8.
14. Schwartz SM, Siscovick DS, Malinow MR, Rosendaal FR, Beverly RK, Hess DL, et al. Myocardial infarction in young women in relation to plasma total homocysteine, folate, and a common variant in the methylenetetrahydrofolate reductase gene. Circulation. 1997; 96: 81-9.
15. Tsai MY, Welge BG, Hanson NQ, Bignell MK, Vessey J, Schwichtenberg K, et al. Genetic causes of mild hyperhomocysteinemia in patients with premature occlusive coronary artery diseases. Atherosclerosis. 1999; 143: 163-70.
16. Bova I, Chapman J, Sylantiev C, Korczyn AD, Bornstein NM. The A677V methylenetetrahydrofolate reductase gene polymorphism and carotid atherosclerosis. Stroke. 1999; 30: 2180-2.
17. Jee SH, Song KS, Shim WH, Kim HK, Suh I, Park JY, et al. Major gene evidence after MTHFR-segregation analysis of serum homocysteine in families of patients undergoing coronary arteriography. Hum Genet. 2002; 111: 128-35.
18. Arruda VR, Siqueira LH, Goncalves MS, von Zuben PM, Soares MC, Menezes R, et al. Prevalence of the mutation C677-->T in the methylenetetrahydrofolate reductase gene among distinct ethnic groups in Brazil. Am J Med. 1998; 78: 332-5.
19. Haddad R, Mendes MA, Hoehr NF, Eberlin MN. Amino acid quantitation in aqueous matrices via trap and release membrane introduction mass spectrometry: homocysteine in human plasma. Analyst. 2001; 126: 1212-5.
20. Arnadottir M, Hultber, B, Vladov V, Nilsson-Ehle P, Thysell H. Hyperhomocysteinemia in cyclosporine-treated renal transplant recipients. Transplantation. 1996; 61: 509-12.
21. Abdel-Rahman SZ, Nouraldeen AM, Ahmed AE. Molecular interaction of 2,3-[14C]-acrylonitrile with DNA in gastric tissues of rat. J Biochem Toxicol. 1994; 9: 121-8.
22. Brogan IJ, Khan N, Isaac K, Hutchinson JA, Pravica V, Hutchinson IV. Novel polymorphisms in the promoter and 5´UTR regions of the human vascular endothelial growth factor gene. Hum Immunol. 1999; 60: 1245-9.
23. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. Nat Med. 2001; 7: 425-9.
24. Srivatsa SS, Edwards WD, Boos CM, Grill DE, Sangiorgi GM, Garratt KN, et al. Histologic correlates of angiographic chronic total coronary artery occlusions. J Am Coll Cardiol. 1997; 29: 955-63.
25. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E , et al. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. Proc Natl Acad Sci USA. 2003; 100: 4736-41.
26. Xu J, Turner A, Little J, Bleecker ER, Meyers DA. Positive results in association studies are associated with departure from Hardy-Weinberg equilibrium: hint for genotyping error? Hum Genet. 2002; 111: 573-4.

27. Song Y, Stampfer MJ, Liu S. Meta-analysis: apolipoprotein E genotypes and risk for coronary heart disease. Am Coll Phys. 2004; 141: 137-47.

28. Wittke-Thompson J, Pluzhnikov A, Cox NJ. Rational inferences about departures from Hardy-Weinberg equilibrium. Am J Hum Genet. 2005; 76: 967-86.

29. Llorca J, Prieto-Salceda D, Combarros O, Dierssen-Sotos T, Berciano J. Competing risks of death and Hardy-Weinberg equilibrium in case-control studies of gene-disease association. Gac Sanit. 2005; 19: 321-4.

30. Williams JK, Armstrong ML, Heistad DD. Vasa vasorum in atherosclerotic coronary arteries: responses to vasoactive stimuli and regression of atherosclerosis. Circ Res. 1988; 62: 515-23.

31. Dedoussis GV, Panagiotakos DB, Pitsavos C, Chrysohoou C, Skoumas J, Choumerianou D, et al. ATTICA Study Group. An association between the methylenetetrahydrofolate reductase (MTHFR) C677T mutation and inflammation markers related to cardiovascular disease. Int J Cardiol 2005;100:409-14.

32. Morita H, Taguchi J, Kurihara H, Kitaoka M, Kaneda H, Kurihara Y, et al. Gene polymorphism of 5,10-methylenetetrahydrofolate reductase as a coronary risk factor. J Cardiol. 1997; 29: 309-15.

33. Couffinhal T, Kearney M, Witzenbichler B, Chen D, Murohara T, Losordo DW, et al. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in normal and atherosclerotic human arteries. Am J Pathol. 1997; 150: 1673-85.

34. Austin RC, Lentz SR, Werstuck GH. Role of hyperhomocysteinemia in endothelial dysfunction and atherothrombotic disease. Cell Death Differ. 2004; 11 (Suppl 1): 56-64.

35. Lima LM, Carvalho MG, Fernandes AP, Sabino Ade P, Loures-Vale AA, da Fonseca Neto CP, et al. Homocysteine and methylenetetrahydrofolate reductase in subjects undergoing coronary angiography. Arq Bras Cardiol. 2007; 88 (2): 167-72.

36. Yilmaz H, Isbir S, Agachan B, Ergen A, Farsak B, Isbir T. C677T mutation of methylenetetrahydrofolate reductase gene and serum homocysteine levels in Turkish patients with coronary artery disease. Cell Biochem Funct. 2006; 24: 87-90.

37. Bonaa KH, Njolstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med. 2006; 354: 1578-88.

38. Lonn E, Yusuf S, Arnold MJ, Sheridan P, Pogue J, Micks M, et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med. 2006; 354: 1567-77.

39. The Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA. 2002; 288: 2015-22.

40. Frederiksen J, Juul K, Grande P, Jensen GB, Schroeder TV, Tybjaerg-Hansen A, et al. Methylenetetrahydrofolate reductase polymorphism (C677T), hyperhomocysteinemia, and risk of ischemic cardiovascular disease and venous thromboembolism: prospective and case-control studies from the Copenhagen City Heart Study. Blood. 2004; 104: 3046-51.

41. Huh HJ, Chi HS, Shim EH, Jang S, Park CJ. Gene-nutrition interactions in coronary artery disease: correlation between the MTHFR C677T polymorphism and folate and homocysteine status in a Korean population. Thromb Res. 2006; 117: 501-6.