

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 diseases¹⁻³. *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 diseases⁶.

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 al⁷ 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 nucleotide⁸. In a study with patients undergoing coronary angiography, the C-2578A polymorphism was considered a risk factor for atherosclerosis development¹. This genetic variation had already been associated with lower *VEGF* expression⁸.

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 individuals¹⁰. The mechanism by which

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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 factors¹². 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 concentrations¹³. The variant allele *MTHFR* 677T has been found at high frequencies in patients with vascular diseases¹⁴⁻¹⁷.

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 them¹⁸. 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)

method¹⁹. Hcy concentrations >15 $\mu\text{mol/ml}$ were considered as characterizing hyperhomocysteinemia²⁰.

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 leukocytes²¹. The *VEGF* C-2578A polymorphism was investigated by the Polymerase Chain Reaction-Single-Strand Conformational Polymorphism (PCR-SSCP) method, according to Brogan et al²². 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% xylene cyanol). 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 digestion¹⁶. 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 \pm 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.

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 ($\chi^2_1=3.12$; $p=0.077$ and $\chi^2_1=0.46$; $p=0.496$, 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 ($\chi^2_1=2.23$; $p=0.135$), but it showed departure from the HWE in the CAD group, with a significant difference between the observed and the expected frequencies ($\chi^2_1=5.76$; $p=0.016$).

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

Polymorphism		One affected artery	Two affected arteries	Three affected arteries	p-value
		N (%)	N (%)	N (%)	
<i>VEGF</i> -2578	CC	15 (44)	14 (41)	5 (15)	0.044
	CA	32 (38)	28 (34)	23 (28)	
	AA	6 (21)	8 (29)	14 (50)	
<i>MTHFR</i> 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				p-value
		<50%	>50-75%	>75-95%	>95%	
		N (%)	N (%)	N (%)	N (%)	
<i>VEGF</i> -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)	
<i>MTHFR</i> 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 al¹, 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 plaque²³, whereas others observed that it acts as an anti-atherosclerotic factor, promoting re-endothelialization, reducing intimal thickening and preventing thrombus formation⁵.

Correlations between genotypes and expression were not examined in our study. However, a study by Shahbazi et al⁸, 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 al¹, 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 wall⁴. 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 inflammation²⁴. Moulton et al²⁵ 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 groups²⁶⁻²⁸. This disequilibrium can be expected for a number of genetic diseases, considering that there probably is a significant genetic contribution in complex diseases²⁹. 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 hyperhomocysteinemia³⁴. In our study, 49.7% of the patients with CAD and 45.2% of the controls showed Hcy concentrations >15 $\mu\text{mol/L}$, 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 CAD³⁵. 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 al³⁶.

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 events³⁹, 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 al⁴⁰, 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 al⁴¹ and Yilmaz et al³⁶ 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 al³⁵, 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 B₆ and B₁₂ 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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