Lack of Association Between Carotid Intima-Media Thickness and Methylenetetrahydrofolate Reductase Gene Polymorphism or Serum Homocysteine in Non–Insulin-Dependent Diabetes Mellitus

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We assessed the contribution of the serum homocysteine (Hcy) level, an independent risk factor for vascular disease, and methylene tetrahydrofolate reductase (MTHFR) gene polymorphism to the variability of intimal-medial thickness (IMT) of the common carotid artery in middle-aged non–insulin-dependent diabetes mellitus (NIDDM) subjects. One hundred thirty NIDDM patients (60 males and 70 females) with a mean age of 53 ± 10 years and a mean diabetes duration of 11.3 ± 7.9 years were enrolled for the study. Exclusion criteria included liver, heart, kidney, or other major-organ disease. Fasting total serum Hcy, folate, and vitamin B_{12} and clinical chemistry analyte levels were measured. MTHFR polymorphism was determined by polymerase chain reaction (PCR). IMT and plaques or stenosis in the common carotid were measured by ultrasonography. Serum Hcy was inversely correlated with vitamin levels and was slightly higher in subjects with the Val/Val genotype versus Ala/Val and Ala/Ala ($P = .02$); no differences in genotype were found in subjects with folate or vitamin B_{12} at or above the median level. In univariate analysis, common carotid IMT was significantly associated with age ($P = .00001$), the body mass index ([BMI] $P = .0003$), uric acid ($P = .004$), systolic blood pressure ($P = .03$), glycemia ($P = .03$), and total cholesterol ($P = .04$). No significant association was found between serum Hcy or MTHFR polymorphism and IMT. In multiple regression analysis, age ($P = .0001$), uric acid ($P = .03$), glycemia, and the BMI ($P = .05$) were independently associated with IMT and explained about 42% of IMT variability. In 130 NIDDM patients without nephropathy, basal levels of serum Hcy, as well as MTHFR polymorphism, did not predict significant changes in common carotid IMT.

CARDIOVASCULAR COMPLICATIONS associated with atherosclerosis are the leading cause of mortality in non–insulin-dependent diabetes mellitus (NIDDM), and several potential genetic factors may interact with the natural history of the disease, contributing to them.[1] In recent years, homocysteine (Hcy) has emerged as an independent risk factor for vascular disease.[2] Subjects with genetically inherited hyperhomocysteinemia and homocystinuria are affected by atherothrombotic complications at an early age.[3] Mild hyperhomocysteinemia occurs in about 5% of the general population, and there is evidence that it represents an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral vasculature.[4]

While severe hyperhomocysteinemia is caused by rare inborn errors of metabolism involving genetic defects in enzymes such as cystathionine β-synthase, a common variant of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is correlated with mild elevations of plasma Hcy in the general population.[5]

The variant originates from a mutation (C-to-T substitution at nucleotide 677 of the MTHFR gene) that converts an alanine to a valine residue[6] and is associated with a defective thermolabile enzymatic activity.[7] The frequency of homozygotes for the mutation is 0.05 to 0.16 among populations. The possible association between MTHFR $C^{677} \rightarrow T$ polymorphism and coronary artery disease or vascular disease has been extensively investigated.[8,9] Furthermore, it has to be considered that the MTHFR-dependent genetic predisposition to hyperhomocysteinemia is modulated by the circulating level of vitamins or cofactors (ie, folate and vitamin B_6 and B_{12}), which in turn reflect different nutritional habits.[10]

NIDDM is characterized by a high incidence of vascular complications,[11] and many factors such as dyslipidemia, endothelial dysfunction, abnormalities in hemostasis, etc., are implicated in the development of vascular damage.[12,13] On the other hand, the mechanisms whereby elevations in Hcy contribute to vascular disease are apparently in common with those described in diabetics.[14]

The present study aimed to assess the possible contribution of the serum Hcy level and MTHFR polymorphism to the variability of intimal-medial thickness (IMT) of the common carotid artery, presumably an early marker of atherosclerotic vascular damage, in a sample of middle-aged Italian NIDDM subjects.

SUBJECTS AND METHODS

Patients

The study population consisted of 130 subjects with NIDDM (60 males and 70 females) recruited among patients attending a diabetes outpatient clinic in Naples, Italy, from November 1996 to December 1997. Inclusion criteria were a diagnosis of NIDDM, defined as fasting overnight serum glucose greater than 7.8 mmol/L on at least 2 different occasions, or a history of hypoglycemic medication. NIDDM patients with a history of coronary disease and/or cerebrovascular accidents, liver or kidney disease (including diabetic nephropathy), diabetic retinopathy, neoplastic pathology, and treatment with calcium-channel blockers for greater than 3 months were excluded from the study. All patients provided informed consent. For each patient, a full clinical examination (including a 12-lead electrocardiogram) was performed. Information was collected about ongoing therapy, duration and/or

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familial history of diabetes, and life-style (alcohol consumption, cigarette smoking, physical activity, etc.).

The patients were middle-aged (53 ± 11 years) and their mean duration of diabetes was 11.0 ± 7.7 years. Height (to the nearest 0.5 cm) and weight (to the nearest 0.5 kg) were recorded. The body mass index (BMI) was calculated as weight divided by height squared. Blood pressure was measured on the right arm after the participant rested in a supine position for at least 5 minutes with a zero-random sphygmomanometer.

Carotid Ultrasonography and IMT Measurement

Echo-Doppler evaluation was performed with an AI 5200 instrument (Acoustic Imaging, Phoenix, AZ) using a 7.5- to 10-MHz frequency linear probe. Plaque and stenosis evaluation was performed as previously described[15] on the common, internal, and external carotid arteries bilaterally. Diagnostic criteria for carotid artery disease based on the integration of spectral analysis and B-mode imaging were as follows: plaque defined as normal Doppler frequency (<4 kHz) and localized lesion with thickness greater than 2.0 mm; stenosis 1% to 49% defined as systolic frequency peak less than 4.0 kHz and spectral broadening; stenosis 50% to 99% defined as systolic frequency peak greater than 4.0 kHz and spectral broadening; and occlusion defined as no Doppler signal. Subjects were classified as pathologic (CA^+) when a plaque and/or stenosis were present in at least 1 of the examined vessels, and as normal (CA^-) when no plaque or stenosis were detected. Echo-Doppler scanning has a sensitivity greater than 90% and specificity greater than 80% for detecting hemodynamically significant carotid stenosis, using angiography as a reference standard.[16] Images for IMT measurement were obtained from video recordings of the ultrasonographic scans, and analyzed as previously reported.[17] Briefly, they were displayed on a computer screen by the use of a scanner and analyzed by the National Institutes of Health software Image 1.31. IMT was measured in the 1-cm segment proximal to dilatation of the carotid bulb, and always in plaque-free segments. The height of the measured carotid segment was never less than 8 mm. For each subject, 3 measurements on both sides were performed on the anterior, lateral, and posterior projection of the far wall. Values for the different projections and for the right and left arteries were then averaged. If a plaque was present in 1 of the projections, that value was excluded from the analysis and IMT was averaged on the remaining 5 values. The sonographer, who also performed the IMT measurements, was the same throughout the study and blind with regard to biochemical measurements and genotype classification. To test the reproducibility of the IMT measurement, 20 nondiabetic subjects were studied twice 4 weeks apart. The correlation coefficient between the 2 measurements was .93.

MTHFR Genotyping

DNA was extracted from whole frozen EDTA blood by a published rapid method[18] and stored at −20°C until analysis. Polymerase chain reaction (PCR) amplification of DNA samples for characterization of the MTHFR $C^{677} \rightarrow T$ polymorphism was performed using GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) following the method described by Frosst et al.[6] Restriction enzyme analysis with the enzyme *Hinf*1 (Boehringer, Mannheim, Germany) was performed according to the manufacturer's recommendation.

Serum Hcy and Other Biochemical Determinations

Blood samples obtained after at least a 12-hour fast were immediately placed on ice and then centrifuged. Serum aliquots for Hcy measurement were stored at −70°C until analysis. Total Hcy was determined using a fully automated high-performance liquid chromatographic method based on single-column (reversed-phase) separation and fluorescence detection. Serum samples were pretreated with sodium borohydride as a reducing agent and derivatized with monobromobimane.[19] Other serum aliquots were used for clinical chemistry measurements, which also included glucose, total and high-density lipoprotein (HDL) cholesterol, triglycerides, uric acid, and creatinine. Serum folate and vitamin B_{12} levels were measured using an automated chemiluminescence system (CIBA Corning, Medfield, MA).

Statistical Analysis

Hardy-Weinberg equilibrium in the distribution of MTHFR genotypes was assessed by χ^2 analysis. All variables showing a non-Gaussian distribution (including serum Hcy) were logarithmically transformed. ANOVA and the Scheffe F test were used to compare mean values among subjects according to MTHFR genotype. Comparisons between genotype subgroups were performed by unpaired Student's *t* test or χ^2 test where appropriate. Study variables were correlated with common carotid IMT values using Pearson's *r* coefficient. Multiple regression analysis was performed using IMT as a dependent variable and different risk factors as independent variables. In this analysis, MTHFR genotypes were designated as follows: Ala/Ala = 0, Ala/Val = 1, and Val/Val = 2. The analyses were performed using the statistical package Statview 4.0 (Abacus, Berkeley, CA).

RESULTS

The frequency of the MTHFR $C^{677} \rightarrow T$ allele was 0.565 for Ala and 0.435 for Val. The genotype frequency distribution was Ala/Ala 0.361, Ala/Val 0.408, and Val/Val 0.231, and was in Hardy-Weinberg equilibrium. Table 1 shows the characteristics of the sample according to MTHFR genotype. There were no significant differences between genotypes in relation to age, sex, duration of disease, smoking, BMI, blood pressure, total

Table 1. Main Clinical and Biochemical Characteristics of Patients Grouped by MTHFR Genotype

Characteristic	MTHFR Genotype			*P*
	Ala/Ala	Ala/Val	Val/Val	
No. of subjects	47	53	30	
Sex ratio (male/female)	22/25	25/28	13/17	.93*
Age (yr)	52.3 ± 11.9	54.2 ± 9.5	53.4 ± 10.7	.67
Diabetes duration (yr)	9.4 ± 7.4	12.4 ± 7.7	12.4 ± 8.6	.11
BMI (kg/m²)	29.0 ± 5.8	28.5 ± 6.1	27.3 ± 4.7	.43
Total cholesterol (mmol/L)	5.00 ± 1.30	5.17 ± 1.02	5.21 ± 1.13	.67
HDL cholesterol (mmol/L)	1.20 ± 0.46	1.25 ± 0.32	1.27 ± 0.61	.77
Triglycerides (mmol/L)	1.60 ± 0.96	1.79 ± 1.12	1.67 ± 0.91	.80
Glucose (mmol/L)	10.74 ± 4.21	10.72 ± 2.9	9.06 ± 4.0	.09
HbA_{1c} (%)	7.8 ± 1.6	8.2 ± 1.1	8.1 ± 1.0	.28
Creatinine (mmol/L)	87.5 ± 10.6	84.0 ± 12.4	87.5 ± 12.4	.25
Uric acid (mmol/L)	255.8 ± 59.5	238.7 ± 53.6	260 ± 65.5	.19
Systolic blood pressure (mm Hg)	138.8 ± 20.7	131.1 ± 17.9	133.4 ± 20.0	.13
Diastolic blood pressure (mm Hg)	82.1 ± 99.4	79.2 ± 10.7	77.5 ± 8.7	.11
Smoker (yes/no)	19/28	21/32	12/18	.99*

NOTE. Data are the mean ± SD.
*χ^2 test.

serum cholesterol, triglycerides, glucose, or hemoglobin A_{1c} (HbA_{1c}).

Serum Hcy levels (mean ± SD) in NIDDM subjects were 7.8 ± 2.1 μmol/L, with significantly higher levels in men (8.4 ± 2.7 μmol/L) versus women (7.3 ± 2.0 μmol/L, $P = .03$). Serum Hcy values were significantly higher in subjects with the Val/Val genotype compared with Ala/Ala and Ala/Val ($P = .02$; Fig 1). Serum folate and vitamin B_{12} levels were 11.21 ± 4.1 nmol/L and 397 ± 190 pmol/L, respectively. Serum Hcy was inversely correlated with serum folate ($r = .331$, $P = .0001$) and vitamin B_{12} ($r = .275$, $P = .0014$). In subjects with serum folate and vitamin B_{12} levels at or above the sample median (10.6 nmol/L for folate and 374 pmol/L for vitamin B_{12}), we found no difference in Hcy levels according to the MTHFR genotype. Among those with serum folate or vitamin B_{12} less than the median, serum Hcy was significantly higher in subjects with the Val/Val genotype versus either Ala/Ala or heterozygotes ($F = 8.22$, $P = .0004$ for folate; $F = 9.11$, $P = .0002$ for vitamin B_{12}). Interestingly, in univariate analysis, serum Hcy was inversely related to glycemia ($P = .02$).

The mean common carotid IMT was 858.1 ± 220 μm, and no significant difference was found among genotypes (Fig 2). No difference in IMT was observed between men and women. In simple regression analysis, IMT was significantly correlated with the age, BMI, uric acid, total cholesterol, systolic blood pressure, and glycemia (Table 2). No significant association was found between common carotid IMT and serum Hcy, folate, or vitamin B_{12}, diastolic blood pressure, triglycerides, HDL cholesterol, duration of diabetes, or cigarette smoking.

In a multiple regression analysis with IMT as the dependent variable, age, uric acid, glycemia, and the BMI appeared to be the major independent predictors of variability, accounting for about 42% of IMT variability.

Plaques were identified in 68 of 130 patients (52.6%). Subjects with plaques were older and exhibited significantly higher total cholesterol, uric acid, and diastolic blood pressure (Table 3). IMT measured in plaque-free segments and adjusted for possible confounders was greater in CA+ versus CA− ($P < .0001$). No significant association between IMT and serum Hcy (even after adjustment for folate and vitamin B_{12}) or MTHFR genotype was found in the CA+ and CA− subgroups.

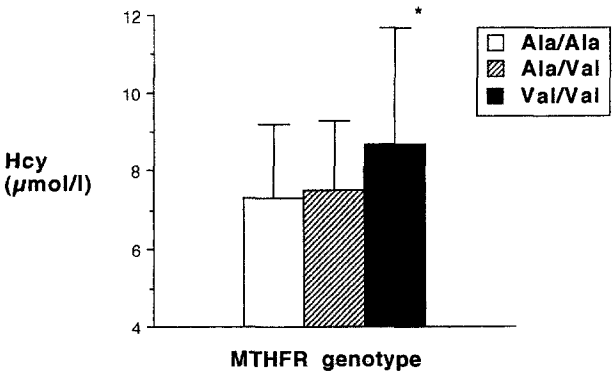

Fig 1. Comparison of serum Hcy in subgroups of the MTHFR genotype. Genotype effect by ANOVA, $P = .02$ (post hoc test, $P < .05$ for Ala/Ala *v* Val/Val and Ala/Val *v* Val/Val).

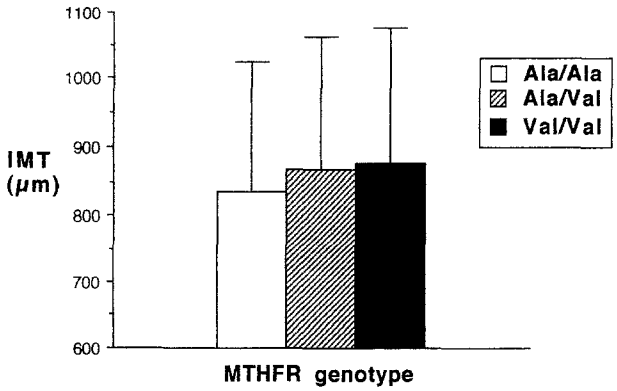

Fig 2. Comparison of carotid IMT in subgroups of the MTHFR genotype.

Also, no association was found when restricting the analysis to patients in the upper quartile of IMT or to those with stenoses of the internal carotid artery (n = 28).

DISCUSSION

Our study aim was to detect any relationship between MTHFR genotype or serum Hcy and subclinical atherosclerosis as assessed by measurement of the common carotid IMT as a surrogate marker of vascular disease in a sample of 130 NIDDM subjects without diabetic nephropathy and retinopathy, in whom a panel of recognized risk factors were also examined. Our results show that in middle-aged Italian NIDDM subjects with a mean duration of disease of 11 years, basal serum Hcy, as well as MTHFR polymorphism, does not predict changes in common carotid IMT either in the whole sample or in subgroups defined by the presence or absence of plaque or stenosis in the carotid district.

Our findings are in contrast to the conclusions of a study in a Japanese population, in which the investigators demonstrated that MTHFR polymorphism is associated with carotid wall thickening and myocardial infarction in NIDDM.[20] This discrepancy may be explained by the different ethnic and nutritional background of the two populations studied. On the other hand, our results support the conclusions of a recent case-control study in European NIDDM patients with or without coronary

Table 2. Pearson's Coefficient for Different Variables and Common Carotid IMT

Variable	*r*	*P*
Age	.590	.00001
BMI	.311	.0003
Uric acid	.253	.004
Systolic blood pressure	.191	.03
Glycemia	.190	.03
Total cholesterol	.180	.04
HDL cholesterol	.147	.09
Triglycerides	.137	.12
Diabetes duration	.038	.67
Diastolic blood pressure	.017	.84
Creatinine	.017	.84
Vitamin B_{12}	.014	.87
Total Hcy	.012	.89
Folate	.011	.90

Table 3. Differences in Variables According to the Presence or Absence of Plaques in the Carotid District

Variable	CA−	CA+	P
No. of subjects	62	68	
Age (yr)	49.3 ± 11.2	56.9 ± 10.0	.0001
Sex ratio (male/female)	25/37	35/33	.27*
Diabetes duration (yr)	10.5 ± 7.5	12.1 ± 8.9	.27
BMI (kg/m^2)	28.3 ± 5.7	28.5 ± 5.7	.84
Systolic blood pressure (mm Hg)	132.5 ± 18.0	136.2 ± 20.2	.27
Diastolic blood pressure (mm Hg)	81.8 ± 9.0	78.0 ± 10.6	.03
Smoker (yes/no)	22/40	30/38	.40*
Total cholesterol (mmol/L)	4.83 ± 0.98	5.37 ± 1.18	.006
HDL cholesterol (mmol/L)	1.25 ± 0.34	1.21 ± 0.28	.34
Triglycerides (mmol/L)	1.70 ± 1.14	1.68 ± 1.06	.91
Glucose (mmol/L)	10.27 ± 3.78	10.39 ± 3.94	.89
Uric acid (mmol/L)	237.9 ± 59.5	261.7 ± 71.4	.04
Hcy (μmol/L)	7.7 ± 2.6	7.9 ± 1.7	.60
MTHFR genotype (n)			
Ala/Ala	23	24	
Ala/Val	27	26	
Val/Val	12	18	.61*

*χ^2 test.

heart disease, in which no significant difference in MTHFR genotype or allele frequency was found between groups.[21]

The role of the MTHFR C677 → T polymorphism as a genetic determinant in the pathogenesis of vascular disease is still a matter of debate.[9,22-24] It shows a strong influence on the basal level of total plasma or serum Hcy dependent upon the actual vitamin levels (folate and vitamin B_{12}). In contrast to previous reports,[22,23] a recent meta-analysis reported that the mutation is not relevant to coronary artery disease in low-risk Western populations despite its association with moderate Hcy elevations.[24] The Val allele has been reported to be associated with diabetic nephropathy and retinopathy in NIDDM[25,26] but not in IDDM.[27] Thus, the lack of an association between MTHFR polymorphism and common carotid IMT in uncomplicated NIDDM is not surprising.

The frequency of the presumed high-risk genotype (Val/Val) in our Italian population of NIDDM subjects is very high (22.9%) in comparison to other reports.[20,28] However, also in nondiabetic individuals, the frequency of Val/Val in Italy seems higher versus other countries.[28] As expected, Val/Val subjects exhibited significantly higher levels of serum Hcy. The observed distribution of the MTHFR genotype frequency was in Hardy-Weinberg equilibrium, ruling out a bias due to sampling. Hcy was inversely correlated with folate and vitamin B_{12} levels and, as in nondiabetics, the increase in Hcy in Val/Val subjects was not present when restricting the analysis to subjects with serum vitamin levels at or above the median value.

High total Hcy may be a strong risk factor for cardiovascular disease in NIDDM.[29] The mean basal level of serum Hcy in our sample of diabetics (7.8 μmol/L) is lower than that described by our group and others in nondiabetic Italian populations (between 12 and 14 μmol/L).[30,31] Furthermore, we found a significant inverse correlation between blood glucose and serum Hcy levels. In a recent report on plasma Hcy and all-cause mortality in diabetes in an Israeli population-based cohort, age-adjusted mean nonfasting total Hcy concentrations were lower for individuals reporting diabetes versus the diabetes-free population, and serum glucose was inversely associated with total Hcy within diabetic patients.[32]

An increase in the urinary excretion of Hcy as a consequence of the hyperosmolar effect of a glucose load at the tubular level has been postulated to explain the observed inverse association.[33] Alternatively, this might reflect a possible interference of hyperglycemia and/or hyperinsulinemia with the metabolic pathways of methionine and/or Hcy.[34] Others found that homocysteinemia (fasting or after a methionine load) was significantly higher in diabetic patients with macroangiopathy than in nondiabetic controls,[34,35] and basal Hcy concentrations measured in plasma were higher in diabetic patients with coexistent nephropathy and elevated plasma creatinine versus healthy controls.[36] Also, in diabetics, Hcy levels are related to the extent of renal dysfunction, but this is unlikely relevant in the context of our study, in which nephropathy was among the exclusion criteria.

Ultrasonographic measurement of the carotid arterial IMT is a noninvasive reliable method to study atherosclerotic lesions at an early stage.[15] It provides a sensitive means for detection of a relationship between presumed risk factors and macroangiography. Atherosclerotic plaques are usually found in the carotid bulb and internal carotid, raising the problem of the relevance of a measurement performed in the common carotid for lesions localized in other districts. IMT measurement in the carotid bulb and internal carotid is presently scarcely reliable. However, cross-sectional studies have demonstrated a strong association between common carotid IMT and cardiovascular risk factors, the prevalence of cardiovascular disease, and the involvement of other arterial beds with atherosclerosis.[37-39] More recently, an association between common carotid IMT and the incidence of myocardial infarction and stroke has also been reported.[40]

Our results do not support an independent role of basal Hcy as a discriminant vascular risk in diabetes. Among the variables investigated, only age, uric acid, glycemia, and the BMI were significantly and independently correlated with IMT and could account for about 42% of its variability. The measurement of serum insulin was not included in the protocol of our study on NIDDM subjects. However, the relationship of carotid IMT to the BMI suggests that insulin resistance may have a role, and hyperinsulinemia is known to influence IMT.[41] This aspect should be better evaluated in future studies.

Evidence of increased carotid IMT in nondiabetic subjects with moderate hyperhomocysteinemia has been reported, indicating that Hcy is an independent risk factor for carotid arterial wall thickening among subjects without clinical atherosclerotic disease.[42-44] On the other hand, increased carotid IMT in subjects with NIDDM compared with age- and sex-matched controls independent of other established risk factors for atherosclerosis has been reported by different groups.[16,45]

Both NIDDM and hyperhomocysteinemia may contribute to vascular disease through various mechanisms. Most likely, the vascular changes induced by NIDDM per se are strong enough to conceal the effects of other risk factors.[46] As also shown in other studies, the range of distribution of Hcy levels in NIDDM without nephropathy is shifted toward lower values, and this might attenuate its effects on changes in the vascular wall. However, despite lower mean Hcy levels in diabetes, a substan-

tially increased risk of death related to total Hcy has been reported in diabetic patients, suggesting that in these high-risk subjects, Hcy-lowering treatments should be considered.[32]

In conclusion, we found that in uncomplicated NIDDM, fasting serum Hcy levels tend to be lower versus other nondiabetic subjects and are not predictive of early vascular changes as evaluated by ultrasonographic assessment of IMT. It is likely that in the progression of NIDDM toward atherosclerosis-related microangiopathy or macroangiopathy complications, Hcy levels may acquire a stronger pathogenetic role.

REFERENCES

1. Stamler J, Vaccaro O, Neaton JD, et al: Diabetes, other risk factors and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care 16:434-444, 1993
2. Clarke R, Daly L, Robinson K, et al: Hyperhomocysteinemia: An independent risk factor for vascular disease. N Engl J Med 324:1149-1155, 1991
3. Mudd SH, Levy HL, Skovby F: Disorders of transsulfuration, in Scriver CR, Beaudet AL, Sly WS, Valle D (eds): The Metabolic Basis of Inherited Disease (ed 6). New York, NY, McGraw-Hill, 1995, pp 1279-1326
4. Boushey CJ, Beresford SAA, Omenn GS, et al: A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: Probable benefits of increasing folic acid intakes. JAMA 274:1049-1057, 1995
5. Goyette P, Sumner JS, Milos R, et al: Human methylenetetrahydrofolate reductase: Isolation of cDNA, mapping and mutation identification. Nat Genet 7:195-200, 1994
6. Frosst P, Blom HJ, Milos R, et al: A candidate genetic risk factor for vascular disease: A common mutation in methylenetetrahydrofolate reductase. Nat Genet 10:111-113, 1995
7. Kang SS, Wong PWK, Susmano A, et al: Thermolabile methylenetetrahydrofolate reductase: An inherited risk factor for coronary artery disease. Am J Hum Genet 48:536-545, 1991
8. Welch GN, Loscalzo J: Homocysteine and atherothrombosis. N Engl J Med 338:1042-1050, 1998
9. Fletcher O, Kessling AM: MTHFR association with atherosclerotic vascular disease? Hum Genet 103:11-21, 1998
10. Motulsky AG: Nutritional ecogenetics: Homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. Am J Hum Genet 58:17-20, 1996
11. Colwell JA, Jokl RL: Vascular thrombosis in diabetes, in Porte D, Sherwin R, Rifkin H (eds): Diabetes Mellitus: Theory and Practice (ed 5). Norwalk, CT, Appleton & Lange, 1996, pp 207-216
12. King GL, Brownlee M: The cellular and molecular mechanism of diabetic complications. Endocrinol Metab Clin North Am 25:255-270, 1996
13. Giugliano D, Ceriello A, Paolisso G: Oxidative stress and diabetic vascular complications. Diabetes Care 19:257-267, 1996
14. McCully KS: Homocysteine and vascular disease. Nat Med 2:386-389, 1996
15. Gnasso A, Irace C, Mattioli PL, et al: Carotid intima-media thickness and coronary heart disease risk factors. Atherosclerosis 119:7-15, 1996
16. Pignoli P, Tremoli E, Poli A, et al: Intima plus medial thickness of the arterial wall: A direct measurement with ultrasound imaging. Circulation 74:1399-1406, 1986
17. Pujia A, Gnasso A, Irace C, et al: Common carotid arterial wall thickness in NIDDM subjects. Diabetes Care 17:1330-1336, 1994
18. Higuchi R: Simple and rapid preparation of samples for PCR, in Erlich HA (ed): PCR Technology. New York, NY, Stockton, 1989, pp 31-38
19. Pastore A, Massoud R, Motti C, et al: Fully automated assay for total homocysteine, cysteine, cysteinylglycine, glutathione, cysteamine, and 2-mercaptopropionylglycine in plasma and urine. Clin Chem 44:825-832, 1998
20. Arai K, Yamasaki Y, Kajimoto Y, et al: Association of methylenetetrahydrofolate reductase gene polymorphism with carotid arterial wall thickening and myocardial infarction in NIDDM. Diabetes 46:2102-2104, 1997
21. Brulhart MC, Dussoix P, Ruiz J, et al: The (Ala/Val) mutation of methylenetetrahydrofolate reductase as a genetic risk factor for vascular disease in non–insulin dependent diabetic patients. Am J Hum Genet 60:228-229, 1997
22. Kluijtmans LAJ, van den Heuvel LPWJ, Boers GHJ, et al: Molecular genetic analysis in mild hyperhomocysteinemia: A common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. Am J Hum Genet 58:35-41, 1996
23. Gallagher PM, Meleady R, Shields DC, et al: Homocysteine and risk of premature coronary heart disease. Evidence for a common gene mutation. Circulation 94:2154-2158, 1996
24. Brattstrom L, Wilcken DEL, Ohrvik J, et al: Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease. The results of a metaanalysis. Circulation 98:2520-2526, 1998
25. Neugebauer S, Baba T, Watanabe T: Methylenetetrahydrofolate reductase gene polymorphism as a risk factor for diabetic nephropathy in NIDDM patients. Lancet 352:454, 1998
26. Neugebauer S, Baba T, Kurokawa K, et al: Defective homocysteine metabolism as a risk factor for diabetic retinopathy. Lancet 349:473-474, 1997
27. Smyth JS, Savage DA, Maxwell AP: MTHFR gene polymorphism and diabetic nephropathy in type 1 diabetes. Lancet 353:1156-1157, 1999
28. Wilcken DEL, Wang XL, Wilcken B: Methylenetetrahydrofolate reductase (MTHFR) mutation, homocyst(e)ine and coronary artery disease. Circulation 96:2738-2740, 1997
29. Hoogeveen EK, Kostense PJ, Beks PJ, et al: Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease, especially in non–insulin-dependent diabetes mellitus: A population-based study. Arterioscler Thromb Vasc Biol 18:133-138, 1998
30. Motti C, Gnasso A, Bernardini S, et al: Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine and other risk factors for vascular disease. Atherosclerosis 139:377-383, 1998
31. Girelli D, Friso S, Trabetti E, et al: Methylenetetrahydrofolate reductase C677T mutation, plasma homocysteine, and folate in subjects from northern Italy with or without angiographically documented severe coronary atherosclerotic disease: Evidence for an important genetic-environmental interaction. Blood 91:4158-4163, 1998
32. Kark JD, Selhub J, Bostom A, et al: Plasma homocysteine and all-cause mortality in diabetes. Lancet 353:1936-1937, 1999
33. Wollesen F, Brattstrom L, Refsum H, et al: Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int 55:1028-1035, 1999
34. Munshi MN, Stone A, Fink L, et al: Hyperhomocysteinemia following a methionine load in patients with non–insulin-dependent diabetes mellitus and macrovascular disease. Metabolism 45:133-135, 1996
35. Araki A, Sako Y, Ito H: Plasma homocysteine concentrations in Japanese patients with non–insulin dependent diabetes mellitus: Effect of parenteral methylcobalamin treatment. Atherosclerosis 103:149-157, 1993
36. Hultberg B, Agardh E, Andersson A, et al: Increased levels of plasma homocysteine associated with nephropathy, but not severe

retinopathy in type I diabetes mellitus. Scand J Clin Lab Invest 51:277-282, 1991

37. Poli A, Tremoli E, Colombo A, et al: Ultrasonographic measurement of the common carotid artery wall thickness in hypercholesterolemic patients; a new model for the quantitation and follow-up of preclinical atherosclerosis in living human subjects. Atherosclerosis 70:253-261, 1988

38. O'Leary DH, Polak JF, Kronmal RA, et al: Distribution and correlates of sonographically detected carotid artery disease in the Cardiovascular Health Study. Stroke 23:1752-1760, 1992

39. Allan PL, Mowbray PI, Lee AJ, et al: Relationship between carotid intima-media thickness and symptomatic peripheral arterial disease: The Edinburgh Arterial Study. Stroke 28:348-353, 1997

40. O'Leary DH, Polak JF, Kronmal RA, et al: Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. N Engl J Med 340:14-22, 1999

41. Kawamori R: Asymptomatic hyperglycaemia and early atherosclerotic changes. Diabetes Res Clin Pract 40:35-42, 1998 (suppl)

42. Malinow RM, Nieto FJ, Szklo M, et al: Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. The Atherosclerosis Risk in Communities Study. Circulation 87:1107-1113, 1993

43. Selhub J, Jacques PF, Bostom AG, et al: Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. N Engl J Med 332:286-291, 1995

44. McQuillan BM, Beilby JP, Nidorf M, et al: Hyperhomocysteinemia but not the C677T mutation of methylenetetrahydrofolate reductase is an independent risk determinant of carotid wall thickening. The Perth Carotid Ultrasound Disease Assessment Study. Circulation 99: 2383-2388, 1999

45. Kawamori R, Yamasaki Y, Matsushima H, et al: Prevalence of carotid atherosclerosis in diabetic patients. Diabetes Care 15:1290-1294, 1994

46. Vauhkonen I, Niskanen L, Ryynanen M, et al: Divergent association of apolipoprotein E polymorphism with vascular disease in patients with NIDDM and control subjects. Diabet Med 14:748-756, 1997