Factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations are not associated with chronic limb ischemia: The Linz Peripheral Arterial Disease (LIPAD) Study

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Objective: **Factor V G1691A (Leiden), prothrombin G20210A, and methylenetetrahydrofolate reductase (MTHFR) C677T mutations are considered risk factors for venous thromboembolism. It remains to be characterized whether the presence of these relatively common mutations poses a risk for peripheral arterial disease (PAD). Therefore, we intended to test, by conducting a case-control study, the hypothesis that PAD was associated with an increased prevalence of factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations.**

Methods: **The study comprised 433 patients admitted for inpatient diagnostics and treatment of PAD in patients with chronic limb ischemia. Patients with acute ischemia or malignancy were excluded. A total of 433 control subjects matched to the patients with PAD in a 1:1 design by sex, age (±2 years), and diabetes mellitus status were recruited. Factor V G1691A, prothrombin G20210A, and MTHFR C677T genotypes were assessed by polymerase chain reaction.**

Results: **For the factor V G1691A polymorphism, the genotype frequencies in PAD patients were 92.8% GG (normal homozygotes = wild type) and 7.2% GA (mutant heterozygotes), and in control subjects they were 94.0% GG and 6.0% GA (χ^2 test; $P = .493$). The distribution of the prothrombin G20210A genotypes was 96.3% GG (normal homozygotes = wild type) and 3.7% GA (mutant heterozygotes) in PAD patients and was 97.2% GG and 2.8% GA in control subjects (χ^2 test; $P = .442$). Genotype frequencies for the MTHFR C677T polymorphism were 47.8% CC (normal homozygotes = wild type), 43.4% CT (mutant heterozygotes), and 8.8% TT (mutant homozygotes) in PAD patients, compared with 47.1% CC, 44.1% CT, and 8.8% TT in control subjects (χ^2 test; $P = .977$). Accordingly, as determined by logistic regression analysis, no significant odds ratios for heterozygous or homozygous genotypes of the three polymorphisms could be observed.**

Conclusions: **PAD was not associated with an increased prevalence of factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations in the population studied. Thus, there is no indication that of one of these mutations may be a risk factor for chronic limb ischemia. However, the role of these mutations in acute limb ischemia remains to be clarified. (J Vasc Surg 2005;41:808-15.)**

Atherosclerotic peripheral arterial disease (PAD) is a progressive condition characterized by arterial stenoses and occlusions in the peripheral arterial bed of the lower limbs; it can be symptomatic or asymptomatic. Symptomatic PAD ranges in severity from intermittent claudication to critical limb ischemia.[1] In the population older than 55 years, PAD is an indicator of systemic atherosclerotic disease. Regardless of whether symptoms are evident, patients with PAD have an increased risk of subsequent myocardial infarction and stroke and are six times more likely to die within 10 years than are patients without PAD.[2] Diabetes mellitus and smoking are the strongest risk factors for PAD.[1,2] Other well-known risk factors are advanced age, male sex, arterial hypertension, dyslipidemia, prior myocardial infarction, history of stroke, and history of transient ischemic attack.[2,3] Emerging risk factors for PAD include increased levels of high-sensitivity C-reactive protein (CRP), total homocysteine (tHcy), and 8-iso-prostaglandin $F_{2\alpha}$ as an indicator of oxidative stress.[3-5] Thrombophilic conditions, such as factor V G1691A (Leiden), prothrombin (factor II) G20210A, and methylenetetrahydrofolate reductase (MTHFR) C677T mutations, have been investigated, but current evidence does not unequivocally support the hypothesis that one of these mutations may be a risk factor for PAD.[6-15]

Activated protein C resistance has been reported to be the most common cause of familial thrombophilia.[16] In most of these cases, the activated protein C resistance is the

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result of a single mutation in the factor V gene, which is known as factor V Leiden. This defect, which is inherited autosomal dominant, yields factor V much more resistant to the proteolytic degradation by activated protein C.[16] This phenomenon results in a hypercoagulable state and is seen in approximately 5% of white populations, in up to 50% in selected thrombophilic families, and in 10% of unselected venous thrombosis patients.[16] The risk for venous thrombosis is also increased in patients who have a mutation in the prothrombin gene (G20210A).[16] This mutation results in increased levels of prothrombin due to increased prothrombin synthesis and is associated with a threefold increase in the risk for venous thrombosis. Approximately 5% to 10% of patients with venous thrombosis and approximately 15% of patients being investigated for thrombophilia will harbor this disorder; up to 4% of individuals in the general population will test positive for this defect.[16] The most common hereditary abnormality associated with hyperhomocysteinemia is a variant that makes the MTHFR gene product thermolabile, thus resulting in functional MTHFR deficiency. The MTHFR C677T mutation is common; in some populations, up to 40% to 50% of unselected patients are heterozygous for this mutation, and approximately 10% to 15% are homozygous.[16] This mutation is not associated with hyperhomocysteinemia in heterozygous patients and is associated with increased tHcy concentrations only in homozygous patients in the presence of coexisting deficiencies of folate, vitamin B_{12}, or vitamin B_6.[16] Certain studies have suggested that increased homocysteine levels roughly double the incidence of venous thrombosis.[17] Overall, however, there seems to be a weak positive association.[18]

Factor V G1691A, prothrombin G20210A, and probably also MTHFR C677T mutations are considered risk factors for venous thromboembolism. However, there is controversy about the role of these mutations in arterial thrombotic disease and atherosclerosis.[19] Because of the small number of published studies, it remains even less well characterized whether the presence of these relatively common mutations poses a risk for PAD. Four studies describing a possible role of the factor V G1691A and/or prothrombin G20210A polymorphisms as risk factors for PAD revealed conflicting results.[6-9] Six studies investigating the association of PAD with hyperhomocysteinemia and the MTHFR C677T polymorphism also do not unequivocally support their hypothesized role as independent risk factors.[10-15] In addition, most of the cited studies on PAD were investigating small populations and had several limitations related to the method of matching cases and controls or related to the spectrum or delineation of cases and controls.[19] Therefore, we intended to address the association of PAD with factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations by conducting a large case-control study with a well-defined study population and with the controls matched to patients with PAD in a 1:1 design by age, sex, and diabetes mellitus status. Using this study design, we tested the hypothesis that PAD was related to an increased prevalence of one of the three mutations.

METHODS

Study population. The Linz Peripheral Arterial Disease (LIPAD) study was performed from April 2000 to April 2002 at the St. John of God Hospital, Department of Surgery, Linz, Austria. Of the patients admitted for inpatient evaluation of suspected or definite PAD during the given time interval, all patients with chronic atherosclerotic occlusive disease of the lower extremities associated with typical symptoms—such as claudication or leg pain on exertion, rest pain, or minor or major tissue loss—were included into this study on the basis of the final clinical diagnosis established by the attending vascular surgeons. The diagnosis was verified by interview, physical examination, noninvasive techniques, and angiography, as detailed below. All cases with acute ischemia (ie, peripheral arterial thrombosis of a native artery or popliteal artery aneurysm or acutely thrombosed peripheral bypass grafts) were excluded. Further exclusion criteria were PAD caused by nonatherosclerotic causes (cardioembolic disease, thromboangiitis obliterans, vasculitis, or congenital or metabolic vascular disease) and the history or presence of any malignancy. The study population comprised 487 consecutive subjects with symptomatic chronic limb ischemia. This series of white patients included 318 nondiabetic and 169 diabetic subjects. Of the 318 nondiabetic patients with PAD, 216 were younger than 75 years of age, and 102 were 75 years of age or older; of the 169 diabetic patients with PAD, 115 were younger than 75 years of age, and 54 were 75 years of age or older. The LIPAD study was designed to evaluate possible phenotypic and genotypic risk factors for PAD by using a case-control design and to determine the predictive value of risk factors on the long-term outcome of patients with symptomatic PAD. The study protocol was approved by the local ethics committee in accordance with the Declaration of Helsinki, and all study participants gave informed consent.

All study subjects (ie, cases and controls) underwent evaluation for the presence of risk factors for atherosclerosis and comorbid conditions, as recommended.[20] Coronary artery disease (CAD) was defined as remote myocardial infarction by history, occult myocardial infarction by electrocardiography, previous coronary bypass surgery or percutaneous transluminal coronary angioplasty, and stable or unstable angina and acute coronary syndrome (cardiac troponin positive or negative). Cerebrovascular disease (CVD) was defined as transient or temporary stroke, completed stroke with permanent neurologic deficit, or acute stroke. Arterial hypertension was defined as the use of any antihypertensive medication, systolic blood pressure of 145 mm Hg or higher, or diastolic blood pressure of 90 mm Hg or higher. Diabetes mellitus was defined as the use of any glucose-lowering medication or as fasting blood glucose levels of 126 mg/dL or higher. Smoking was classified according to recommended standards.[20] The subjects' height and weight were routinely measured when they were

hospitalized, and the body mass index was obtained from the ratio of weight to height squared.

In patients with symptomatic PAD and control subjects, Doppler segmental blood pressure of the lower limbs, including continuous-wave spectral analysis and resting ankle-brachial index (ABI) measurements, as well as color duplex ultrasound scanning of the carotid bifurcation and the internal carotid artery, were performed as previously described.[21,22] In addition to these measurements, intra-arterial aortofemoral angiography was performed in all patients to confirm the presence of PAD and to determine the location and extent of wall changes.

Control subjects matched to the patients with PAD in a 1:1 design by sex, age (±2 years), and diabetes mellitus status were recruited for all nondiabetic patients (n = 319) and for diabetic patients younger than 75 years of age (n = 115). All control subjects were patients in our hospital and fulfilled the following criteria: no clinical indication of PAD by history and physical examination; systolic brachial blood pressure equal to or less than the blood pressure in of each of the right and left anterior tibial and posterior tibial arteries (ie, ABI ≥1.0); no pathologic pattern of pulse waves in lower limbs by continuous-wave spectral analysis; no CAD; no CVD; no previous vascular surgery or stenting of the internal carotid arteries; no stenosis of the internal carotid artery greater than 50% by color duplex ultrasound scans; no history of venous thromboembolism; and no history or presence of any malignancy. All control subjects were generally in good health and were admitted for treatment of minor health problems, such as cataract surgery, vertebragenic pain, or nonvascular surgery (eg, herniotomy or varicose vein extirpation).

Biochemical analyses. Blood was collected at venipuncture in Vacuette polyethylene terephthalate glycol clot activator tubes (Greiner Bio-One, Kremsmuenster, Austria) after the patient had fasted overnight. Creatinine, fasting glucose, glycohemoglobin A_{1c}, total cholesterol, and triglycerides were analyzed with standard assays on a COBAS Integra analyzer (Roche Diagnostics, Mannheim, Germany). For determination of high-density lipoprotein cholesterol and low-density lipoprotein cholesterol, quantitative electrophoresis with enzymatic staining (Helena BioSciences, Europe, Sunderland, UK) was used. Total Hcy, folate, and vitamin B_{12} assays were performed on an AxSYM analyzer (Abbott Diagnostics, Abbott Park, Ill). The concentration of CRP was measured by a high-sensitivity assay (N High Sensitivity CRP) on a BN ProSpec analyzer (Dade Behring, Marburg, Germany) with polystyrene particles coated with monoclonal mouse antibodies to CRP.

Genotyping. Genomic DNA was isolated from buffy coat prepared from ethylenediaminetetraacetic acid whole-blood samples by using the commercially available Purgene DNA isolation kit (Gentra Systems, Minneapolis, Minn) and was frozen at −80°C until further assessment. Real-time multiplex fluorescence polymerase chain reaction (PCR) was performed for simultaneous detection of factor V G1691A and prothrombin G20210A polymorphisms by using primer sequences described previously.[23,24] The two probes targeting several gene-specific amplicons were labeled with two different reporter dyes to achieve multiplex genotyping in a single glass capillary. For the MTHFR C677T polymorphism, a separate PCR was performed that used slightly modified primers and probes of validated methods.[25,26] PCR reactions were performed according to standardized procedures on a LightCycler (Roche Diagnostics). In each run, an H_2O control and known homozygotes and heterozygotes for the tested polymorphisms were included to check for unspecific reactions and to confirm correct genotyping, respectively.

Statistical methods. Statistical analysis was performed with the SPSS version 10.0 software (SPSS Inc, Chicago, Ill). Dichotomous variables are given as prevalence in number, and continuous data are expressed as median (25th-75th percentiles). Univariate comparisons of risk factors and other dichotomous variables between the two study groups were calculated with Fisher exact tests, and continuous variables were evaluated with the nonparametric Mann-Whitney *U* test (respective *P* values were not adjusted for multiple comparisons and are therefore only descriptive). Hardy-Weinberg equilibrium for all three polymorphisms was tested for by the χ^2 test with 1 *df* separately in cases and controls. The χ^2 test was also used for comparing the distributions of the factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations in cases and controls. To determine univariate and multivariate odds ratios for PAD, logistic regression analysis was performed; polymorphisms were coded with a dummy variable of 1 for having the homozygous or heterozygous condition and 0 for being wild type. Probabilities are 2 tailed, and $P < .05$ is regarded as statistically significant.

RESULTS

Patients with PAD included in this study (n = 433) were admitted because of mild to severe claudication or leg pain on exertion (n = 359), ischemic rest pain (n = 15), and minor or major tissue loss (n = 59). Of the 433 patients with PAD, 131 (30%) had concomitant CAD, and 80 (18%) had concomitant CVD. Furthermore, 101 patients with PAD had 50% or greater internal carotid stenosis. Another 18 patients with PAD were classified as having a stenosis 50% or greater as well, because they had undergone previous carotid surgery to treat stenosis. At enrollment, 116 patients with PAD had undergone remote percutaneous transluminal angioplasty with or without stenting, 86 PAD patients had undergone vascular surgery, and 22 patients with PAD had undergone minor or major amputations. Of the 318 nondiabetic patients with PAD, 273 (86%) had an ABI of 0.90 or less, and 45 (14%) had an ABI greater than 0.90; of the 115 diabetic patients with PAD, 94 (82%) had an ABI of 0.90 or less, and 21 (18%) had an ABI greater than 0.90. Per definition, none of the 433 control subjects matched to the patients with PAD for sex, age (±2 years), and diabetes mellitus had either CAD and CVD or an internal carotid stenosis 50% or greater. However, many control subjects (n = 365) had carotid plaques

Table I. Clinical and biochemical data of patients with PAD and control subjects

Variable	*PAD group (n = 433)*	*Control group (n = 433)*	P *value**
Male/female (n)	306/127	306/127	0.999†
Age (y)	68 (59-75)	68 (60-75)	.692†
BMI (kg/m^2)	26 (24-29)	26 (24-29)	.211
Current smoking (n)‡	193	51	<.001
Arterial hypertension (n)	251	178	<.001
Diabetes mellitus (n)	115	115	0.999†
CAD (n)	131	0	NA
CVD (n)	80	0	NA
Carotid stenosis ≥50% (n)	119	0	NA
PAD relevant data			
Previous PTA/stenting (n)	116	0	NA
Previous vascular surgery (n)	86	0	NA
Previous amputation (n)	22	0	NA
ABI (mm Hg/mm Hg)	0.63 (0.47-0.79)	1.18 (1.09-1.29)	NA
Biochemical markers			
Creatinine (mg/dL)	1.0 (0.9-1.1)	0.9 (0.8-1.0)	<.001
Total cholesterol (mg/dL)	229 (195-258)	215 (182-243)	<.001
LDL cholesterol (mg/dL)	150 (121-178)	136 (108-158)	<.001
HDL cholesterol (mg/dL)	50 (39-61)	52 (42-62)	.057
Triglycerides (mg/dL)	132 (99-201)	117 (88-160)	<.001
Fasting glucose (mg/dL)§	96 (88-104)	93 (86-102)	.003
HbA_{1c} (%)§	5.8 (5.5-6.2)	5.7 (5.4-5.9)	<.001
hs-CRP (mg/L)	4.1 (1.8-9.2)	2.1 (0.9-6.0)	<.001
tHcy (μmol/L)	16.0 (13.0-21.1)	14.3 (11.9-18.0)	<.001
Folate (ng/mL)	7.0 (5.1-9.1)	7.2 (5.4-9.6)	.125
Vitamin B_{12} (pg/mL)	341 (245-474)	349 (254-495)	.556

ABI, Resting ankle-brachial index; *BMI,* body mass index; *CAD,* coronary artery disease; *CVD,* cerebrovascular disease; *HbA_{1c},* glycohemoglobin A_{1c}; *HDL,* high-density lipoprotein; *hs-CRP,* high-sensitivity C-reactive protein; *LDL,* low-density lipoprotein; *NA,* not applicable; *PAD,* peripheral arterial disease; *PTA,* percutaneous transluminal angioplasty; *tHcy,* total homocysteine.

Age, BMI, ABI, and biochemical markers are presented as median (25th-75th percentiles).

*Nonparametric Mann-Whitney *U* test or Fisher exact test as appropriate.

†Matched variables.

‡Current smoking was defined as any amount of tobacco use, including abstinence less than 1 year.[19]

§Subjects with diabetes mellitus were excluded.

as a sign of a mild, but not clinically relevant, atherosclerosis. At enrollment, of the patients with PAD, 102 were receiving a lipid-lowering medication, 10 took a folate supplement, and 18 took B vitamins (usually in combination); none of the control subjects took any of these medications. The clinical and biochemical characteristics of all individuals included in the study are described in Table I.

After enrollment into the study and as a consequence of the clinical evaluation, the therapy for the 433 patients with symptomatic PAD was vascular surgery, including open revascularization procedures or bypass graft placement, in 88 patients; endovascular techniques, such as percutaneous transluminal angioplasty with or without stenting, in 164 patients; lumbar sympathectomy in 4 patients; major amputation in 1 patient; minor amputation in 6 patients; vascular surgery plus endovascular techniques in 14 patients; vascular surgery plus minor amputation in 3 patients; vascular surgery plus endovascular techniques plus lumbar sympathectomy in 1 patient; vascular surgery plus lumbar sympathectomy in 1 patient; endovascular techniques plus minor amputation in 2 patients; endovascular techniques plus lumbar sympathectomy in 1 patient; and minor amputation plus lumbar sympathectomy in 1 patient. Also, 147 PAD patients received conservative treatment only (eg, because of no indication for revascularization procedures, missing patient consent to perform revascularization procedures, or impossibility to perform revascularization procedures because of comorbidity).

Genotyping for factor V G1691A, prothrombin G20210A, and MTHFR C677T polymorphisms was performed successfully in 100% of the study subjects. The distributions of all three genotypes of the overall study population (cases, n = 433; controls, n = 433) are depicted in Table II and were in Hardy-Weinberg equilibrium (factor V G1691A in cases: frequency of allele G, 0.964; frequency of allele A, 0.036; $\chi^2 = 0.597$; $P > .200$; factor V G1691A in controls: frequency of allele G, 0.970; frequency of allele A, 0.030; $\chi^2 = 0.415$; $P > .200$; prothrombin G20210A in cases: frequency of allele G, 0.982; frequency of allele A, 0.018; $\chi^2 = 0.153$; $P > .200$; prothrombin G20210A in controls: frequency of allele G, 0.986; frequency of allele A, 0.014; $\chi^2 = 0.085$; $P > .200$; MTHFR C677T in cases: frequency of allele C, 0.695; frequency of allele T, 0.305; $\chi^2 = 0.258$; $P > .200$; MTHFR C677T in controls: frequency of allele C, 0.693; frequency of allele T, 0.307; $\chi^2 = 0.327$; $P > .200$). As shown in Tables III and IV, we additionally assessed the distribution of the three genotypes in different subgroups

Table II. Overall genotype frequencies in patients with PAD and matched control subjects

Polymorphism	*Genotype*	*All cases n = 433 (100%)*	*All controls n = 433 (100%)*	*χ2 (P-value)*	*Univariate odds ratios (95% CI; p-value)*
Factor V G1691A	GG	402 (92.8%)	407 (94.0%)	0.469 (*P* = .493)	1.000
	GA	31 (7.2%)	26 (6.0%)		1.207 (95%CI,0.704to2.070;p = .494)
	AA	0 (0%)	0 (0%)		—
Prothrombin G20210A	GG	417 (96.3%)	421 (97.2%)	0.591 (*P* = .442)	1.000
	GA	16 (3.7%)	12 (2.8%)		1.346 (95%CI,0.629to2.880;p = .444)
	AA	0 (0%)	0 (0%)		—
MTHFR C677T	CC	207 (47.8%)	204 (47.1%)	0.046 (*P* = .977)	1.000
	CT	188 (43.4%)	191 (44.1%)		0.970 (95%CI,0.734to1.282;p = .831)
	TT	38 (8.8%)	38 (8.8%)		0.986 (95%CI,0.604to1.608;p = .986)

Table III. Genotype frequencies in patients with PAD <75 years of age and matched control subjects

Polymorphism	*Genotype*	*Cases n = 331 (100%)*	*Controls n = 331 (100%)*	*χ2 (P-value)*	*Univariate odds ratios (95% CI; p-value)*
Factor V G1691A	GG	307 (92.7%)	311 (90.4%)	0.390 (*P* = .640)	1.000
	GA	24 (7.3%)	20 (6.0%)		1.126 (95%CI,0.658to2.246;p = .533)
	AA	0 (0%)	0 (0%)		—
Prothrombin G20210A	GG	317 (95.8%)	322 (97.3%)	1.126 (*P* = .396)	1.000
	GA	14 (4.2%)	9 (2.7%)		1.580 (95%CI,0.674to3.703;p = .292)
	AA	0 (0%)	0 (0%)		—
MTHFR C677T	CC	157 (47.4%)	156 (47.1%)	0.206 (*P* = .902)	1.000
	CT	142 (42.9%)	146 (44.1%)		0.966 (95%CI,0.702to1.331;p = .834)
	TT	32 (9.7%)	29 (8.8%)		1.096 (95%CI,0.633to1.899;p = .743)

Table IV. Genotype frequencies in patients with PAD ≥75 years of age and matched control subjects

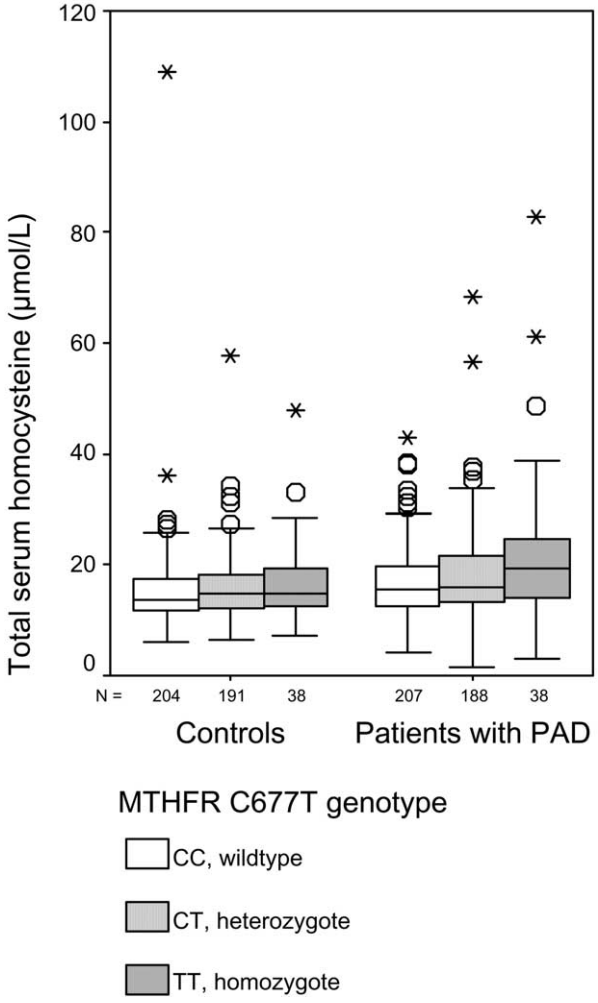
Polymorphism	*Genotype*	*Cases n = 102 (100%)*	*Controls n = 102 (100%)*	*χ2 (P-value)*	*Univariate odds ratios (95% CI; p-value)*
Factor V G1691A	GG	95 (93.1%)	96 (94.1%)	0.082 (*P* = .774)	1.000
	GA	7 (6.9%)	6 (5.9%)		1.179 (95%CI,0.382to3.638;p = .775)
	AA	0 (0%)	0 (0%)		—
Prothrombin G20210A	GG	100 (98.0%)	99 (97.1%)	0.205 (*P* = .651)	1.000
	GA	2 (2.0%)	3 (2.9%)		0.660 (95%CI,0.108to4.036;p = .653)
	AA	0 (0%)	0 (0%)		—
MTHFR C677T	CC	50 (49.0%)	48 (47.1%)	0.652 (*P* = .722)	1.000
	CT	46 (45.1%)	45 (44.1%)		0.981 (95%CI,0.555to1.737;p = .948)
	TT	6 (5.9%)	9 (8.8%)		0.640 (95%CI,0.212to1.935;p = .429)

of the study population (patients <75 years of age and patients ≥75 years of age). Neither in the entire study population nor in the subgroup analyses was there a significant difference between cases and controls with respect to the distribution of factor V G1691A, prothrombin G20210A, and MTHFR C677T genotypes as tested by χ^2 test. Accordingly, no significant odds ratios (assessed by logistic regression analysis) for heterozygous or homozygous genotypes of the three polymorphisms could be observed in the population studied (Tables II-V). Given the distribution of the factor V G1691A and prothrombin G20210A mutations determined in the entire study population, sample-size calculations showed that approximately 8000 cases and controls would have been necessary to reveal a statistically significant difference of the prevalence in cases and controls. On the basis of the prevalence of the MTHFR C677T polymorphism in our study population, another sample-size calculation indicated that more than 50,000 cases and controls would have been necessary to obtain a statistically significantly different distribution of heterozygote and homozygote genotypes for this mutation in cases and controls. The Figure details the association

Table V. Multivariate odds ratios of PAD for factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations (433 patients with PAD vs 433 control subjects)

Risk factor	*Multivariate odds ratios of PAD**	P *value*
Current smoking (vs not)†	7.76 (5.24-11.48)	<.001
Arterial hypertension (vs not)	2.81 (2.03-3.90)	<.001
Creatinine (median value of ≥0.9 mg/dL)	1.80 (1.27-2.54)	.001
LDL cholesterol (median value of ≥142 mg/dL)	1.90 (1.39-2.61)	<.001
Triglycerides (median value of ≥126 mg/dL)	1.05 (0.76-1.44)	.767
hs-CRP (median value of ≥3.2 mg/L)	1.88 (1.34-2.56)	<.001
tHcy (median value of ≥15.0 μmol/L)	1.68 (1.22-2.31)	.001
Factor V G1691A polymorphism		
GG genotype	1.00	
GA genotype	1.26 (0.67-2.36)	.472
Prothrombin G20210A polymorphism		
GG genotype	1.00	
GA genotype	1.47 (0.62-3.49)	.379
MTHFR C677T polymorphism		
CC genotype	1.00	
CT genotype	0.98 (0.71-1.35)	.885
TT genotype	0.85 (0.48-1.51)	.581

Statistical model with continuous variables dichotomized according to the median values of the entire study population (n = 866).
hs-CRP, High-sensitivity C-reactive protein; *LDL,* low-density lipoprotein; *PAD,* peripheral arterial disease; *tHcy,* total homocysteine; *MTHFR,* methylenetetrahydrofolate reductase.
*Data are expressed as odds ratio (95% confidence internal); multivariate odds ratios were calculated with logistic regression analysis without variable selection techique (all variables were included simultaneously into the model). Inasmuch as patient and control groups were matched for age, sex and diabetes mellitus, these variables were not included into the analysis.
†Current smoking was defined as any amount of tobacco use, including abstinence less than one year.[19]



Box-and-whisker plots for total serum homocysteine in patients with peripheral arterial disease (*PAD*; n = 433) and control subjects (n = 433) according to their methylenetetrahydrofolate reductase *(MTHFR)* C677T genotype. In box-and-whisker plots, the central box represents values from the lower to upper quartile, and the middle line represents the median; whiskers extend from the minimum to the maximum value, excluding outside (>1.5 box lengths from box) and far-out (>3 box lengths from box) values, which are displayed as open circles and stars, respectively. Median total homocysteine (25th-75th percentile) values according to the MTHFR C677T genotype in control subjects were 13.8 μmol/L (11.6-17.3 μmol/L) in CC (normal homozygotes = wild type), 14.8 μmol/L (12.0-18.2 μmol/L) in CT (mutant heterozygotes), and 14.8 μmol/L (12.4-19.4 μmol/L) in TT (mutant homozygotes); in patients with PAD, they were 15.6 μmol/L (12.6-19.7 μmol/L) in CC, 16.0 μmol/L (13.1-21.7 μmol/L) in CT, and 19.2 μmol/L (13.9-24.9 μmol/L) in TT.

between tHcy and the MTHFR C677T polymorphism in cases and controls.

DISCUSSION

The results of the LIPAD study showed that atherosclerotic PAD was not associated with an increased prevalence of factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations. Thus, considering our sample size calculations, there is no indication that one of these mutations may be a clinically relevant risk factor for chronic ischemia of the lower limbs. The prevalence of the three mutations in both cases and controls was similar to the prevalence described in previous epidemiologic reports on white populations.[16]

As detailed previously, the factor V G1691A and prothrombin G20210A mutations are widely accepted as risk factors for the development of venous thromboembolism.[15] Their role in arterial thrombotic disease and atherosclerosis, however, is controversial,[19] and the association of the factor V G1691A and prothrombin G20210A polymorphisms with PAD is even less well characterized. Four

case-control studies investigating whether the factor V G1691A mutation is associated with PAD have been published.[6-9] The two earlier studies suggested that the factor V G1691A mutation may be a risk factor for PAD,[6,7] but the two studies published more recently did not find such evidence.[8,9] The prothrombin G20210A polymorphism has been evaluated as a risk factor of PAD by two published studies that also found conflicting results.[8,9] Only one study indicated a significant association between PAD and the prothrombin G20210A mutation. The studies investigating the association of PAD with hyperhomocysteinemia and the MTHFR C677T polymorphism are raising doubts about the putative causal relationship between the MTHFR C677T mutation and PAD.[10-15] These six studies, similar to the studies on factor V G1691A and prothrombin G20210A polymorphisms, differ considerably in size and in the selection of patients and controls. In addition, as indicated in the introduction, some of these studies did not provide an appropriate spectrum or delineation of cases and controls.[19] As a consequence, on the basis of these publications, a definite statement related to the association of PAD with factor V G1691A, prothrombin G20210A, and MTHFR C677T polymorphisms has not been possible until now.

The pathogenesis of arterial thrombotic disease is complex and involves multiple genetic and environmental factors related to atherosclerosis and thrombosis, as well as their interaction.[27] Classically, acute thrombosis at the site of a ruptured, lipid-rich atherosclerotic plaque is understood as the precipitating event in the transition from stable or subclinical atherosclerotic disease to acute myocardial infarction or stroke.[28] Similar mechanisms resulting in peripheral arterial thrombosis are considered causative for acute ischemia of the lower limbs.[28] Conversely, chronic ischemia of the lower limbs is the consequence of the progression of atherosclerosis with the occurrence of hemodynamically relevant stenoses and occlusions of the arterial tree, but without clinically relevant thromboembolic events.[29,30] Although atherosclerosis is pathologically a continuously progressive disease (assuming that the promoting factors continue unabated), the clinical pattern in PAD consists of episodes of deterioration followed by resolution with long periods of stability or apparent improvement due to compensatory mechanisms—particularly the development of collateral circulatory pathways around obstructed segments.[31]

These differences in the natural history of acute ischemia and chronic ischemia in patients with PAD may be related to distinct risk profiles including specific genes that may be responsible for the occurrence of thrombotic events. The current evidence supports the hypothesis that factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations may be a risk factor for acute arterial occlusive events, including myocardial infarction and stroke, by modest but significant associations.[19] Assuming thrombophilic conditions, similar associations may also hold true for acute ischemic events in PAD patients (eg, peripheral arterial thrombosis of a native artery or popliteal artery aneurysm; thrombosed peripheral bypass grafts; or cardioembolic disease). This would be in accordance with observations in smaller populations that peripheral bypass graft thrombosis may be related to one of these three mutations.[7,32-34] However, considering the natural history of chronic ischemia of the lower limbs, as detailed previously, there is no rationale for premising that a thrombophilic state caused by a factor V G1691A, prothrombin G20210A, or MTHFR C677T mutation should be associated with PAD. Accordingly, the results of the LIPAD study, demonstrating no relationship of chronic ischemia of the lower limbs and the three polymorphisms, are conclusive. These considerations may, at least in part, explain the conflicting results on factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations in previous studies as potential risk factors for PAD, as described previously, because these studies obviously included patients with both acute and chronic ischemia of the lower limbs and thus possibly included different proportions of these two disease states.

The LIPAD study included a series of 487 consecutive patients with symptomatic PAD. However, matched controls were recruited only for nondiabetic patients and for those with diabetes mellitus but who were younger than 75 years of age (n = 433). This may be considered a limitation, but given our very stringent study design, it may have been impossible to enroll the required 54 diabetic controls 75 years of age or older without any relevant atherosclerotic manifestation, as detailed in the Methods section. Another limitation of this study may be that the study population was a selected subgroup of the overall population of PAD, as described in the Methods section (white patients admitted for inpatient diagnostics and treatment of atherosclerotic PAD). Thus, the findings cannot be generalized to non-Caucasian patients, asymptomatic patients with PAD, or patients who do not meet criteria for hospitalization. However, if there were an association between PAD and thrombophilic risk factors, this would be expected to be most easily detected in the most severely diseased patients (ie, the symptomatic patients admitted to the hospital). Thus, although the findings of the LIPAD study may not be generalizable, we probably did not miss a significant association.

In conclusion, the LIPAD study demonstrated that PAD was not associated with an increased prevalence of the factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations. Thus, considering the natural history of chronic ischemia of the lower limbs, there is no indication that one of these mutations may be a risk factor for chronic limb ischemia. Accordingly, routine screening for factor V G1691A, prothrombin G20210A, and MTHFR C677T mutations is not justified in this patient group, in our opinion. However, the role of these mutations in acute limb ischemia remains to be clarified by further studies.

REFERENCES

1. Hiatt WR. Medical treatment of peripheral arterial disease and claudication. N Engl J Med 2001;344:1608-21.

2. Belch JJ, Topol EJ, Agnelli G, Bertrand M, Califf RM, Clement DL, et al. Critical issues in peripheral arterial disease detection and management: a call to action. Arch Intern Med 2003;163:884-92.
3. Criqui MH, Denenberg JO, Langer RD, Fronek A. The epidemiology of peripheral arterial disease: importance of identifying the population at risk. Vasc Med 1997;2:221-6.
4. Blake GJ, Ridker PM. Inflammatory bio-markers and cardiovascular risk prediction. J Intern Med 2002;252:283-94.
5. Mueller T, Dieplinger B, Gegenhuber A, Haidinger D, Schmid N, Roth N, et al. Serum total 8-iso-prostaglandin F2alpha: a new and independent predictor of peripheral arterial disease. J Vasc Surg 2004;40:768-73.
6. Foley PW, Irvine CD, Standen GR, Morse C, Smith FT, McGrath C, et al. Activated protein C resistance, factor V Leiden and peripheral vascular disease. Cardiovasc Surg 1997;5:157-60.
7. Sampram ES, Lindblad B, Dahlback B. Activated protein C resistance in patients with peripheral vascular disease. J Vasc Surg 1998;28:624-9.
8. Renner W, Koppel H, Brodmann M, Pabst E, Schallmoser K, Toplak H, et al. Factor II G20210A and factor V G1691A gene mutations and peripheral arterial occlusive disease. Thromb Haemost 2000;83:20-2.
9. Reny JL, Alhenc-Gelas M, Fontana P, Bissery A, Julia PL, Fiessinger JN, et al. The factor II G20210A gene polymorphism, but not factor V Arg506Gln, is associated with peripheral arterial disease: results of a case-control study. J Thromb Haemost 2004;2:1334-40.
10. Verhoeff BJ, Trip MD, Prins MH, Kastelein JJ, Reitsma PH. The effect of a common methylenetetrahydrofolate reductase mutation on levels of homocysteine, folate, vitamin B12 and on the risk of premature atherosclerosis. Atherosclerosis 1998;141:161-6.
11. Todesco L, Angst C, Litynski P, Loehrer F, Fowler B, Haefeli WE. Methylenetetrahydrofolate reductase polymorphism, plasma homocysteine and age. Eur J Clin Invest 1999;29:1003-9.
12. Rassoul F, Richter V, Janke C, Purschwitz K, Klotzer B, Geisel J, et al. Plasma homocysteine and lipoprotein profile in patients with peripheral arterial occlusive disease. Angiology 2000;51:189-96.
13. Fowkes FG, Lee AJ, Hau CM, Cooke A, Connor JM, Lowe GD. Methylene tetrahydrofolate reductase (MTHFR) and nitric oxide synthase (ecNOS) genes and risks of peripheral arterial disease and coronary heart disease: Edinburgh Artery Study. Atherosclerosis 2000;150:179-85.
14. Stricker H, Soldati G, Balmelli T, Mombelli G. Homocysteine, vitamins and gene mutations in peripheral arterial disease. Blood Coagul Fibrinolysis 2001;12:469-75.
15. Ciccarone E, Di Castelnuovo A, Assanelli D, Archetti S, Ruggeri G, Salcuni N, et al. Homocysteine levels are associated with the severity of peripheral arterial disease in Type 2 diabetic patients. J Thromb Haemost 2003;1:2540-7.
16. Crowther MA, Kelton JG. Congenital thrombophilic states associated with venous thrombosis: a qualitative overview and proposed classification system. Ann Intern Med 2003;138:128-34.
17. Bauer KA, Rosendaal FR, Heit JA. Hypercoagulability: too many tests, too much conflicting data. Hematology 2002;353-68.
18. Key NS, McGlennen RC. Hyperhomocyst(e)inemia and thrombophilia. Arch Pathol Lab Med 2002;126:1367-75.
19. Kim RJ, Becker RC. Association between factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations and events of the arterial circulatory system: a meta-analysis of published studies. Am Heart J 2003;146:948-57.
20. Rutherford RB, Baker JD, Ernst C, Johnston KW, Porter JM, Ahn S, et al. Recommended standards for reports dealing with lower extremity ischemia: revised version. J Vasc Surg 1997;26:517-38.
21. Sacks D, Bakal CW, Beatty PT, Becker GJ, Cardella JF, Raabe RD, et al. Position statement on the use of the ankle brachial index in the evaluation of patients with peripheral vascular disease. J Vasc Interv Radiol 2003;14:S389.
22. Grant EG, Benson CB, Moneta GL, Alexandrov AV, Baker JD, Bluth EI, et al. Carotid artery stenosis: gray-scale and Doppler US diagnosis—Society of Radiologists in Ultrasound Consensus Conference. Radiology 2003;229:340-6.
23. Lay MJ, Wittwer CT. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. Clin Chem 1997;43:2262-7.
24. von Ahsen N, Schutz E, Armstrong VW, Oellerich M. Rapid detection of prothrombotic mutations of prothrombin (G20210A), factor V (G1691A), and methylenetetrahydrofolate reductase (C677T) by real-time fluorescence PCR with the LightCycler. Clin Chem 1999;45:694-6.
25. Bernard PS, Lay MJ, Wittwer CT. Integrated amplification and detection of the C677T point mutation in the methylenetetrahydrofolate reductase gene by fluorescence resonance energy transfer and probe melting curves. Anal Biochem 1998;255:101-7.
26. Endler G, Kyrle PA, Eichinger S, Exner M, Mannhalter C. Multiplexed mutagenically separated PCR: simultaneous single-tube detection of the factor V R506Q (G1691A), the prothrombin G20210A, and the methylenetetrahydrofolate reductase A223V (C677T) variants. Clin Chem 2001;47:333-5.
27. Voetsch B, Loscalzo J. Genetic determinants of arterial thrombosis. Arterioscler Thromb Vasc Biol 2004;24:216-29.
28. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115-26.
29. Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR. Large vessel and isolated small vessel disease. In: Fowkes FGR, editor. Epidemiology of peripheral vascular disease. New York: Springer-Verlag; 1991. p. 85-96.
30. Mohler ER III. Peripheral arterial disease: identification and implications. Arch Intern Med 2003;163:2306-14.
31. Ruckley CV. Symptomatic and asymptomatic disease. In: Fowkes FGR, editor. Epidemiology of peripheral vascular disease. New York: Springer-Verlag; 1991. p. 97-108.
32. Ouriel K, Green RM, DeWeese JA, Cimino C. Activated protein C resistance: prevalence and implications in peripheral vascular disease. J Vasc Surg 1996;23:46-51.
33. Sampram ES, Lindblad B. The impact of factor V mutation on the risk for occlusion in patients undergoing peripheral vascular reconstructions. Eur J Vasc Endovasc Surg 2001;22:134-8.
34. Kibbe MR, Hassett AL, McSherry F, Conner P, Bontempo FA, Williford W, et al. Can screening for genetic markers improve peripheral artery bypass patency? J Vasc Surg 2002;36:1198-206.

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