Original Article

Influence of genetic and environmental factors in peripheral arterial disease natural history: Analysis from six years follow up

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Abstract

Background: Peripheral arterial disease (PAD) is a disease affecting million of patients worldwide. Though traditional cardiovascular risk factors have been associated with the development of PAD, the possible existence of an inherited genetic predisposition to PAD has been investigated in few familial aggregation studies. A link between genetics and PAD may open new avenues for the prevention of this morbid and mortal disorder. **Aim**: The aim of this study is to investigate a possible role of some genetic determinant involving into coagulation and homocysteine metabolism in the progression of PAD. **Materials and Methods:** We follow one-hundred patients affected by PAD for six years. We evaluated Ankle-Brachial Index (ABI) two times; first at the time of recruitment and then after six years, in order to assess the progression of disease. Genotypes for the genes of Factor V Leiden, Prothrombin or Factor II G20210A, Cystathionine Beta-Synthase 844ins68bp and Methylenetetrahydrofolate Reductase C677T was ascertained after taking blood samples. Chi-square test was performed to determinate the possible correlation of these genes and the most common environmental factors in the progression of PAD. **Results:** Genetic disorders resulting in high level of homocysteina or thrombophilic phenotype are not so frequent. None among the genetic factors we considered were correlated with PAD. **Conclusion:** PAD is a chronic disease whose course can be slowed down especially with the control of environmental risk factors. Genetic analyses are not useful to determine the disease progression or its tendency to remain stable.

Key words: Peripheral arterial disease, cystatyonina beta-synthase 844ins68bp, factor V leiden, factor II G20210A, methylene tetra hydro folate reductase C677T

Introduction

Peripheral artery disease (PAD) is a chronic obstruction of the arteries of the lower extremities. In most cases the stenosis is due to the development of atherosclerotic plaques within the vessel. PAD could be considered a marker of widespread atherosclerosis, in fact the presence of the disease is a marker of future cardiovascular events.[1] With the growing elderly population, there is a significant increase in the burden of PAD. This disease affects from 12 to 20% of American >65 years old.[2] Clinical manifestations of PAD are wide and present the disease progression. They include an asymptomatic stage and a symptomatic one ranging from *Intermittent claudication* (IC) to critical limb ischemia with rest pain, ulcers or gangrene. Quality of life of people suffering of IC is reduced especially in terms of mobility and level of independence.[3] Although 3-6% of 60-70 years old men in western countries suffers from *Intermittent claudication*,[4] the majority of patients with PAD are asymptomatic. For this reason PAD remains under-recognized by clinicians and the real prevalence of the disease remains unknown,[5] in particular among patients classified at intermediate or low cardiovascular risk according to the current risk scores.

Ankle-brachial index (ABI) <0.90 is the worldwide accepted cut-off for the PAD diagnosis. The American

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College of Cardiology (ACC) / American Heart Association (AHA) guidelines recommend screening for symptomatic and asymptomatic PAD, by physical examination and ABI measurement, in order to identify patients with asymptomatic lower limb PAD, offer an appropriate therapeutic intervention and monitoring the progression of disease. Several risk factors play a role in the onset and progression of atherosclerosis. Most of these are environmental factors such as age, race, sex, hypertension, smoking habit, dyslipidemia and diabetes. One of the mainstays of PAD treatment is the risk factors management. Aggressive medical treatment of risk factors, in fact, has been shown to significantly decrease morbidity and mortality associated with the disease.[6]

In the last years, apart from the traditional risk factors, a lot of new possible mediators have been studied. In particular, clinical research has been focused on the role of thrombophilic factor with conflicting results. Thrombophilia has long been recognized as contributing to venous thrombosis, but is increasingly associated with arterial disease. Activated protein C (APC) resistance has been reported to be the most common cause of familial thrombophilia. In most of these cases, the activated protein C resistance is the result of a single mutation (G1691A) in the Factor V gene, which is known as Factor V Leiden (FVL). This defect was described for the first time in 1994 by Bertina *et al*.,[7] which inherited autosomal dominant yields Factor V much more resistant to the proteolytic degradation by activated protein C. This phenomenon results in a hypercoagulable state and is seen in approximately 5% of Caucasians, in up to 50% in selected thrombophilic families, and in 10% of unselected venous thrombosis patients. The risk of venous thrombosis is also increased in patients who have a mutation in the Prothrombin gene (G20210A). This mutation results in increased levels of blood Prothrombin due to increased Prothrombin synthesis and is associated with a threefold increase in the risk of venous thrombosis. Approximately 5% to 10% of patients affecting by 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. Many case-control and several large epidemiological studies have demonstrated that mild hyperhomocysteinemia is an important risk factor for cerebral and coronary artery disease.[8-10] Hyperhomocysteinemia is involved in endothelial dysfunction, an important step in atherosclerosis development.[11] The relation between hyperhomocysteinemia and PAD has been recently confirmed.[12] In the absence of overt deficiencies of vitamin B_{12}, folate, or pyridoxal phosphate, the most important causes of mild hyperhomocysteinemia are genetic defects affecting either the Cystathionine Beta Synthase (CBS) in the transsulfuration pathways or Methylenetetrahydrofolate Reductase (MTHFR), a critical enzyme in the remethylation of homocysteine to methionine. The most common hereditary abnormality associated with hyperhomocysteinemia is a variant that makes MTHFR a thermolabile enzyme, thus resulting in functional MTHFR deficiency. In some populations the MTHFR C677T variant is common (40 - 50%) among healthy subjects in heterozygous form and the homozygous carriers are approximately 10 - 15%. Many studies suggested that there is a weak positive association between hyperhomocysteinemia and MTHFR C677T only in homozygous carriers.

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. 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. Some studies describing a possible role of Factor V G1691A and/or Prothrombin G20210A polymorphisms as risk factors for PAD revealed conflicting results.[13-15] An interesting work lead by Sartori presents Prothrombin G20210A as a genetic marker predisposing critical ischaemia in patients with PAD suggesting a limited role of the mutation in the aetiology of the disease.[16] Others investigating the association of PAD with hyperhomocysteinemia and the MTHFR C677T polymorphism also do not unequivocally support their hypothesized role as independent risk factors.[17-19] In addition, most of the cited studies on PAD were investigated in 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.

The aim of this study is to determinate the probable role of some genetic and environmental factors in the progression of PAD like FVL, Prothrombin G20210A, CBS 84ins68bp, MTHFR C677T. In particular, for the first time, we intended to study a possible role of these factors in the progression of PAD by evaluating six years follow up of one hundred patients. Demonstration of the roles of these factors in the prognostic evaluation of PAD may open new prospective into the predictive diagnosis of patients affected by PAD.

Materials and Methods

Our study was divided in two parts. The first had been performed from July 2003 to July 2004 and the second one from April 2009 to February 2010. In the first phase we recruited one hundred patients affected by PAD at the Clinical Surgery of Vascular Disease of University of Brescia, Italy. PAD was diagnosed when patient had typical sign of *Intermittent claudication* and/or an ABI at rest less than 0.90 calculated according to AHA recommendations.[20]

The patients older than 80 years were excluded. Further exclusion criteria were - PAD caused by non-atherosclerotic disease; history of acute ischemia of lower extremities; a Fontaine's Stage upper than IIb; and history or presence of any malignancies.

In this phase, after signing the informed consensus, all studied patients underwent to an evaluation for the presence of risk factors for atherosclerosis. Every patient was subjected to clinical biochemistry (glycaemia, HBA1c%, C Reactive Protein, IL6, creatinine levels, total cholesterol, LDL, HDL, uric acid, and triglycerides) and blood count analysis (data not shown). In particular we investigated the presence of obesity, diabetes, hypertension, hypercholesterolemia and smoke habit. If these problems were present, we investigated for the drug therapy. Patients with a BMI >25 were considered overweight and those with BMI >30 as obese. Then we evaluated every patient with ABI, physical examination and assessed the blood sample for genetic analysis.

The hypertension, diabetes and dyslipidemia diagnosis were in agreement with the guidelines of European Society of Hypertension/European Society of Cardiology, with the Third Report of the National Cholesterol Education program (NECP), and with the American Diabetes Association.[21-23]

After six years, all patients re-evaluated for their clinical condition, pharmacological anamnesis and a new ABI measurement. At the end of this part, living patients were classified into different groups based on the stability or progression of their disease. Reduction of ABI by ≥0.2 was considered as worsening of PAD. Patients who required surgical intervention were classified in progress group.

ABI measurements

ABI measurement technique was performed according to ACC/AHA guidelines for the management of patients with PAD.[24,25] We recorded the systolic blood pressure from left and right brachial arteries and from both the dorsalis pedis and posterior tibial arteries after the patient had been at rest in the supine position for some minutes. The blood pressure cuffs were always of the appropriate size and were positioned immediately above the ankle. For ABI calculation the higher of dorsalis pedis and posterior tibial pressure in each ankle were selected. The ABI values were calculated by dividing the higher of the two ankle systolic pressure in one leg with the greater brachial artery value of systolic pressure. ABI values were calculated up to two decimal places.

Genotyping

Blood was collected at venipuncture in Ethylene DiamineTetra Acetic Acid (EDTA) tubes, after the patient had fasted overnight and genomic DNA isolated from whole blood using the commercially available DNA isolation system (QIAamp DNA Blood Kit, Qiagen SPA, Milano; Italy) as described by the manufacturer. The remaining DNA was stored at -80°C.

Aliquots of the same DNA samples were tested for FVL, Prothrombin G20210A and MTHFR C677T using the Light Cycler (LC®) and the Roche Light Cycler Mutation Detection. Real-Time fluorescence Polymerase Chain Reaction (PCR) was performed for simultaneous detection of factor V and Prothrombin G20210A polymorphisms by using primer sequences described previously.[26,27] The two probes targeting several gene-specific amplicons were labelled with two different reporter dyes to achieve multiplex genotyping in a single glass capillary. For the MTHFR C677T polymorphism, a separated PCR was performed that used slightly modified primers and probes of validated methods.[28] PCR reactions were performed according to the method described and provided with the kits by the manufacturer. In each run, an H_2O control and known homozygous and heterozygous for the tested polymorphisms were included to check for unspecific reactions and to confirm correct genotyping, respectively.

The CBS 844ins68 bp polymorphism determination was carried out by a PCR followed by electrophoresis on 2% Agarose gel stained with Ethidium Bromide. In each run an H_2O control and a known heterozygous internal control was used to confirm correct results of analyse.

Statistical analysis

All the statistical analyses were performed using χ^2 test. We correlated the environmental risk factors (such as diabetes, hypertension, hypercholesterolemia, obesity) and genetic risk factors with the progression of PAD.

Results

One hundred patients were recruited in the first phase of our study. The average age of the patients in our study was 69 years. Eighty six of them were male. We studied the prevalence of the most important atherogenic determinant within the population. Most of them were smokers (74%) and no one, still living at the follow up, stopped the habit. The prevalence of diabetic disease was only 29% while hypertension, dyslipidemia and obesity were the most represent risk factors with 74%, 48% and 63% of patient affected respectively [Table 1]. The pharmacologic anamnesis has shown that every patient with one or more risk factors had also the right therapy in order to control diabetes hypertension or dyslipidemia, while none still living at follow up reduced his BMI.

The first measurement of ABI showed that the majority (61%) of patients had a Windsor's Index between 0.7–0.5, 21% of patients had the index bigger then 0.7 and 18% smaller then 0.5.

Genotyping for factor V G1691A, prothrombin G20210A, MTHFR C677T and CBS 844ins68 bp polymorphisms was performed successfully in 100% of the study subjects. Few people were positive for FVL (10% heterozygous and nobody homozygous) and Prothrombin variant (5% heterozygous and nobody homozygous); reflecting the low prevalence of these gene's mutations in general population (3-5% for FVL and 2% for Prothrombin). Distribution of MTHFR C677T (60% heterozygous and 16% homozygous) and CBS 844ins68 bp (26% heterozygous and nobody homozygous) genotypes in the studied population was more [Table 2].

At follow up six years later, twenty-two patients were not alive. In eight cases cardiac origin was the cause of the death, five patients died from neoplasm, three died after a stroke, and six for unknown reason. Of the 78 patients still living at the follow up, only 24 presented with a stable clinical condition; the other 54 patients had worsening of their ABI ≥0.2. During these six years, worsening of clinical conditions forced 20 patients to choose for surgical intervention.

The distribution of four genotypes in the different subgroups of the studied population was assessed at the six year follow-up i.e., in stable patients (n = 24), worsened patients (n = 54) and patients who underwent surgical procedures (n = 20). These results showed that there were not much differences of the distribution of the four mutations in the subgroup analyses [Table 3].

On univariate analysis, the correlation of PAD progression was found only with smoking habit, with a P value of 0.025. No other environmental or genetic variable was found to be correlated with the progression of the PAD [Table 4].

Table 1: Demographic profile of study population

Variables	Patients (n=100)	Alive (n=78) %	Dead (n=22) %
Males	86	66 (85)	20 (91)
Females	14	12 (15)	2 (9)
Diabetes	29	22 (28)	7 (32)
Hypertension	74	56 (72)	18 (82)
Dyslipidemia	48	40 (51)	8 (36)
Obesity	63	50 (64)	13 (59)
Smoking Habit	74	56 (72)	18 (82)

Table 2: Percentage prevalence of genetic thrombophilic risk factors

Genotype	Wild type	Heterozigosity	Homozigosity
CBS 84ins68bp	74	26	0
MTHFR C677T	24	60	16
FV Leiden	90	10	0
Prothrombin G20210A	95	5	0

CBS: Cystathionine Beta Synthase; MTHFR: Methylene Tetrahydro Folate Reductase; FV: Factor V

Table 3: Prevalence of genetic thrombophilic risk factors after six years of follow up in alive patients

Genotype	Wild type (%)	Heterozigosity (%)	Homozigosity (%)
Stable (n=24)			
CBS 84ins68bp	16 (67)	8 (33)	0 (0)
MTHFR C677T	7 (29)	13 (54)	4 (17)
FV Leiden	23 (96)	1 (4)	0 (0)
Prothrombin G20210A	24 (100)	0 (0)	0 (0)
Worsened (n=54)			
CBS 84ins68bp	41 (76)	13 (24)	0 (0)
MTHFR C677T	13 (25)	32 (59)	9 (17)
FV Leiden	47 (87)	7 (13)	0 (0)
Prothrombin G20210A	49 (91)	5 (9)	0 (0)
Surgical cases (n=20)			
CBS 84ins68bp	16 (80)	4 (20)	0 (0)
MTHFR C677T	4 (20)	12 (60)	4 (20)
FV Leiden	18 (90)	2 (10)	0 (0)
Prothrombin G20210A	18 (90)	2 (10)	0 (0)

CBS: Cystathionine Beta Synthase; MTHFR: Methylene Tetrahydro Folate Reductase; FV: Factor V

Table 4: Univariate analysis

Variable	χ^2	P
Hypertension	0.176	>0.05
Dyslipidemia	0.115	>0.05
Obesity	1.789	>0.05
Smoking Habit	5.319	0.025*
Diabetes	1.497	>0.05
CBS 84ins68bp	0.724	>0.05
MTHFR C677T	0.226	>0.05
FV Leiden	1.396	>0.05
Prothrombin G20210A	2.374	>0.05

*Statistically significant

Discussion

FVL is an important heritable cause of hypercoagulability. FVL is a G1619A nucleotide transition resulting in an R506Q amino-acid missense mutation. This mutation causes the resistance of Factor V to the proteolysis by the APC. It accounts for 90% to 95% of cases of APC resistance and it has an incidence of 4.8% in the general population.[29] FVL seems to play a role in arterial thrombosis and in its complication such as myocardial infarction,[30] but the data available in literature do not confirm the higher prevalence of FVL in patient with PAD.[31,32] In our study, though only one patient with FVL heterozygous status remained stable at follow up, while seven patients showed worsening of their condition; statistical analysis proved that there is no influence of this mutation on PAD progression.

The G20210A Prothrombin mutation is the second most common genetic condition that results in thrombosis. It has a prevalence of 2.7% in the general population.[33] This mutation is associated with both elevated plasma prothrombin level and increased risk of venous thrombosis.[34] This mutation is frequently studied with FVL because the combination of the two mutations cause an additive risk of venous thrombosis.[35] It is not well know whether the mutation with or without FVL increase the risk of arterial thrombosis. Its role in PAD remains far from clear. In fact in one case control study, PAD was not associated with an increase prevalence of the mutation;[31] but in another one the researcher found an independent association with Prothrombin G20210A variant gene.[32] In our study, all carriers of the mutation showed a worsening of the disease; though statistical analysis does not correlate genetic defect with the progression of PAD.

CBS and MTHRF are two regulating genes involved in homocysteine metabolism. In particular MTHFR is important for re-methylation of homocysteine (HCY); a process in which HCY is transformed in methionine by the transfer of a methyl group from Me-THF to HCY. In 1988 Kang *et al.* described a MTHFR "thermolabile" variant characterized by enzyme lability at 46°C and a reduced activity.[36] Frosst *et al.* identified the genetic disorder of this variant, consisting of cytosine-thymine transversion at cDNA nucleotide 677 that lead to an amino-acid substitution from valine to cytosine, on translation.[37] In presence of genotype C/C MTHFR activity is 100%, but reduced when genotype is C/T (65%) or T/T (30%). Many studies have shown that elevated HCY plasma levels are considered a risk factor for vascular disease.[38-40] One study reported an increased prevalence of hyper-HCY in PAD patients.[8] The association between PAD and MTHFR polymorphism has been studied in literature with controversial results; the Linz Peripheral Arterial Disease (LIPAD) study doesn't correlate MTHFR and PAD, while a meta-analysis of 9 appropriate studies showed that being homozygous for the C677T allele was associated with an increased risk of PAD.[31,41]

CBS is involved in transsulfuration pathway in which HCY is converted in Cysteine. The 844ins68bp is an evolutionary conserved polymorphism of the CBS gene that segregates with the pathogenic T833C mutation and consists of a 68bp insertion, duplicating the 3' splice site between intron 7 and exon 8. The gene rearrangement brought two GGGG runs close to each other and generated a splicing control element that allows the constitutive selection of the more distal 3' splice site in the 844ins68 carriers. Heterozygosity for CBS deficiency is considered a minor cause of hyperhomocysteinaemia. There is no study that showed a correlation between CBS mutation and PAD. In our study, both CBS 844ins68 and MTHFR C677T have no affect on the progression of the disease.

Statistical analysis showed that of all the environmental and genetic variables studied; only the smoking habit plays a role in the progression of PAD. The fact that the common atherogenic risk factors such as diabetes, hypertension, dyslipidemia, have no statistical significance could be explained by a good compliance to drug therapy. Moreover, in the first stage, our advice to stop the smoking habit and reduce body weight has been widely rejected. It would appear that patients prefer to control their disease with medication and are less willing to correct their habits. No correlation exists between the genetic thrombophilic factors (FVL and Prothrombin G20210A) and genetic determinants of hyperhomocysteinemia (CBS and MTHFR) studied with the progression of PAD.

In conclusion, we can also argued that it does not seem useful to undertake more investigation in order to associate the presence of mutations we have studied to the common methods of prevention and control of chronic progression PAD. The way of a possible influence of these factors in developing acute events at the lower limb remain open.

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