Novel Risk Factors for Premature Peripheral Arterial Occlusive Disease in Non-Diabetic Patients: A Case-Control Study

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Abstract

Background: This study aimed to determine the prevalence of genetic and environmental vascular risk factors in non diabetic patients with premature peripheral arterial disease, either peripheral arterial occlusive disease or thromboangiitis obliterans, the two main entities of peripheral arterial disease, and to established whether some of them are specifically associated with one or another of the premature peripheral arterial disease subgroups.

Methods and Results: This study included 113 non diabetic patients with premature peripheral arterial disease (diagnosis <45-year old) presenting either a peripheral arterial occlusive disease (N = 64) or a thromboangiitis obliterans (N = 49), and 241 controls matched for age and gender. Both patient groups demonstrated common traits including cigarette smoking, low physical activity, decreased levels of HDL-cholesterol, apolipoprotein A–I, pyridoxal 5’-phosphate (active form of B6 vitamin) and zinc. Premature peripheral occlusive disease was characterized by the presence of a family history of peripheral arterial and carotid artery diseases (OR 2.3 and 5.8 respectively, 95% CI), high lipoprotein (a) levels above 300 mg/L (OR 2.3, 95% CI), the presence of the factor V Leiden (OR 5.1, 95% CI) and the glycophorin la807T,837T,873A allele (OR 2.3, 95% CI). In thromboangiitis obliterans group, more patients were regular consumers of cannabis (OR 3.5, 95% CI) and higher levels in plasma copper has been shown (OR 6.5, 95% CI).

Conclusions: According to our results from a non exhaustive list of study parameters, we might hypothesize for 1) a genetic basis for premature peripheral arterial occlusive disease development and 2) the prevalence of environmental factors in the development of thromboangiitis obliterans (tobacco and cannabis). Moreover, for the first time, we demonstrated that the 807T/837T/873A allele of platelet glycoprotein la may confer an additional risk for development of peripheral atherosclerosis in premature peripheral arterial occlusive disease.

Introduction

Among peripheral arterial diseases (PAD), lower limb peripheral arterial occlusive disease (PAOD) is considered as the disease process resulting from obstruction of large peripheral arteries, exclusive of the coronary and intracranial cerebrovascular system, most commonly due to atherosclerosis. PAOD which affects up to 20% of adults older than 50–55 years [1], is an important healthcare problem in Western countries and is associated with considerable morbidity and mortality [2]. The course of atherosclerosis is even more aggressive in young patients than in older ones with more frequent surgical interventions and death, and also more frequent coronary artery disease (CAD) [3,4]. The prevalence of premature PAOD is estimated at 1% of the
population. A number of vascular risk factors for premature PAOD have been demonstrated including cigarette smoking [5], high level of lipoprotein (a) (Lp(a)) [6], or fibrinogen [6] and other prevalent conditions such as chronic renal insufficiency [7], CAD [6,8] or hypertension [6]. However, these risk factors fail to explain all the cases of PAOD in young adults, where the disease is thought to be multifactorial. The concerted action of both genetic and environmental factors has been incriminated and a family history of vascular disease is noted in most of the patients [9]. On the other hand, although PAOD concerns large peripheral arteries, thromboangiitis obliterans (TAO) affects small and medium sized peripheral blood vessels. Prevalence of TAO as a percentage of all cases of premature PAD (PPAD) is 0.5 to 5.6% in Western countries. Smoking is considered as the major risk factor [10], and a higher frequency of prothrombin gene mutation and endothelial dysfunction [11,12], have also been reported in these patients. But TAO etiology remains mostly unelucidated.

The aim of the present study was to investigate genetic and environmental factors in two groups of young patients with either PAOD or TAO (diagnosis before 45-year old) and in one group of controls matched for age and gender. The prevalence of traditional and novel risk factors was compared in the three groups of subjects to define which of them are altered in PPAD when compared to controls and to recognize whether some of them are specifically associated with one or another of the PPAD subgroups.

Methods

Ethics Statement

The study protocol was approved by the Committee to Protect Persons (CPP) from Bordeaux hospital in 1999 (Nb 99-35) and by the French Ministry of Health and Solidarity in 2001 (Nb DGS:2001-0058). Written informed consent was obtained from all study participants.

Participants

A French, multicenter, age- and sex-matched case-control study was conducted between 2002 and 2007 including 113 patients with known PPAD (first observed before 45-year old) and 241 controls aged from 18 to 50 years. Since the definition of PPAD is controversial with respect to extracranial carotid circulation, upper extremity arteries, and mesenteric and renal circulation, we restricted our study on patients with chronic arterial occlusive disease in the arteries of the lower limbs. At the time of inclusion, all PPAD patients had symptoms of intermittent claudication (Fontaine stage II) or chronic critical limb ischemia (Fontaine stage IV) and an ankle-brachial index (ABI) at rest of less than 0.9, calculated according to AHA recommendations [13] or toe systolic pressure below 50 mm Hg or transcutaneous oxygen measurement below 30 mm Hg in case of uncompressible arteries. All patients had an angiographically confirmed diagnosis of PPAD. Patients with TAO were included according to Adar’s criteria, i.e. possible diagnosis in the presence of sub-popliteal ischemia and tobacco consumption but in the absence of connective tissue disease, embolic disease, diabetes, dyslipidemia or hematologic disease; and likely diagnosis if recurrent venous thrombosis or Raynaud’s phenomenon or upper limb ischemia. Unlike original Adar’s criteria, we decided to include female patients as well. Workers in the Ford factory located in Blanquefort (33290– France) were enrolled as controls with no evidence of cardiovascular disease and an ABI above 1.0. All participants were asked to complete the same standardized questionnaire on alcohol consumption, use of cannabis (interview completed by 24 h-

DNA Analysis

Genomic DNA was isolated from buffy coat prepared from EDTA whole-blood samples by using the commercially available
Qiagen DNA isolation kit (Courtaboeuf, France) and was kept at +4°C until further assessment. We selected candidate genes encoding proteins that are potentially contributing to the atherosclerotic process as known in premature CAD. We particularly focus on the biological aspects of variants and mutations of these genes which impact on: J) lipid and lipoprotein metabolism via the intracellular incorporation of lipids in lipoproteins during synthesis (microsomal triglyceride transfer protein: \(MTTP^{\text{E9G/C/T}}\), (rs1800591)), the remodelling of plasma lipoproteins through the plasma transfer of cholesterol from HDL to triglyceride-rich lipoproteins (cholesterol ester transfer protein: \(CETP \; \text{TaqI} \; (\text{rs}7082722)\), specific membrane transporter systems as ABCA1 which allows the transfer of intracellular cholesterol to plasma HDL (\(ABCA1^{R219K}\) (rs2230880)), molecule-mediated hepatic uptake of atherogenic lipoproteins as apoE \(\text{APOE}^{2/2/3/4} (\text{rs} \; 7412; \; \text{rs} \; 429358)\), or transcription factors involved in lipid metabolism and atherogenesis as peroxisome proliferator-activated receptor \(\gamma\) (\(PPARG^{A/G/C/T}\) (rs3856806)); 2) cell adhesion to vascular endothelium via E selectin (\(SELE^{57E/58R}\) (rs5361)); and 3) thrombosis via coagulation factors II and V (\(F2^{382106C/A}\) (rs1799963); \(F5^{R506Q \; \text{Leiden}}\) (rs6025)) as well as platelet glycoproteins Ia and IIIa (\(ITGAI^{2/1007C/T}\), \(C3\) (rs1126643, rs1139484 and rs1062535) and \(ITGB3^{4/638P}\) (HPA-1, rs5918)).

Except for \(MTTP, F2\) and \(F3\) gene polymorphisms, DNA fragments containing the mutational sites were amplified by polymerase chain reaction (PCR) and digested with allele-specific restriction enzymes (specific primers and restriction enzymes are shown in Table S1 in Supplementary data). Fragments were separated on polyacrylamide \(\text{APOE}^{2/2/3/4}\) or agarose gels according to their size, and visualized by use of ethidium bromide. An example of observed alleles is illustrated in Figure 1. As quality control of the method, 5 randomly chosen subjects were additionally genotyped by sequencing of PCR products using an ABI sequencer (Applied Biosystems, Courtaboeuf, France). For \(MTTP\) gene polymorphism \(\text{MTTP}^{493G/T} (\text{rs} \; 1800591)\), amplified selected DNA fragments were sequenced. No new gene variation has been found by sequencing in our study. For the \(F2^{G1007A \; \text{Leiden}}\) and \(F2^{G28491A}\) (rs1799963) polymorphism determination, the TaqMan® fluorogenic \(5'\rightarrow3'\) nuclease assay (PE/Applied Biosystems, Foster City, CA) was carried out using the primers and probes of the human pre-designed TaqMan® SNP Genotyping Assays. In each run, blanks and known homozygotes and heterozygotes were included.

**Statistical Analysis**

We compared risk factors distribution \(J\) in controls versus PPAD patients, \(J\) in PAOD versus TAO, \(J\) in controls versus PAOD and \(J\) in controls versus TAO. For comparing the values of continuous quantitative variables between groups of subjects, \(t\) tests were used. For comparing the percentages of qualitative categorical variables between groups of subjects, \(x^2\) tests were used. Quantitative variables (expressed in tables as mean ± SD) had a normal distribution except in the case of cotinine and cigarette consumption (expressed as pack x years) since zero values were found in non-smokers. Qualitative variables are expressed in tables in percentages. Hardy-Weinberg equilibrium of allele frequencies was tested for each SNP in controls by \(x^2\) statistics. A Pearson \(x^2\) test was performed to detect differences in SNP allele frequency distribution between patients and controls.

**General Linear Models.** Disease state (no disease, PPAD, PAOD, TAO), allele frequencies, and some other qualitative variables were defined as independent ( explicative) variables. The effects of all categorical variables, including allele frequencies, on the dependent quantitative variables were tested systematically for the whole subject population using univariate general linear models. Age and sex were included as an adjustment factor. Before testing the effect of independent categorical variables on the dependent variables, interfering covariables (to be used as adjustment factors in the linear models) were identified by 2 types of tests: \(J\) Quantitative variables chosen as adjustment factors: linear correlations were performed between all possible pairs of quantitative variables in the whole subject population on one hand, and in either controls, patients, PAOD or TAO separately on the other hand. All independent variables showing correlations significant either at the 0.01 level or at the 0.05 level with a clinical or biological significance, were retained as quantitative adjustment factors to be included in the linear models. This procedure was chosen to avoid the presence of too many covariables in the general linear models. \(J\) Qualitative variables chosen as adjustment factors: all independent qualitative variables showing correlations at the 0.05 were retained as qualitative adjustment factors specifically for each studied dependent variable. Results of the General Linear Models include adjusted \(p\) values, means, and SD. For each of the biallelic polymorphisms, 100% of patients and controls were successfully genotyped. The allelic model was chosen for testing association between polymorphisms and the diseased or

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**Figure 1. PCR analysis of genomic DNA encoding the glycoprotein Ia gene surrounding the 807, 837 and 873 polymorphisms.** (A) Amplified products (1332 bp) were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1: molecular weight marker; lane 2: blank; lanes 3 to 11: different genotyped individuals. (B) Analysis of \(ITGAI^{2/1007C/T}\), \(C3\) (rs1126643, rs1139484 and rs1062535) and \(ITGB3^{4/638P}\) (HPA-1, rs5918). doi:10.1371/journal.pone.0037882.g001
control phenotype. Alleles were in Hardy-Weinberg equilibrium and the lower degree of freedom (df) required made it more powerful than the general genetic model. The general model, or allele-based test, requiring 2 df was used. SNP were then analyzed by comparing differences in allele frequencies between cases and controls using a $\chi^2$ test. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

Confidence intervals (CI) for proportions were calculated using the Wilson score method without continuity correction [17]. CI for the odds ratios (OR) were calculated using the methods described by Armitage and Berry [18].

**Results**

**Population Characteristics**

Population characteristics are summarized in Table 1. $P$ values from comparison between controls and PPAD and between PAOD and TAO are directly given in Table 1. $P$ values from comparison between PAOD or TAO and controls are given in the text.

Age at the inclusion time was higher in PPAD patients than in controls, with a significant difference between PAOD patients and controls. Age was thus included in the adjustment for statistical analysis. At diagnosis time, a 5-year difference was present between PAOD and TAO patients showing the earlier diagnosis of the latter (38.3±5.7 vs 33.4±7.6 years, $p<0.03$). Women represented $\approx21\%$ in all groups of subjects with no difference between the groups. This result is new compared to other studies, probably because there is no difference in the degree of feminization between the two groups.

According to Fontaine’s stages, most of PPAD had intermittent claudication (stage II) at the time of inclusion as well as at the time of diagnosis. But the distribution of the stages was significantly different between patients with TAO with a large predominance of rest ischemia (stage IV), and PAOD cases mainly at a claudication stage (stage II). This distribution between the two groups of patients was the same at the inclusion and diagnosis times with no difference. Moreover, 30% of the 113 patients had other arterial diseases (occlusive carotid artery disease, coronary disease) compared to controls (1.3%). However, the prevalence of arterial diseases, mainly carotid and coronary artery diseases, was higher in patients with PAOD than in those with TAO (46.8 vs 8.2%). On the other hand, the prevalence of Raynaud’s disease and venous thromboembolism was significantly higher in PAOD patients vs controls ($p<0.001$) but a marked predominance was found in patients with TAO (61.2 vs 18.8%, $p<0.001$). Because of their vascular disease, most of PAOD and patients with TAO underwent a vascular surgery or amputation and used medication ($p<0.001$). For this last point, according to their pathology and other diseased vascular districts, statins and clopidogrel were mainly used by PPAD patients whereas iloprost was predominantly given to patients with TAO. No difference was demonstrated for aspirin intake between the two patient groups.

**Cardiovascular Risk Factor Analysis**

Table 2 shows the distribution of vascular risk factors among individuals with or without PPAD as well as among PAOD and patients with TAO. $P$ values from comparison between controls and PPAD and between PAOD and TAO are directly given in Table 2. $P$ values from comparison between PAOD or TAO and controls are given in the text.

The prevalence of early cardiovascular events occurring in first-degree relatives of patients with PPAD was determined. Interestingly, peripheral and carotid arteries were more often altered in first-degree relatives of PPAD patients than in controls, while coronary arteries were not. Moreover, when first-degree relatives of PAOD and patients with TAO were separately compared to controls, the former had more peripheral and carotid artery diseases ($p<0.02$ and $p<0.002$ respectively), while the latter had fewer coronary diseases ($p<0.035$). Finally, the prevalence of venous thromboembolism in first-degree relatives was not significantly different among study groups.

Among all other evaluated vascular risk factors, hypertension and hyperhomocysteinemia were not more prevalent in both groups of patients compared to controls. Lack of physical exercise, cigarette smoking, low plasma HDL-cholesterol and apoA-I, and high sensitive CRP (hsCRP) were found in PPAD more frequently than in controls. Absence of physical activity was significantly more frequent in both patient groups compared to controls ($p<0.006$ and $p<0.001$ respectively), but no significant difference was detected for BMI. Active cigarette smoking was far more prevalent in both PAOD and TAO groups than in controls as assessed by claimed consumption ($p<0.001$ for both groups) or urinary cotinin evaluation ($p<0.004$ and $p<0.05$ respectively). In addition, cannabis consumption was higher in TAO group than in PAOD patients and controls as assessed by interview and positive urinary tetrahydrocannabinol (THC) detection ($p<0.015$). On the other hand, HDL-cholesterol and apoA-I were significantly lower in both groups of patients compared to controls ($p<0.001$ for both variables in PAOD patients, $p<0.004$ and $p<0.05$ respectively in patients with TAO) while Lp(a) showed higher levels in PAOD patients only ($p<0.02$). It is noteworthy that there was no difference in LDL-cholesterol probably due to statin medication. Two inflammatory factors, hsCRP and fibrinogen, were also evaluated and showed higher plasma concentrations in patients with TAO only, when compared to controls ($p<0.02$ and $p<0.03$ respectively); this is presumably due to local infection found in many patients with toe necrosis. Finally, homocystein metabolism evaluation demonstrated a decrease in pyridoxal 5′-phosphate (active form of B6 vitamin) in both groups of patients compared to controls ($p<0.005$). On the other hand, as copper, selenium and zinc are essential cofactors for antioxidative enzymes, i.e. Cu-Zn superoxide dismutase and glutathione peroxidase, we investigated the relationship between these trace-elements and PPAD. We showed an increase in plasma copper levels in patients with TAO ($p<0.001$) and a decrease in plasma zinc levels in PAOD ($p<0.02$) and TAO ($p<0.004$) patients compared to controls so that both groups of patients had diminished zinc/copper ratio ($p<0.001$). Nevertheless, concentrations in copper and zinc remained in the normal range. At last, no difference in the frequency of antiphospholipid antibodies was shown among the groups (data not shown).

**Polymorphism Frequency**

The distribution of all genotypes in the different study groups is depicted in Table 3 and was in Hardy-Weinberg equilibrium. When comparing differences in allele frequencies between cases and controls, only $F_{5\text{Leiden}}^{rs126643}$ ($F_{5\text{Leiden}, ITG42^{rs9118}/ITG42}^{rs126643/ITG42}$, $F_{5\text{Leiden}, ITG42}^{rs126643}$, $ITG42$ and $F_{5\text{Leiden}}^{ITG42}$) in PAOD and controls were different. Interestingly, a statistically significant different distribution was found between PAOD and controls with an overrepresentation of $F_{5\text{Leiden}}^{rs126643}$ in PAOD patients compared to controls. Conversely, a statistically significant different distribution was found between controls and PAOD with an overrepresentation of $F_{5\text{Leiden}}^{rs126643}$ in controls compared to PAOD patients.
Discussion

This case-control study clearly demonstrated common and particular PAD risk factors (Table 4), at least among those determined in this study, for patients presenting either a PAOD or a TAO, after adjustment for potential confounders.

Both patient groups demonstrated common clinical conditions associated with PPAD including low physical activity, cigarette smoking, decreased HDL-cholesterol and apoA-I levels, lower pyridoxal 5'-phosphate (active form of B6 vitamin) and zinc concentrations. Both patients with PAOD and TAO had reduced physical activity compared with controls but both populations of patients had a high frequency of claudication, rest pain, and lower extremity ulcers which represents a bias to conclude about this recognized risk factor. Cigarette smoking is widely accepted as a major risk for PAD [5] even in young patients [5,9]. The underlying mechanisms explaining the tobacco-induced risk include alterations in HDL-cholesterol [19] and pyridoxal 5'-phosphate [20] levels, hemostatic factors [21] and endothelial function [22], all of them known as playing an important role in cardiovascular diseases. In both PPAD groups, we demonstrated a significant decrease in HDL-cholesterol, apoA-I and pyridoxal 5'-phosphate levels associated with a prothrombotic status. Indeed HDL lipoproteins play a pivotal role in the reverse cholesterol transport, known to have athero-protective effects. Nevertheless, their role is unknown in TAO. In addition, there was a significant inverse association between pyridoxal 5'-phosphate and hsCRP (data not shown, p<0.03), independently of homocystein level so far showing no significant difference. This relationship was also demonstrated elsewhere for ischemic stroke and coronary artery disease [23,24]. Finally, patients with PPAD had significant lower zinc/copper ratio. This imbalance in zinc/copper metabolism

Table 1. Clinical Characteristics of the Study Sample.

| Study characteristics       | Controls | PPAD  | p-value | PAOD  | TAO  | p-value |
|-----------------------------|---------|-------|---------|-------|------|---------|
|                             | n = 241 | n = 113 |         | n = 64 | n = 49 |         |
| Age at the inclusion time (years) | 33.1±6  | 39±7.8 | <0.001  | 41±6.9 | 36.4±8.2 | 0.002   |
| Sex (% men)                 | 77.2    | 78.8  | ns      | 78.1  | 79.6 | ns      |
| Fontaine’s stage (diagnostic time) |         |       |         |       |       | <0.001  |
| - II (%)                    | -       | 54.8  | 79.7    | 22.5  |       |         |
| - III (%)                   | -       | 19.5  | 14      | 26.5  |       |         |
| - IV (%)                    | -       | 25.7  | 6.3     | 51    |       |         |
| Fontaine’s stage (inclusion time) |         |       |         |       |       | <0.001  |
| - II (%)                    | -       | 46    | 71.9    | 12.2  |       |         |
| - III (%)                   | -       | 17.7  | 14.1    | 22.4  |       |         |
| - IV (%)                    | -       | 36.3  | 14      | 65.4  |       |         |
| Associated arterial diseases (%) |         |       | <0.001  |       |       | <0.001  |
| - 0                         | 98.7    | 70    | 53.2    | 91.8  |       |         |
| - 1                         | 1.3     | 26.5  | 40.6    | 8.2   |       |         |
| - 2 or more                 | 0       | 3.5   | 6.2     | 0     |       |         |
| - Occlusive carotid artery disease | 0       | 25.9  | <0.001  | 40.6  | 6.2   | <0.001  |
| - Coronary disease          | 1.2     | 8.8   | <0.001  | 14.1  | 2     | 0.03    |
| - Aortic abdominal anevrysm | 0       | 0.9   | ns      | 1.6   | 0    | ns      |
| Other associated vascular diseases (%) |         |       |         |       |       | <0.001  |
| - Raynaud’s disease         | 1.7     | 24.7  | <0.001  | 6.3   | 49    | <0.001  |
| - Venous thromboembolism    | 2.5     | 19.5  | <0.001  | 12.5  | 28.6  | 0.03    |
| - None                      | 95.9    | 71.7  | <0.001  | 81.2  | 38.8  | <0.001  |
| Vascular surgery history (%) | 2.5     | 53.1  | <0.001  | 62.5  | 40.8  | ns      |
| Medication use (%)          |         |       |         |       |       | <0.001  |
| - Statins                   | 2       | 30    | <0.001  | 40.6  | 8.2   | <0.001  |
| - Anti-platelet agents:     |         |       |         |       |       |         |
| - Aspirin                   | 0       | 48.7  | <0.001  | 42.1  | 57.1  | ns      |
| - Clopidogrel               | 0       | 26.5  | <0.001  | 39.1  | 10.2  | 0.001   |
| - Aspirin + Clopidogrel     | 0.4     | 3.5   | ns      | 6.25  | 0    | ns      |
| - Iloprost                  | 0       | 9.7   | <0.001  | 1.6   | 20.4  | 0.001   |
| - Statins + antiplatelet agents | 0.4       | 23.9  | <0.001  | 37.5  | 6.1   | <0.001  |
| - none                      | 97.5    | 16.8  | <0.001  | 12.5  | 22.4  | ns      |

Results as mean ± SD; ns: no significant different when p>0.05.

(PPAD, premature peripheral arterial diseases; PAOD, peripheral arterial occlusive disease; TAO, thromboangiitis obliterans).

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| Study characteristics          | Controls n = 241 | PPAD n = 113 | PAOD n = 64 | TAO n = 49 | PAOD vs TAO p-value |
|-------------------------------|-----------------|-------------|-------------|------------|---------------------|
| **Family history (1st-degree relatives)** |                 |             |             |            |                    |
| Arterial diseases (%)        |                 |             |             |            |                    |
| – Peripheral arterial disease| 10              | 17.8        | 0.04        | 21.3       | 13                 | ns                  |
| – Occlusive carotid artery disease| 2.1            | 7.5         | 0.015       | 11.4       | 2.17               | ns                  |
| – Coronary artery disease    | 28.2            | 26.2        | ns          | 36.1       | 13                 | 0.007               |
| – Aortic abdominal aneurysm  | 1.3             | 1.9         | ns          | 0          | 4.4                | ns                  |
| – None                        | 68.9            | 63.7        | ns          | 56.3       | 73.5               | ns                  |
| Others vascular diseases (%) |                 |             |             |            |                    |
| – Raynaud’s disease           | 2.1             | 2.8         | ns          | 1.7        | 4.3                | ns                  |
| – Venous thromboembolism      | 15              | 16.8        | ns          | 16.4       | 17.4               | ns                  |
| – None                        | 83              | 82.3        | ns          | 82.8       | 81.6               | ns                  |
| **Patient characteristics**  |                 |             |             |            |                    |
| Physical exercice (%)        |                 |             |             |            | <0.001             | ns                  |
| – Never                      | 24.9            | 52          | 47.6        | 58.6       |                    | ns                  |
| – Sometimes                  | 57.7            | 27          | 33.3        | 17.2       |                    |                    |
| – Intense                    | 17.4            | 21          | 19.1        | 24.2       |                    |                    |
| Body mass index (kg/m²)      |                 |             |             |            |                    |
|                              | 24.5±3.5        | 24.5±3.6    | ns          | 24.6±3.8   | 24.4±3.5           | ns                  |
| Hypertension (%)             |                 |             |             |            |                    |
|                              | 48.5            | 32.1        | 0.004       | 42.6       | 18.8               | 0.008               |
| Smoking status (%)           |                 |             |             |            | <0.001             | ns                  |
| – Never                      | 31.1            | 6.2         | 4.7         | 8.2        |                    |                    |
| – Former                     | 34              | 27.4        | 32.8        | 20.4       |                    |                    |
| – Current                    | 34.9            | 66.4        | 62.5        | 71.4       |                    |                    |
| Pack years                   |                 |             |             |            |                    |
|                              | 6.7±7.4         | 24.6±16.6   | <0.001      | 26.4±17.7  | 22.4±15            | ns                  |
| Urinary cotinine (µg/dL)     |                 |             |             |            |                    |
|                              | 58±99           | 103±127     | 0.013       | 113±122    | 94±136             | ns                  |
| Urinary THC* (%)             |                 |             |             |            |                    |
|                              | 5.5             | 9           | ns          | 2.5        | 17.1               | 0.03                |
| **Biology**                  |                 |             |             |            |                    |
| Triglycerides (mg/dL)        |                 |             |             |            |                    |
|                              | 140±96          | 149±114     | ns          | 166±140    | 140±52             | ns                  |
| Total cholesterol (mg/dL)    |                 |             |             |            |                    |
|                              | 209±39          | 197±46      | 0.013       | 197±50     | 201±39             | ns                  |
| HDL-cholesterol (mg/dL)      |                 |             |             |            |                    |
|                              | 54.2±11.6       | 46.4±11.6   | <0.001      | 42.5±11.6  | 46.4±11.6          | ns                  |
| LDL-cholesterol (mg/dL)      |                 |             |             |            |                    |
|                              | 131.6±31        | 124±38.7    | ns          | 120±38.7   | 127.7±35           | ns                  |
| Total chol/HDL-chol          |                 |             |             |            |                    |
|                              | 4.1±1           | 4.6±1.5     | ns          | 4.7±1.6    | 4.6±1.2            | ns                  |
| ApoA-I (mg/dL)               |                 |             |             |            |                    |
|                              | 150±20          | 130±30      | 0.001       | 130±30     | 130±30             | ns                  |
| ApoB (mg/dL)                 |                 |             |             |            |                    |
|                              | 90±20           | 90±30       | ns          | 90±30      | 100±30             | ns                  |
| ApoA-1/ApoB                  |                 |             |             |            |                    |
|                              | 1.7±0.5         | 1.5±0.6     | ns          | 1.5±0.6    | 1.5±0.5            | ns                  |
| Lp(a) (mg/dL)                |                 |             |             |            |                    |
|                              | 25.7±25.4       | 46.8±25.4   | ns          | 57.5±63.4  | 32.5±32.3          | 0.02                |
| hsCRP* (µg/L)                |                 |             |             |            |                    |
|                              | 2.3±4.8         | 6.6±9.2     | 0.015       | 4.7±6      | 9.3±12             | ns                  |
| Fibrinogen (mg/dL)           |                 |             |             |            |                    |
|                              | 330±60          | 360±10      | ns          | 340±90     | 380±120            | ns                  |
| Homocystein (µmol/L)         |                 |             |             |            |                    |
|                              | 12.8±5.8        | 12±5.6      | ns          | 12.1±6.1   | 11.4±4.9           | ns                  |
| B6 vitamin (active form) (nmol/L) | 50.6±20.4    | 27.5±15.8   | <0.001      | 28.1±15.1  | 25.1±15.9          | ns                  |
| B9 vitamin (nmol/L)          |                 |             |             |            |                    |
|                              | 12.2±4.8        | 12.3±7.1    | ns          | 12.4±8.2   | 12.1±5.8           | ns                  |
| B12 vitamin (pmol/L)         |                 |             |             |            |                    |
|                              | 337±123         | 387±217     | 0.016       | 381±178    | 393±257            | ns                  |
| Plasma Copper (µmol/L)       |                 |             |             |            |                    |
|                              | 16.1±4.5        | 17.8±4.5    | 0.001       | 16.6±3.4   | 19.2±5.2           | 0.03                |
| Plasma Selenium (µmol/L)     |                 |             |             |            |                    |
|                              | 1.1±0.2         | 0.9±0.2     | ns          | 1.0±0.2    | 0.9±0.2            | ns                  |
| Plasma Zinc (µmol/L)         |                 |             |             |            |                    |
|                              | 14.6±2.4        | 13.2±2.7    | 0.003       | 13.3±2.7   | 13.2±2.7           | ns                  |

Results as mean ± SD; ns: no significant different when p > 0.05.

*THC: tetrahydrocannabinol, 1 Lp(a): lipoprotein(a), 2 hsCRP: ultrasensible C-reactive protein.

(PPAD, premature peripheral arterial diseases; PAOD, peripheral arterial occlusive disease; TAO, thromboangiitis obliterans).

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may be a consequence of an acute phase response known in these patients and might contribute to the pathological process as mentioned elsewhere [25].

In PAOD patients, specific PAD risk factors such as higher Lp(a) levels, presence of F5 Leiden and ITGA2807T,837T,873A allele as well as a family history of peripheral and carotid artery diseases were demonstrated. As Lp(a) level is mostly genetically determined and is unaffected by statins, we were able to confirm the correlation demonstrated. As Lp(a) level is mostly genetically determined and as a family history of peripheral and carotid artery diseases were described by others [26]. The pathophysiological role of Lp(a) is not well understood, but at least two properties may be attributed to Lp(a): 1) once oxidized, Lp(a) contributes to foam cell formation; 2) Lp(a) attenuates fibrinolysis and promotes coagulation [27]. Thrombophilic conditions could be also promoted by the presence of F5 Leiden, F220210A, ITGA2807T,837T,873A and ITGB3PlA2 alleles. In our study, premature PAD in PAOD patients was associated with an increased prevalence of F5 Leiden and ITGA2807T,837T,873A but not of F220210A or ITGB3PlA2 alleles. A recent meta-analysis on genetic association studies in PAD including 2,466 patients, selected independently to their age-of-onset and to the presence or not of diabetes, and 2,686 controls demonstrated that neither F5 nor F2 Leiden mutations were risk factors for PAD [28]. In the present study, inclusion of non-diabetic patients with a mean age-of-onset under 45 years may explain the difference. The increased frequency observed for the F5 heterozygous mutation indicates that, in young patients, these common thrombophilia factors participate in uncovering underlying vessel alterations. In the older patients included in the meta-analysis, the prevalence (and probably the severity) of vessel alteration exceeds largely that of thrombophilia whose additive contribution in symptoms onset is no more detectable. F5 Leiden is a common gain-of-function mutation, which leads to resistance to activated protein C: it modifies one of the cleavage sites in factor V, where activated protein C inactivates factor Va. The delay in factor Va destruction facilitates overproduction of thrombin leading to excess fibrin generation and excess clotting. On the other hand, we demonstrated for the first time that polymorphisms leading to excess fibrin generation and excess clotting. On the other hand, we demonstrated for the first time that polymorphisms in platelet glycoprotein Ia (ITGA2807T,837T,873A) are associated with peripheral arterial disease in premature PAOD. The ITGA2807T,837T,873A allele has been associated with increased levels

Table 3. Gene Polymorphisms in the All Study Population.

|              | Controls | PPAD | p-value | PAOD | p-value | TAO | p-value |
|--------------|----------|------|---------|------|---------|-----|---------|
| MTPP<sup>305G>T</sup> (rs1800591) |          |      |         |      |         |     |         |
| G allele     | 27.6     | 29.0 | ns      | 31   | ns      | 27.2| ns      |
| T allele     | 72.4     | 71.0 |         | 69   |         | 72.8|         |
| CETF-TaqI (rs708272) |          |      |         |      |         |     |         |
| B1 allele    | 43.5     | 36.5 | ns      | 34.5 | ns      | 39.2| ns      |
| B2 allele    | 56.5     | 63.5 |         | 65.5 |         | 60.8|         |
| ABC-A1<sup>19021G>A</sup> (rs2230806, intron 8) |          |      |         |      |         |     |         |
| G allele     | 71.9     | 72.9 | ns      | 74.2 | ns      | 71.8| ns      |
| A allele     | 28.1     | 27.1 |         | 25.8 |         | 28.2|         |
| APOE<sup>c2</sup> (rs7412) |          |      |         |      |         |     |         |
| c2 allele    | 6.3      | 5.6  | ns      | 4.3  | ns      | 6.5 | ns      |
| c3 allele    | 82.1     | 83.6 |         | 84.5 |         | 83.7|         |
| c4 allele    | 11.6     | 10.8 |         | 11.2 |         | 9.8 |         |
| PPARG<sup>1139484,1139984,1062535</sup> |          |      |         |      |         |     |         |
| T allele     | 88.6     | 88.3 | ns      | 89.7 | ns      | 87.0| ns      |
| C allele     | 11.4     | 11.7 |         | 10.3 |         | 13.0|         |
| SELP<sup>1799963</sup> |          |      |         |      |         |     |         |
| A allele     | 88.0     | 85.5 | ns      | 82.8 | ns      | 89.1| ns      |
| C allele     | 12.0     | 14.5 |         | 17.2 |         | 10.9|         |
| F2<sup>200100A</sup> (rs1799963) |          |      |         |      |         |     |         |
| G allele     | 98.4     | 96.8 | ns      | 97.4 | ns      | 95.8| 0.03    |
| A allele     | 1.6      | 3.2  |         | 2.6  |         | 4.2 |         |
| F5<sup>873G>A</sup> Leiden (rs6025) |          |      |         |      |         |     |         |
| G allele     | 99.0     | 95.5 | 0.003   | 95.0 | 0.003   | 97.9| ns      |
| A allele     | 1.0      | 4.5  |         | 5.0  |         | 2.1 |         |
| ITGA2 (gpIIb/IIIa) |          |      |         |      |         |     |         |
| allele 1     | 35.7     | 47.6 | 0.001   | 50.0 | 0.001   | 43.5| ns      |
| allele 2     | 50.2     | 44.4 | ns      | 44.8 | ns      | 45.6| ns      |
| allele 3     | 14.1     | 8.0  | 0.02    | 5.2  | 0.01    | 10.9| ns      |
| ITGB3<sup>385</sup> (rs5918) |          |      |         |      |         |     |         |
| P<sup>A1</sup> | 80.4 | 81.4 | ns     | 78.4 | 0.01     | 83.7| ns    |
| P<sup>A2</sup> | 19.6 | 18.6 |        | 21.6 |         | 16.3|         |

*allele 1 = 807T/837T/873A, allele 2 = 807C/837T/873G, allele 3 = 807C/837C/873G

ns: no significant different when p>0.05.

(PPAD, premature peripheral arterial diseases; PAOD, peripheral arterial occlusive disease; TAO, thromboangiitis obliterans).
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of glycoprotein Ia-IIa (α2β1 integrin) on platelets which could enhance platelet adhesion to collagen and thus contribute to an increased risk of thrombosis. Indeed, patients with atherothrombotic manifestations have been demonstrated to have a higher frequency of ITGA2807T associated with higher levels of platelet glycoprotein Ia-IIa [29]. In addition, higher platelet activation has been shown in patients with lower limb ischemia [30] and the highest activation rate was found in patients with the most severe PAD [31]. Involving platelet pathophysiology in PAD is in line with the recognition of the importance of platelets in chronic inflammatory processes such as atherosclerosis. Altogether, our findings could explain the use of clopidogrel in PAOD patients. On contrary, the use of aspirin is more controversial but it may be explained more by its pleiotropic effects, including protection of LDL and fibrinogen from oxidation [32].

### Table 4. Risk Factors for PAOD Patients and TAO.

| Parameters                          | PAOD (n = 64) vs controls (n = 241) OR [95% CI] | TAO (n = 49) vs controls (n = 241) OR [95% CI] |
|------------------------------------|---------------------------------------------|---------------------------------------------|
| Physical exercise (never)          | 2.7† [1.4–5.4]                             | 4.3† [1.9–9.5]                             |
| Smoking (current or former)        | 9.2† [2.8–30.2]                            | 5.1† [1.8–14.6]                            |
| Package x year number              | 17.1† [8.7–33.6]                           | 15.9† [7.4–34.3]                           |
| Urinary cotinin >50 ng/mL          | 3.3† [1.7–6.3]                             | 3.6† [1.7–7.7]                             |
| HDL-cholesterol <40 mg/dL          | 5.2† [2.7–10.1]                            | 5.2† [2.5–10.6]                            |
| ApoA-I <130 mg/dL                  | 4.9† [2.7–8.9]                             | 4.3† [2.2–8.1]                             |
| ApoA-I/ApoB <1                   | 5.7† [2.15–15.2]                           | 6.7† [2.5–18.5]                            |
| B6 vitamin (active form) <20 nmol/L | 23.4† [5.14–107]                          | 38.3† [8.1–18]                             |
| Plasma zinc <12 μmol/L             | 3.4† [1.63–7]                              | 3.9† [2–7.4]                               |
| Plasma zinc/copper ratio <0.7     | 2.6† [1.4–5.1]                             | 5.1† [2.6–10]                              |
| Family history Peripheral arterial disease Occlusive carotid artery disease | 2.3† [1.1–4.8]                             | ns                                          |
| Lp(a) >30 mg/dL                    | 2.3† [1.3–4]                               | ns                                          |
| Factor V Leiden presence          | 5.1† [1.5–17.4]                            | ns                                          |
| gpIa allele 1 presence            | 3.0† [1.5–5.9]                             | ns                                          |
| gpIa allele 3 presence            | 0.3† [0.13–0.8]                            | ns                                          |
| Urinary THC >50 ng/mL             | ns                                         | 3.5† [1.3–10]                              |
| hsCRP >0.24 mg/dL                  | ns                                         | 8.2† [4.1–16.3]                            |
| Fibrinogen >370 mg/dL              | ns                                         | 3.4† [1.7–7.7]                             |
| Plasma copper >18 μmol/L           | ns                                         | 6.5† [3.3–12.7]                            |

*p < 0.001, † p < 0.01, ‡ p < 0.05; ns: no significant different when p > 0.05.
(PAOD, peripheral arterial occlusive disease; TAO, thromboangiitis obliterans).
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(cigarette smoking, lack of physical activity, decreased HDL-cholesterol and apoA-I, high Lp(a) levels, hypercoagulable states) cannot explain the development of premature PAOD. Several lines of evidence indicate that susceptibility to premature atherosclerosis of the coronary and cerebral vasculature is heritable. In our study, premature symptomatic vascular disease (peripheral and occlusive carotid artery diseases) was significantly more prevalent in family members of premature PAOD patients than in family members of healthy individuals from the control group. Our results provide strong evidence for familial aggregation of arterial diseases in the first-degree relatives of these patients as described elsewhere [9]. It may therefore reflect the clustering in some families of genes (such as genes encoding hemostatic factors, Lp(a) or platelet glycoproteins) that confer susceptibility to atherosclerosis. Thus premature PAOD patients seem to be
genetically susceptible to the effects of environmental factors such as cigarette smoking and sedentary lifestyle.

TAO patients were shown to present specific vascular risk factors such as cannabis consumption, higher copper concentration as well as higher hsCRP and fibrinogen levels. This last point, i.e. inflammation, has already been discussed above with a possible bias due to their pathology and associated with diminished functional capacity. In respect of cannabis consumption, cannabis arterial disease has been described [33] and a vasoconstrictor effect of THC seems probable. Furthermore cannabis consumption is often associated to tobacco smoking [34] and a synergistic deleterious effect of cannabis and tobacco seems likely, along with that of a probable common contaminant such as arsenic. As a matter of fact, a positive relationship between plasmatic arsenic levels and the severity of TAO has been demonstrated [35]. In addition, Cannabis sativa has a very high capability to absorb and accumulate heavy metals such as copper [36]. In the present study, patients with TAO presented higher plasma copper concentrations. Thus, environmental factors such as cannabis consumption associated with cigarette smoking may play a major role in the development and progression of the vascular disease in patients with TAO.

In conclusion, our present study demonstrated that the risk of developing premature PAD was significantly increased in current or former smokers versus non-smokers, in cases with low plasma concentrations in HDL-cholesterol (below 0.40 mg/dL) and apoA-I (below 130 mg/dL), active form of B6 vitamin (below 20 nmol/L) and zinc (below 12 μmol/L) with a zinc/copper ratio less than 0.7). Moreover each group of patients showed specific risk factors. In one hand, our results support the importance of environmental factors in the development of TAO. Particularly our case-control study shows more frequent cannabis consumption in patients with TAO compared with controls or patients with premature PAOD. This result is particularly relevant even if more scientific reports need to be forthcoming to support the hypothesis of cannabis being a causative factor or co-factor of TAO. For this group of patients, it is also noticeable that female patients with PPAD may have clinical characteristics of TAO. It might be proposed that Adar’s criteria for inclusion of patients with TAO should take the inclusion of female patients into account. On the other hand, high Lp(a) levels above 300 mg/L as well as the presence of a family history of peripheral and carotid artery diseases, the F5 Leder and the HLA2G087/T07F874 allele were specifically linked to premature PAOD. These results led us to hypothesize for a generic basis for premature PAOD development. Moreover and for the first time, our study demonstrated differences in allelic frequencies of platelet glycoprotein Ia which seem to be associated with a different predisposition to atherothrombosis in premature PAOD.

### Supporting Information

#### Table S1  List of Used Specific Primers and Restriction Enzymes.

(DOC)

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### Author Contributions

Conceived and designed the experiments: AMB AB RL GF AN SC VG MCV FB GC LB PL JPC ABR JE MD AMD SM BC VV JC. Performed the experiments: AMB RLT GF SC VG MCV FB GC LB PL JPC ABR JE SM BC VV JC. Analyzed the data: AMB AB RL GF AN SC VG MCV FB GC LB PL JPC ABR JE MD AMD SM BC VV JC. Contributed reagents/materials/analysis tools: AMB AB GF AN SC VG MCV FB GC LB PL JPC ABR JE MD AMD SM BC VV JC. Wrote the paper: AMB AB RL GF AN SC VG MCV FB GC LB PL JPC ABR JE MD AMD SM BC VV JC.

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