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Mitochondrial genome association study with peripheral arterial disease and venous thromboembolism

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ABSTRACT

Background and aims: Peripheral arterial disease (PAD) and venous thromboembolism (VTE) are vascular traits sharing common modifiable and non-modifiable risk factors. These vascular pathologies have known nuclear-encoded genetic risk factors and the mitochondrial DNA may account for part of the missing heritability. To determine if PAD and VTE have a dual genetic control (mitochondrial and nuclear), we hereby investigated the association of mitochondrial DNA polymorphisms and haplogroups with these vascular traits.

Methods: The association of mitochondrial single nucleotide polymorphisms (mtSNPs) and haplogroups was tested in 1652 PAD cases and 1629 controls from the eMERGE PAD genome-wide association study (GWAS), and 1241 VTE cases and 1278 controls from the GENEVA GWAS of venous thrombosis (dbGaP accession numbers phs000203.v1.p1 and phs000289.v2.p1, respectively).

Results: 66 and 72 mtSNPs passed quality control filters and were tested for association with PAD and VTE, respectively. Significant evidence of population stratification could not be detected in both datasets. Three mtSNPs (m.477T > C, m.9667A > G, and m.10915T > C) were nominally associated ($3.01 \times 10^{-3} \leq p_a \leq 3.96 \times 10^{-2}$) with PAD in the logistic regression adjusted for confounding factors, and m.11914G > A was nominally associated ($p_a = 4.14 \times 10^{-2}$) with VTE. None of the nine major mitochondrial haplogroups were associated with either PAD or VTE.

Conclusion: Unlike other vascular diseases such as stroke and diabetes, these results suggest that common mitochondrial variants individually or in combination do not play a major role in PAD and VTE susceptibility.

1. Introduction

Peripheral arterial disease (PAD) and venous thromboembolism (VTE) are frequent vascular pathologies with considerable morbidity and/or mortality. PAD is most commonly an atherosclerotic disease characterized by progressive arterial narrowing in the limbs while VTE, consisting of deep vein thrombosis (DVT) and pulmonary embolism (PE), is a thrombotic disorder resulting from hypercoagulation, vascular endothelial damage and reduction of the blood flow (known as the Virchow triad). These complex phenotypes share risk factors such as age and obesity, and genetics is estimated to account for approximately half of their phenotypic variance [1–4]. The genetic architecture of PAD and VTE has been investigated through candidate gene and genome-wide association studies (GWAS). Numerous nuclear-encoded genetic variants have been associated with these phenotypes, including the 9p21, *PAX2* and *ATXN2-SH2B3 loci* for PAD [5,6], and the Factor V Leiden, prothrombin and ABO blood group for VTE [7–9]. However, these *loci* explain only a fraction of the estimated heritability (e.g. population-attributable risk of 40% for VTE) [8], suggesting that other genetic factors must be involved.

The mitochondrial DNA (mtDNA) and/or its interaction with the nuclear genome may partially explain the missing heritability.

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Human mitochondria are extranuclear organelles which contain circular, haploid, and maternally inherited genomes with 16.6 kilobases, encoding for thirteen genes involved in oxidative phosphorylation, two ribosomal RNA genes, and twenty-two transfer RNA genes. Mitochondrial primary function is the production of energy through the oxidative phosphorylation. Notably, these organelles play a vital role also in other cellular processes including cholesterol synthesis, fatty acid oxidation, ammonia detoxification, calcium homeostasis, and apoptosis, suggesting that mitochondrial dysfunction could have far-reaching effects. Indeed, mitochondrial dysfunction and oxidative stress are observed in vascular aging [10], inflammation and plaque rupture [11], tobacco smoke and alcohol exposure [12], and in late onset vascular and inflammatory disorders such as myocardial infarction and stroke [13], hypertension [14], diabetes [15], PAD [16,17], and thrombosis [18]. There is also increasing evidence that these organelles are implied in the vascular cell growth and function by favoring apoptosis of endothelial cells, macrophages and/or vascular smooth muscle cells, along with promoting thrombosis and inflammation [19].

Common mtDNA variants have been associated with risk for vascular diseases such as stroke [20–22] and diabetes [23], as well as with quantitative traits variance for phenotypes known to play a crucial role in vascular disorders such as triglycerides [24] and HDL-C [25] levels. Even though most of the microarrays used in PAD and VTE GWAS include mitochondrial single nucleotide polymorphisms (mtSNPs), their association was not investigated or reported and remains unexplored. To determine if the mitochondrial genome accounts for part of the unknown heritability in PAD or VTE, we hereby tested the association of mtSNPs and haplogroups in 1652 PAD cases and 1629 healthy controls and in 1241 VTE cases and 1278 controls, respectively, from the GWAS data deposited in the NCBI Database of Genotypes and Phenotypes (dbGaP).

2. Methods

2.1. Study subjects and genotyping

The criteria and procedures for the recruitment of patients and controls are described in detail elsewhere [6,8]. Succinctly, PAD cases had: 1) an ankle brachial index (ABI) $\leq$ 0.9 at rest or 1 min after exercise, with an abnormal continuous wave Doppler signal in one of the lower extremity arteries; 2) a normal ABI with a history of lower extremity revascularization; and 3) ABI $\geq$ 1.4 or ankle systolic blood pressure > 250 mmHg. Patients with PAD secondary to vasculitis, abdominal or lower extremity radiation, lower extremity trauma, thrombophilia and arterial thrombosis were excluded. PAD controls were individuals with negative exercise electrocardiogram, older than 50 years of age, normal ABI and without PAD history. VTE patients were adults with deep vein thrombosis and/or pulmonary embolism confirmed by either: 1) venography or pulmonary angiography, or pathology examination of thrombus removed at surgery; 2) at least one non-invasive positive test (compression duplex ultrasonography, lung scan, CT scan, or MRI). Patients with VTE related to active cancer, a mechanical cause for DVT, an antiphospholipid syndrome, vasculitis, vascular anomalies, autoimmune disorders, prior bone marrow or liver transplantation were excluded. VTE controls were individuals frequency-matched for the age group, gender, state of residence distribution and myocardial infarction (MI)/stroke status of the cases, without a previous diagnosis of VTE or superficial vein thrombosis, active cancer, an antiphospholipid antibody syndrome, rheumatologic or other autoimmune disorder, prior bone marrow or liver transplant.

DNA samples from the PAD and VTE datasets were genotyped using the Illumina 660W-Quad_v1_A BeadChip (Illumina, San Diego, California, USA) at the Broad Institute and the John Hopkins University Center for Inherited Diseases Research, respectively. Full genotyping details can be found in Kullo et al. [6] and Heit et al. [26]. The genotype and phenotype data investigated in this study are deposited in the Database of Genotypes and Phenotypes [27], respectively under the dbGaP accession numbers phs000203.v1.p1 and phs000289.v2.p1 (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gap). Additional quality control (QC) was performed: call rate $\geq$ 0.90; only nuclear SNPs in Hardy-Weinberg equilibrium (HWE) ($p > 1.00 \times 10^{-5}$ in the control group) were considered for stratification analysis; heterozygote mitochondrial genotypes were considered as missing; individuals found to be duplicated in the datasets, with missing affection status, gender discrepancies, and with missing or non-European ancestry were excluded.

2.2. Population stratification

The presence of population stratification in each dataset under study was assessed using identity-by-state (IBS) multidimensional scaling (MDS) implemented in PLINK 1.9 (http://pngu.mgh.harvard.edu/purcell/plink/) [28]. For the MDS analysis, all nuclear SNPs that passed quality control and had a minor allele frequency (MAF) $\geq$ 0.05 were pruned such that all SNPs within a given window size of 50 had a variance inflation factor (VIF) of 2.0. VIF is defined as $1/(1\text{-}R^2)$, where R^2 is the multiple correlation coefficient between a SNP and all other SNPs in the window based on allele counts. Pairwise IBS distances were calculated using all autosomal SNPs that remained after pruning. Five nearest neighbors were identified for each individual based upon the pairwise IBS distance. IBS distance to each of the five nearest neighbors was then transformed into a Z score. Individuals with a minimum Z score among the five nearest neighbors < -4 were excluded from analysis as population outliers. MDS dimensions were extracted using the mds-plot option and population structure visualized using the R v.3.2.1 freeware (http://cran.r-project.org/) with or without HapMap reference samples (http://www.hapmap.org, release #23) from four populations (60 CEU – U.S. Caucasians with Northern and Western European ancestry; 60 YRI – Yoruba individuals from Ibadan, Nigeria; 45 CHB – Han Chinese individuals from Beijing, China; and 45 JPT – Japanese individuals from Tokyo, Japan). The genomic inflation factor λ was computed using PLINK 1.9 and is used to correct chi-square association tests when stratification exists ($\lambda > 1.0$) [29].

2.3. mtDNA haplogroup classification

Given the non-recombining nature of mtDNA, haplogroups are defined by the specific arrangement of alleles at multiple mtSNPs throughout the molecule, rather than the status at any particular genetic variation. For both datasets under study, we classified each individual into mtDNA haplogroups using genotypes available at all mtSNPs in each dataset, including polymorphisms not passing quality control for association testing (MAF < 1.0% in the control group and/or call rate < 90%) since they may be of phylogenetic relevance. Haplogroup classification was performed automatically using the HaploGrep [30] software (http://www.haplogrep.uibk.ac.at) and checked manually using the PhyloTree mtDNA tree (build 16) as a reference [31].

2.4. Statistical analyses

Chi-square and T-tests were used to compare discrete and continuous variables, respectively, between cases and their respective controls. For haplogroups' analyses, only those present in $\geq$1.0% of the controls were tested for association and each

haplogroup was compared with all others pooled together. Rare haplogroups (<1.0%) were included in the "others" category but, since no phylogeographic reasoning exists for this category, its association was not assessed.

Multivariate logistic regression with backward elimination of risk factors was performed in R to explore the association of each mtDNA SNP (with MAF $\geq$ 0.01 in the control group) or haplogroup with PAD or VTE (adjusted *p*-value p_a). In the PAD dataset, hypertension, history of coronary heart disease (CHD), history of cerebrovascular disease (CVD), diabetes (type 1 or type 2), dyslipidemia, and ever smoking were included as covariates. For VTE, prior stroke or myocardial infarction (stroke/MI) and state of residence in the USA (Minnesota versus other states) was included as a covariate in adjusted analyses. In each dataset, the interaction i among the covariates in the regression model was not strong ($-0.5 < i < 0.5$, data not shown).

Results were considered nominally significant below the conventional level of 5.00×10^{-2}. Since the haplogroup comparisons are not independent and some mtSNPs are in linkage disequilibrium, corrections for multiple testing were not performed in the mtSNP and haplogroup association analyses and uncorrected *p-values* are reported.

2.5. Ethical considerations

This study was granted approval by the ethics committee from the Centro Hospitalar Lisboa Norte, E.P.E./Faculdade de Medicina de Lisboa, Lisboa, Portugal.

3. Results

3.1. Original dbGaP datasets

After our quality controls (e.g. ancestry, HWE, minimum call rate and MAF), the eMERGE PAD dbGaP database (accession number phs000203.v1.p1) includes clinical and demographic data from 1662 PAD cases and 1633 controls, with respective genotype data at 525081 nuclear-encoded and 66 mitochondrial SNPs, while the GENEVA VTE dbGaP database (accession number phs000289.v2.p1) includes clinical and demographic data from 1252 VTE cases and 1286 controls, with respective genotype data at 494507 nuclear-encoded and 72 mitochondrial SNPs.

3.2. Population stratification

A major concern of association studies is genetic heterogeneity that may lead to false positive results or failure to detect true associations. The effect of population stratification is particularly problematic in mtDNA studies owing to its smaller effective population size when compared to autosomal markers [32]. To rule out the presence of hidden ancestry and/or geographic substructure in the datasets under investigation, we performed MDS analyses using 147371 and 122440 autosomal pruned SNPs for the PAD and VTE datasets, respectively. Using the criteria defined in the methods section, 14 PAD and 19 VTE individuals were excluded as outliers. Figs. 1 and 2 depict the first two dimensions of MDS analyses for PAD and VTE, respectively, with (Figs. 1A and 2A) or without (Figs. 1B and 2B) HapMap samples. For both conditions, the MDS distribution of cases and controls seems to overlap, with no major outliers. Furthermore, a genomic inflation factor λ of 1.04 for PAD and of 1.01 for VTE after removal of the outliers supports the inexistence of major stratification in both datasets and therefore adjustment for ancestry in association tests is not required.

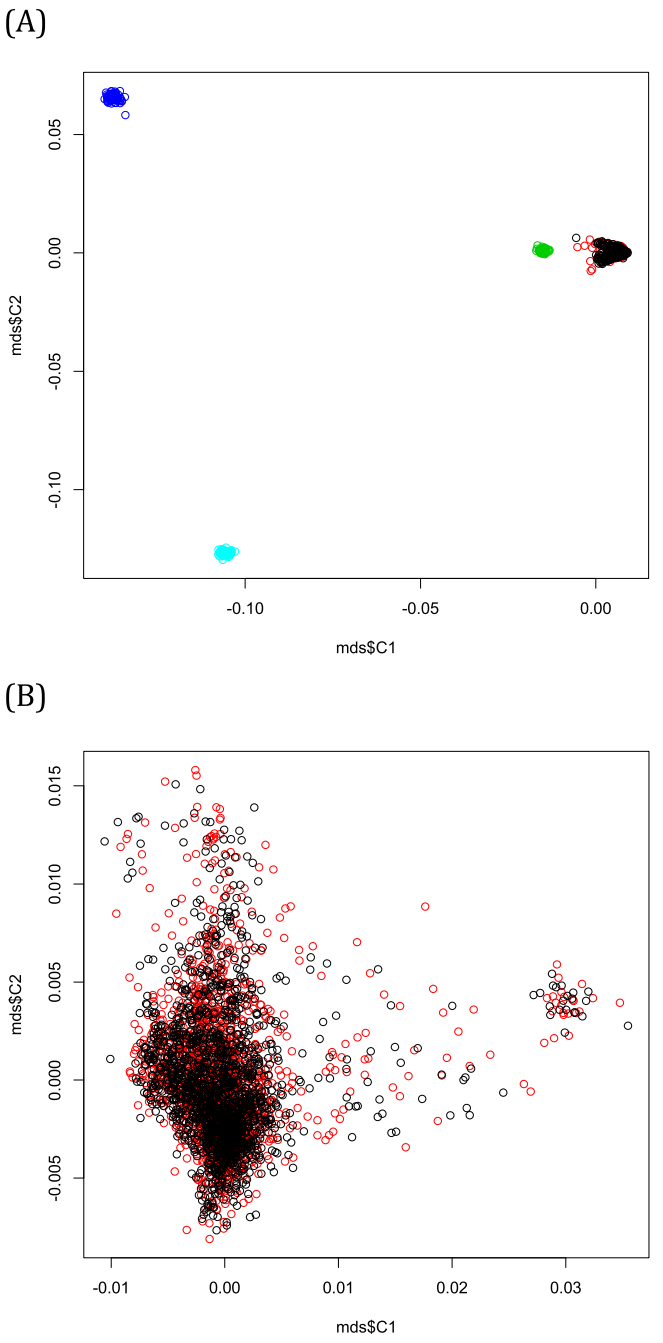

Fig. 1. Population stratification analysis in the peripheral arterial disease (PAD) dataset. The first two MDS (multidimensional scaling) dimensions (mds$C1 and mds$C2) are plotted for the 1652 PAD cases and 1629 controls (after outliers' exclusion) with (A) or without (B) HapMap reference samples (60 CEU – US Caucasians with Northern and Western Europe ancestry; 60 YRI – Yoruba people of Ibadan, Nigeria; 45 CHB – individuals from Beijing, China; 45 JPT – individuals from Tokyo, Japan). PAD cases, PAD controls, CEU, YRI, CHB and JPT HapMap samples are shown in black, red, green, turquoise and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Characterization of datasets

The main demographic and clinical characteristics of PAD (1652 cases and 1629 controls) and VTE (1241 cases and 1278 controls) samples remaining after QC and population stratification exclusions are summarized in Table 1. Even though PAD patients and controls have significantly different gender and age distributions

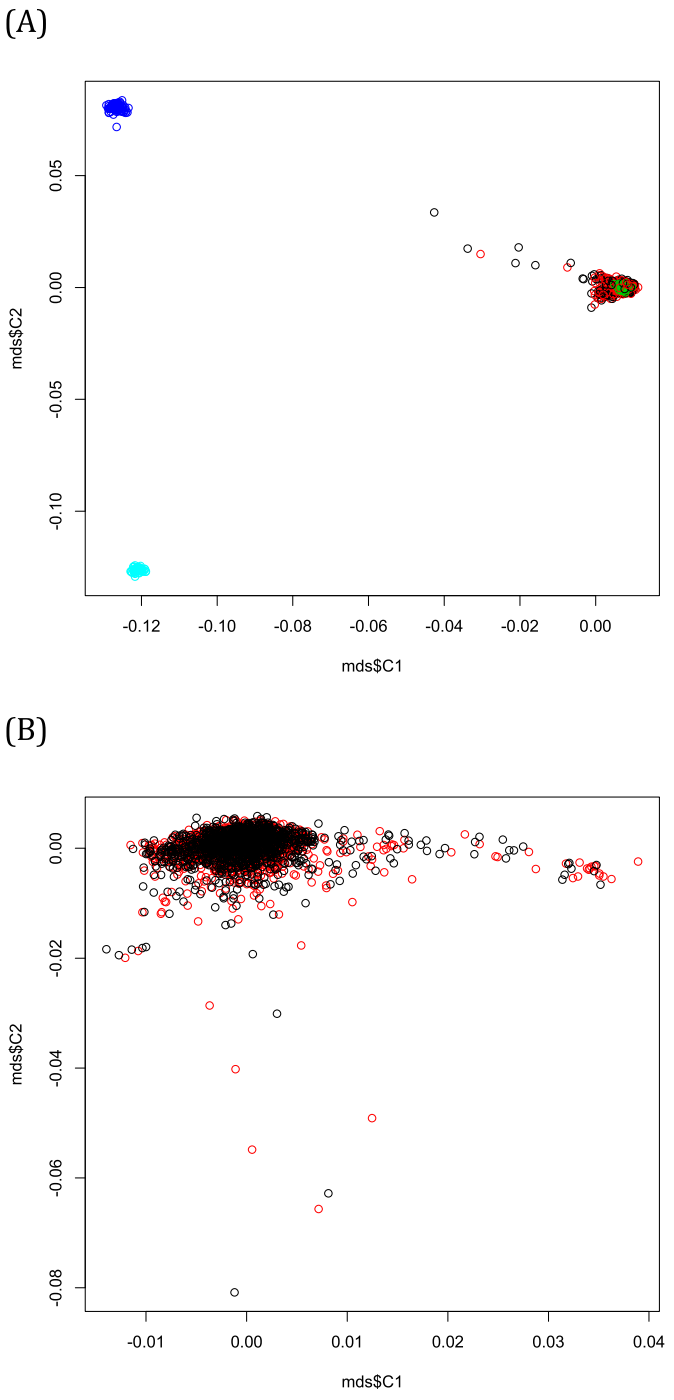

Fig. 2. Population stratification analysis in the venous thromboembolism (VTE) dataset. The first two MDS (multidimensional scaling) dimensions (mds$C1 and mds$C2) are plotted for the 1241 VTE cases and 1278 controls (after outliers' exclusion) with (A) or without (B) HapMap reference samples (60 CEU – US Caucasians with Northern and Western Europe ancestry; 60 YRI – Yoruba people of Ibadan, Nigeria; 45 CHB – individuals from Beijing, China; 45 JPT – individuals from Tokyo, Japan). VTE cases, VTE controls, CEU, YRI, CHB and JPT HapMap samples are shown in black, red, green, turquoise and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

($p = 2.40 \times 10^{-2}$, and $p < 2.20 \times 10^{-16}$, respectively), gender was not significant in the multivariate logistic regression model and therefore was not included as a covariate in the analysis. As expected, risk factors for PAD (hypertension, CHD, CVD, diabetes, dyslipidemia, and ever smoking) are significantly ($p < 1.00 \times 10^{-7}$) more frequent in cases than in controls and were included as covariates in adjusted association tests. On the other hand, VTE patients and controls have similar gender distributions ($p = 4.20 \times 10^{-1}$) and smoking status ($p = 1.40 \times 10^{-1}$) but differ in the average of age-at-examination ($p = 4.25 \times 10^{-2}$), prior stroke or myocardial infarction ($p = 1.94 \times 10^{-8}$), and state of residence within the USA ($p = 4.00 \times 10^{-10}$). In the multivariate logistic regression with backward elimination of factors, only the former two variables were significant and remained in the final model of the adjusted association analyses.

3.4. Association analyses

Since the post-QC datasets and analyses in this study differ slightly from those used in the published GWASs [6,26], we first validated our sample set by confirming the association of top GWAS findings. SNPs rs653178, rs11726269, and rs131408 were found associated with PAD in the discovery cohort of Kullo et al. [6] with p-values ranging from 4.30×10^{-5} to 7.94×10^{-5}, while in the current dataset they are associated with $6.40 \times 10^{-5} \leq p_a \leq 1.91 \times 10^{-3}$. SNPs rs495828 and rs16861990 were reported to be associated with VTE in the discovery dataset of Heit et al. [26] with $2.96 \times 10^{-16} \leq p \leq 1.69 \times 10^{-12}$, while in the current dataset the associations ranged from 1.52×10^{-13} to $< 2.00 \times 10^{-16}$. The confirmation of top GWAS findings therefore validates the subgroups of samples used in the present study.

Out of the 119 mtSNPs for which genotype data was available in the PAD dataset, 66 passed QC with a mean genotype call rate of 99.9% and were further tested for the association with PAD risk using an adjusted logistic regression (log-additive model) (Supplementary Table 1). Table 2 shows the mtSNPs nominally associated with PAD, namely m.477T > C, m.9667A > G, and m.10915T > C ($3.01 \times 10^{-3} \leq p_a \leq 3.96 \times 10^{-2}$), of which only m.477T > C was also associated ($p = 3.79 \times 10^{-2}$) in the unadjusted model.

Among the 128 mtSNPs for which genotype data was available in the VTE dataset, 72 passed QC with a mean genotype call rate of 99.8% and were further tested for the association with VTE risk using an adjusted logistic regression (Supplementary Table 2). Only m.11914G > A was nominally associated ($p_a = 4.14 \times 10^{-2}$) with VTE in the adjusted log-additive model (Supplementary Table 2), and its association remained nominal ($p = 3.16 \times 10^{-2}$) in an unadjusted log-additive model (Table 2).

To test the association of particular combinations of mtSNPs with phylogenetic relevance, PAD and VTE study individuals were first classified into the major European mtDNA haplogroups (Table 3), their frequencies in the control groups being in agreement with those previously reported for American populations of white ancestry [33,34]. No mtDNA haplogroup was associated with PAD or VTE after adjusting for the appropriate risk factors (Table 3). Since mitochondrial haplogroups have been shown to exert sex-specific effects as risks factors for certain diseases [33], we tested their association with PAD or VTE stratified by gender. In sex-specific haplogroup analyses, X2 was protective for PAD in females ($p_a = 3.24 \times 10^{-2}$, OR [95% CI] = 0.31 [0.11–0.92]), and U was protective for VTE in males ($p_a = 4.05 \times 10^{-2}$, OR [95% CI] = 0.72 [0.52–0.99]). Furthermore, we also performed gender-specific tests for the SNPs nominally associated with PAD and VTE. m.477T > C and m.10915T > C were associated with PAD in males only ($p_a = 2.36 \times 10^{-2}$, OR_C [95% CI] = 2.16 [1.11–4.19], and $p_a = 4.13 \times 10^{-3}$, OR_C [95% CI] = 0.14 [0.04–0.54], respectively), while m.9667A > G was protective for PAD in males ($p_a = 2.81 \times 10^{-2}$, OR_G [95% CI] = 0.35 [0.14–0.89]) and females ($p_a = 3.47 \times 10^{-2}$, OR_G [95% CI] = 0.21 [0.05–0.89]). m.11914G > A was marginally associated with VTE in males only

Table 1
Characterization of peripheral arterial disease (PAD) and venous thromboembolism (VTE) datasets.

Characteristic	PAD dataset		VTE dataset	
	Controls	Cases	Controls	Cases
N	1629	1652	1278	1241
Gender (*n*/*N*, % men)	982/1629 (60.3)	1059/1652 (64.1)[a]	620/1278 (48.5)	622/1241 (50.1)
Age-at-examination (mean ± SD, years)	60.7 ± 7.4	65.6 ± 10.7[a]	55.8 ± 15.8	54.5 ± 16.2[a]
Risk factors (*n*/*N*, %)				
Hypertension	855/1629 (52.5)	1368/1652 (82.8)[a]	–	–
DM	151/1629 (9.3)	562/1652 (34.0)[a]	–	–
Dyslipidemia	784/1629 (48.1)	954/1652 (57.7)[a]		
CHD	254/1629 (15.6)	909/1652 (55.0)[a]	–	–
Cerebrovascular disease	72/1629 (4.4)	544/1652 (32.9)[a]		
Ever smoking	977/1544 (63.3)	1336/1526 (87.5)[a]	536/1275 (42.0)	546/1214 (45.0)
Stroke/MI	–	–	68/1278 (5.3)	143/1241 (11.5)[a]
State of residence (MN versus others)	–	–	704/1278 (55.1)	529/1241 (42.6)[a]

SD: Standard deviation; DM: *Diabetes mellitus* type 1 or type 2; CHD: Coronary heart disease; MI: Myocardial infarction; MN: Minnesota, USA.
[a] Significant ($p < 5.00 \times 10^{-2}$) difference between cases and controls using a T-test or a chi-square test for continuous and discrete data, respectively.

Table 2
Results of mitochondrial single nucleotide polymorphisms (mtSNPs) nominally associated with peripheral artery disease (PAD) and venous thromboembolism (VTE).

Phenotype	mtSNP	dbSNP	Allele	Controls (n/N, %)	Patients (n/N, %)	p_a OR [95% CI]	p OR [95% CI]
PAD	m.477T > C	rs41442247	C	38/1627 (2.3)	59/1652 (3.6)	**2.32×10^{-2}** 1.81 [1.08–3.04]	**3.79×10^{-2}** 1.55 [1.02–2.34]
	m.9667A > G	rs41482146	G	29/1627 (1.8)	18/1652 (1.1)	**3.01×10^{-3}** 0.30 [0.14–0.67]	**9.88×10^{-2}**
	m.10915T > C	rs2857285	C	20/1617 (1.2)	12/1652 (0.7)	**3.96×10^{-2}** 0.36 [0.14–0.95]	**1.49×10^{-2}**
VTE	m.11914G > A	rs2853496	A	27/1278 (2.1)	44/1241 (3.5)	**4.14×10^{-2}** 1.67 [1.02–2.73]	**3.16×10^{-2}** 1.70 [1.05–2.77]

p_a: *p*-values adjusted for hypertension, coronary heart disease, cerebrovascular disease, diabetes, dyslipidemia, and ever smoking in the PAD dataset, and adjusted for stroke/myocardial infarction and USA state of residence in the VTE dataset; *p*: Unadjusted *p*-values (logistic regression log-additive model). Nominally significant *p*-values are highlighted in bold and their odds ratios (OR) and 95% confidence intervals (CI) are indicated.

Table 3
Results of mitochondrial haplogroup association testing with peripheral artery disease (PAD) and venous thromboembolism (VTE).

Haplogroup	PAD		p_a[a]	Haplogroup	VTE		p_a[b]
	Controls (N,%)	Patients (N,%)			Controls (N,%)	Patients (N,%)	
H	696 (42.7)	742 (44.9)	1.91×10^{-1}	H	539 (42.2)	543 (43.7)	4.66×10^{-1}
R0/HV	79 (4.8)	69 (4.2)	6.79×10^{-1}	HV	61 (4.8)	54 (4.4)	4.33×10^{-1}
I	55 (3.4)	53 (3.2)	5.53×10^{-1}	I	33 (2.5)	29 (2.3)	8.04×10^{-1}
J	186 (11.4)	177 (10.7)	3.93×10^{-1}	J	129 (10.1)	122 (9.8)	9.87×10^{-1}
K	75 (4.6)	67 (4.1)	3.06×10^{-1}	K	106 (8.3)	110 (8.9)	5.48×10^{-1}
T	193 (11.8)	199 (12.0)	6.93×10^{-1}	T	130 (10.2)	129 (10.4)	9.46×10^{-1}
U	236 (14.5)	242 (14.6)	7.93×10^{-1}	U	205 (16.0)	167 (13.5)	1.05×10^{-1}
W	28 (1.7)	34 (2.1)	2.31×10^{-1}	W	22 (1.7)	26 (2.1)	5.22×10^{-1}
X2	29 (1.8)	19 (1.2)	1.23×10^{-1}	X2	20 (1.6)	20 (1.6)	9.00×10^{-1}
Others[c]	52 (3.2)	50 (3.0)	–	Others[d]	33 (2.6)	41 (3.3)	–

[a] p_a: *p*-values adjusted for hypertension, history of coronary heart disease (CHD), history of cerebrovascular disease, diabetes (type 1 or type 2), dyslipidemia, and ever smoking are shown for all PAD haplogroups.
[b] p_a: *p*-values adjusted for prior stroke/myocardial infarction and state of residence in the USA are shown for all VTE haplogroups. Only clades with frequency above 1% in the controls were tested for association.
[c] Includes minor clades A, B, C, D, G, M, N, P, R and Z.
[d] Includes minor clades A, B, C, D, G, L, M, N, R, V and Z. Since these classes include an assortment of lineages not phylogenetically related, association tests were not performed.

($p_a = 6.67 \times 10^{-2}$).

The main steps and results of this study are summarized in the graphical abstract shown in Fig. 3.

4. Discussion

In this study, we investigated for the first time the association of the mitochondrial genome with PAD and VTE risk. In datasets with negligible population structure, we found nominal associations for m.477T > C (located in HVS3 - hypervariable segment 3), m.9667A > G (N154S in *COX3*-cytochrome c oxidase III), and m.10915T > C (C52X in *ND4* - NADH dehydrogenase, subunit 4) with PAD ($3.01 \times 10^{-3} \leq p_a \leq 3.96 \times 10^{-2}$) and for m.11914G > A (T385T in *ND4*) with VTE ($p_a = 4.14 \times 10^{-2}$) in adjusted logistic regressions. Considering that these findings do not withstand any correction for multiple testing and that the samples had enough power to detect the typical associations found in complex diseases, the mitochondrial genome does not appear to harbor frequent genetic risk factors for either PAD or VTE in populations of European descent, despite considerable biological evidence suggesting an important

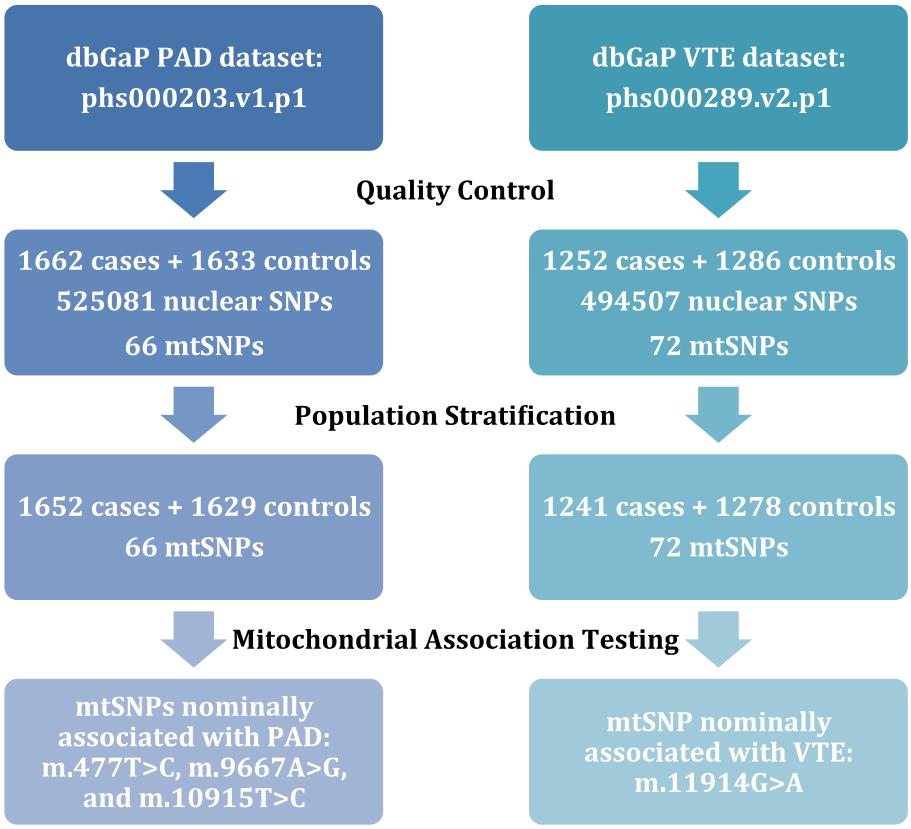

Fig. 3. Graphical abstract for mtSNP (mitochondrial single nucleotide polymorphism) association study with peripheral arterial disease (PAD) and venous thromboembolism (VTE).

role of mitochondrial dysfunction in vascular pathologies.

GWAS studies have pinpointed as main genetic risk factors for PAD nuclear *loci* such as *SH2B3* [6], with pleiotropic roles in immune and inflammatory signaling pathways, hematopoietic cell lineage regulation, and platelet production, adhesion and migration. Mitochondria and their genome may be involved in the development of atherosclerotic plaques, either directly given their influence in inflammatory processes and apoptosis of endothelial cells [19] or indirectly, by influencing the plasma levels of triglycerides and HDL-C [24,25]. Human and animal model research has produced firm evidence that reactive species of oxygen (ROS) control several signaling pathways of the vascular inflammation in atherogenesis, from the initiation of fatty streak development through lesion progress to ultimate plaque rupture [35]. Additional support for the role of oxidative stress in PAD is given from clinical studies with effective use of certain classes of antioxidants for reducing oxidative stress [36,37], and because this mechanism is central to many of its risk factors [35].

Variants within nuclear genes coding for agents of the coagulation and fibrinolytic pathways have been highlighted in VTE association studies and are responsible for a fraction of the disease risk. Vascular endothelial damage leading to platelet activation and coagulation may also lead to high plasma levels of free radicals and evidence supports a role for oxidative stress in the physiopathology of VTE [38,39]. These anucleated cellular fragments have a key role in hemostasis and multiple inflammatory responses, by recognizing vascular lesions and secreting microparticles with thrombotic and proinflammatory mediators [40,41]. Besides supplying energy for platelet aggregation and secretion of procoagulant molecules and regulating apoptosis of these cytoplasts, new and relevant functions for platelet mitochondria have emerged in more recent years and may underlie PAD and/or VTE risk: i) activated platelets release respiratory-competent mitochondria, both within membrane-encapsulated microparticles and as free organelles [42] and ii) mitochondrial cyclophilin D (CypD) and mitochondrial permeability transition pore regulate early platelet activation mechanisms such as phosphatidylserine externalization, fibrinogen retention and membrane vesiculation [43]. The first findings suggest that platelet-released mitochondria constitute a highly potent inflammatory trigger, since the hydrolysis of mitochondrial membrane by secreted phospholipase A2 releases inflammatory mediators, including mtDNA, ATP, ROS, lipids and N-formylated peptides which may act as auto-pathogens [44,45], and thus promote leukocyte activation and neutrophil adhesion to the endothelial wall [42]. In the latter, experiments with thrombin and H_2O_2 agonists pushed a subset of platelets to a highly activated state, which can be genetically controlled by nuclear and/or mtDNA variation. Although highly activated platelets have a high phosphatidylserine externalization, they exhibit several antithrombotic features such as slower clot retraction, high surface levels of anticoagulant proteins and a decreased ability to support adhesive interactions and thrombus growth [46] and may contribute to VTE risk. Consistent with this view are CypD−/− mice, which develop thrombosis more rapidly than CypD+/+ [43], and the successful experimental use of reversible inhibitors of mitochondrial respiratory chain, which reduces platelet-activated blood coagulation [47], and therefore open a window to their possible therapeutic use.

mtDNA variants and haplogroups may have not been established as relevant etiopathogenic drivers of PAD or VTE due the study limitations. We recognize that the groups of cases include very heterogeneous subsets of patients with potentially different etiopathogenic mechanisms (VTE cases include DVT in limbs and

thrombosis in the gastrointestinal tract, kidneys, brain, and in the vena cava, as well as pulmonary embolism; PAD patients include cases with atherosclerotic disease and with fibromuscular dysplasia). GWAS data for mitochondrial studies has the advantage that population stratification can be accounted for if necessary with available nuclear SNPs data, but mtSNPs in GWAS arrays represent only a small subset of the existing mitochondrial genetic variants, with most low and rare frequency variants being excluded. Conversely, sequencing of the full mitochondrial genome in large datasets allows a deeper analysis of mtDNA variation, but additional genotyping of a few hundred nuclear-encoded ancestry informative markers would be required to adequately control for population substructure. In comparison with other commercially available arrays, the chips used in the PAD and VTE GWAS had a quite extensive coverage of the most common and phylogenetically-relevant mtSNPs for the population under study. These mtSNPs allowed the classification of all samples into their haplogroups. Since we did not exhaustively analyze all mitochondrial genetic variants, we cannot completely exclude a role for the mitochondrial genome in these two disorders. However, our study suggests that the common mitochondrial variation is unlikely to be associated with PAD and VTE in populations of European ancestry. A second limitation of this study is that the samples tested here were of European origin only (the cases and controls were collected in the United States of America). Even though the dbGaP datasets included samples of other ancestries, they were excluded in our analyses due to the very small number of individuals in these other groups. In association studies of moderate size, power is an important concern. Power calculations indicate that ~6000 cases and 6000 controls of European ancestry are required to have 90% power to detect a 10% change in the frequency of the most common haplogroup H, and even greater samples sizes are needed for all the other less frequent subclades [48]. Furthermore, approximately 10000 cases and controls are required to have 80% power to detect a mitochondrial allele with a relative risk of 1.5 [49], well above the PAD and VTE sample sizes. As for nuclear SNPs, replication studies for mtSNPs are hindered by widely varying frequencies among populations, with some variations (e.g. haplogroups) being unique to given ancestral groups [50], but replication studies must undertaken to validate any positive findings.

To conclude, we investigated for the first time the association of common mitochondrial genetic variants with risk for peripheral arterial disease and venous thromboembolism in populations of European ancestry. We found nominally significant mtSNPs associations that do not withstand correction for multiple testing, and no associations with mitochondrial haplogroups were observed, suggesting that the missing heritability for PAD and VTE does not appear to reside in the non-nuclear genome. Given the compelling biological evidence for mitochondrial involvement in PAD and VTE etiopathogenesis, future lines of research may include an in-depth evaluation of mitochondrial variation by deep-sequencing in more clinically homogeneous sets of patients and of diverse ancestries, or focus on nuclear-encoded mitochondrial genes and their interaction with previously reported genetic, clinical and environmental risk factors.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.07.920.

List of abbreviations

ABI:	Ankle brachial index
CEU:	U.S. Caucasians with Northern and Western European ancestry
CHB:	Han Chinese individuals from Beijing, China
CHD:	Coronary heart disease
CI:	Confidence interval
CVD:	Cerebrovascular disease
dbGaP:	National Center for Biotechnology Information (NCBI) Database of Genotypes and Phenotypes
DM:	*Diabetes mellitus* type 1 or type 2
DVT:	Deep vein thrombosis
eMERGE:	Electronic Medical Records and Genomics Consortium
GENEVA:	Gene Environment Association Studies
GWAS:	Genome-wide association study

HWE: Hardy-Weinberg equilibrium
IBS: Identity-by-state
JPT: Japanese individuals from Tokyo, Japan
MAF: Minor allele frequency
MDS: Multidimensional scaling
MI: Myocardial infarction
MN: Minnesota, USA
mtDNA: Mitochondrial DNA
mtSNP: Mitochondrial single nucleotide polymorphism
OR: Odds ratio
PAD: Peripheral arterial disease
PE: Pulmonary embolism
QC: Quality control
ROS: Reactive species of oxygen
SNP: Single nucleotide polymorphism
VIF: Variance inflation factor
VTE: Venous thromboembolism
YRI: Yoruba individuals from Ibadan, Nigeria

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