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RESEARCH ARTICLE

Cross-sectional study of risk factors for atherosclerosis in the Azorean population

Teresa Cymbron[1,2,3], Mafalda Raposo[1,2,3], Nadiya Kazachkova[1,2,3], Conceição Bettencourt[1,2,3,4], Francisca Silva[1,2,3], Cristina Santos[5], Yahya Dahmani[6], Paula Lourenço[3,7], Rita Ferin[1,8], Maria Leonor Pavão[1,8] & Manuela Lima[1,2,3]

[1]Center of Research in Natural Resources (CIRN), [2]Department of Biology, University of the Azores, Ponta Delgada, Portugal, [3]Molecular and Cellular Biology Institute (IBMC), University of Porto, Porto, Portugal, [4]Lab. de Diagnostico Molecular del Banco de Tejidos para Investigaciones Neurológicas (BTIN), Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain, [5]Unitat Antropologia Biològica, Dep. Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain, [6]Department of Biochemistry, Molecular and Cellular Biology, Miguel Servet 177, University of Zaragoza, 50013 Zaragoza, Spain, [7]Specialized Service of Epidemiology and Molecular Biology (SEEBMO), Hospital of Santo Espírito, Angra do Heroísmo, and [8]Department Technological Sciences and Development, University of the Azores, Ponta Delgada, Portugal

***Background*: Atherosclerosis—a major cause of vascular disease, including ischemic heart disease (IHD), is a pathology that has a two-fold higher mortality rate in the Azorean Islands compared to mainland Portugal.**

***Aim*: This cross-sectional study investigated the role of genetic variation in the prevalence of atherosclerosis in this population.**

***Subjects and methods*: A total of 305 individuals were characterized for polymorphisms in eight susceptibility genes for atherosclerosis: ACE, PAI1, NOS3, LTA, FGB, ITGB3, PON1 and APOE. Data were analysed with respect to phenotypic characteristics such as blood pressure, lipid profile, life-style risk factors and familial history of myocardial infarction.**

***Results*: In the total sample, frequencies for hypercholestrolemic, hypertensive and obese individuals were 63.6%, 39.3% and 23.3%, respectively. The genetic profile was similar to that observed in other European populations, namely in mainland Portugal. No over-representation of risk alleles was evidenced in this sample.**

***Conclusions*: One has to consider the possibility of an important non-genetic influence on the high cholesterolemia present in the Azorean population. Since diet is the most important life-style risk factor for dyslipidemia, studies aiming to evaluate the dietary characteristics of this population and its impact on serum lipid levels will be of major importance.**

Keywords: Polymorphisms, susceptibility genes, atherosclerosis, ischemic heart disease, Azores

INTRODUCTION

Atherosclerosis (AT), the major cause of cardiovascular disease, including Ischemic Heart Disease (IHD), is a multifactoral pathological process where inflammation and oxidative processes are key components from fatty streak formation to plaque rupture and thrombosis (Roy et al. 2009). The death rate due to IHD is about twice as high in the Azores Archipelago as in mainland Portugal (Direcção-Geral da Saúde 2009). However, the extent of the contribution of genetic risk factors to this prevalence remains unstudied. We developed a cross-sectional study of risk factors for AT, including characterization of the polymorphisms in a series of established susceptibility genes for AT, namely *ACE*—angiotensin converting enzyme (Rigat et al. 1990; Samani et al. 1996; Sabbagh et al. 2007), *PAI1*—plasminogen activator inhibitor 1 (Onalan et al. 2008), *NOS3*—nitric oxide synthase (Casas et al. 2004), *LTA*—lymphotoxin α (Schreyer et al. 2002; Trompet et al. 2008), *FGB*—fibrinogen β (Renner et al. 2002; Gialeraki et al. 2008), *ITGB3*—integrin β-3 (Marian et al. 1996; Ridker et al. 1997; Knowles et al. 2007), *PON1*—paraoxonase 1 (Mackness et al. 1998; Salonen et al. 1999; Garcés et al. 2008) and *APOE*—apolipoprotein E (Burman et al. 2009).

These genes are mainly involved in physiological processes which have been shown to be related to endothelial dysfunction (*NOS3*), inflammation (*LTA*), dyslipidemia (*APOE* and *PON1*), dysfunctional coagulation

Correspondence: Teresa Cymbron, Department of Biology/CIRN, University of the Azores, Rua da Mãe de Deus, Apartado 1422, 9501-801 Ponta Delgada, Azores, Portugal. Tel: +351 296650477. Fax: +351 296650100. E-mail: tcymbron@uac.pt

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and fibrinolysis (*PAI1*, *FGB* and *ITGB3*), as well as blood pressure deregulation (*ACE* and *NOS3*), all of which can ultimately be involved in the atherogenic process (Roy et al. 2009).

The Azores Archipelago (Portugal) is a group of nine islands, with an area of 2344 Km^2 and a total population of 237 315 (INE 2006). According to historical records, these islands were uninhabited until the 15th century, when the first settlers arrived, mostly from mainland Portugal and the island of Madeira. However, people of different origins, such as Spanish, French, Italian, English, German and Flemish were also among the early settlers (Matos 1989; Mendonça 1996). There is clear evidence that Jews also contributed to the peopling of the archipelago and the presence of African and Moorish slaves in the islands is also documented (Matos 1989; Mendonça 1996). Research conducted in the Azorean populations, using various types of genetic system, has shown considerable levels of diversity (Santos et al. 2003; 2009; Montiel et al. 2005); given the particular characteristics of the population size and composition of the Azores, genetic studies have been conducted for several diseases (see, for example, Bruges-Armas et al. 2002; Lima et al. 2005).

To gain insights into the determinants of atherosclerosis in the Azorean population we analysed variation in the previously listed loci, in a large sample of individuals of Azorean background and related it to specific phenotypic characteristics, such as blood pressure and serum lipid profile. Furthermore, other individual and life-style derived risk factors for AT were also analysed.

MATERIALS AND METHODS

Sampling

After informed consent, a total of 305 apparently healthy unrelated individuals were sampled by collecting peripheral blood and buccal swabs. Individuals filled in a genealogical questionnaire, which confirmed their Azorean ancestry. The samples were from the islands of São Miguel ($n = 224$), Graciosa ($n = 64$) and Flores ($n = 17$).

For all participants, data concerning age, gender, height, weight, systolic and diastolic blood pressure (BP) were registered. Body mass index (BMI) was calculated. Individuals were considered hypertensive if they presented an average systolic BP of $\geq$ 140 mm Hg and/or diastolic BP of $\geq$ 90 mm Hg (Chobanian et al. 2003) and/or if they were taking anti-hypertensive medication. Serum total cholesterol (TC), HDL cholesterol (HDL-C) and triglycerides (TG) were determined by collaborating laboratories, using standardized protocols. LDL cholesterol (LDL-C) concentration was calculated by the Friedewald formula (Friedewald et al. 1972). Individuals were considered hypercholesterolemic if presenting at least one of the following situations: (a) total fasting cholesterol concentration of $\geq$ 200 mg/dl; (b) LDL-C concentration of $\geq$130 mg/dl and (c) use of cholesterol-lowering medication. Individuals were hypertriglyceridemic if triglycerides levels were $\geq$ 150 mg/dl and/or if they were being medicated for this alteration (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001). Information was collected on life-style habits such as smoking. Subjects were considered smokers if they smoked or had stopped smoking in the last year. The presence of family history of myocardial infarction was considered whenever at least one-first-degree relative had a myocardial infarction.

Genotyping

DNA was extracted from buccal swabs using JETQUICK Tissue DNA SPIN Kit and from blood samples using JETQUICK Blood & Cell Culture DNA SPIN Kit, for Genomic DNA (GENOMED), according to the manufacturer's specifications. Samples were genotyped by the Spanish National Genotyping Centre (CeGen) for seven polymorphic positions at the following candidate genes for AT: *ACE* (rs1799752), *PAI1* (rs1799889), *NOS3* (rs1799983), *LTA* (rs1041981), *FGB* (rs1800790) and *ITGB3* (rs5918). Polymorphisms at the *PON1* (rs854560) and *APOE* (rs429358; rs7412) loci were genotyped by PCR-RFLP, at CIRN's laboratories (University of the Azores), in accordance with Zhang et al. (2006) and Bettencourt et al. (2006), respectively.

Statistical analysis

Allele and genotype frequencies were estimated for all loci. Hardy-Weinberg equilibrium (HWE) was tested using an exact test, based on a Markov chain approach. An unbiased estimate of heterozygosity was computed according to Nei (1978). The statistical significance of the variance components of the AMOVA (considering the island as the main group) and the paired comparisons were determined by non-parametric procedures. Pairwise population differentiation exact tests, using allele frequencies, were carried out to compare the data obtained for the three islands. Allele frequencies for the total sample ($n = 305$) were compared with the available data for other European and non-European populations. Data from mainland Portugal were taken from David et al. (2004), Ferrer-Antunes et al. (1998), Magro et al. (2003), Medeiros et al. (2002), Mendonça et al. (2008) and Rodrigues et al. (2005). For the remaining populations the data used in the differentiation tests were taken from the ALlele FREquency Database (http://alfred.med.yale.edu/alfred), SNPedia (http://www.snpedia.com), Entrez SNP database (http://www.ncbi.nlm.nih.gov/SNP). All population analyses were performed using Arlequin software version 3.0 (Excoffier et al. 2005).

A one-way between-groups multivariate analysis of covariance was conducted to compare lipid profile in accordance with *APOE* and *PON1* 55 genotypes with *p*-values adjusted for age (42.6), gender and BMI (27.4). To relate lipid profile with *APOE* genotype the sample was reduced to 262 individuals, since those taking lipid lowering medication were excluded from this analysis; genotypes $\varepsilon 2^{\star}\varepsilon 4$ ($n = 7$) and $\varepsilon 4^{\star}\varepsilon 4$ ($n = 1$) were also excluded due to the small group size. Analysis for *PON1* 55 vs lipid profile included a sample of 269 subjects, since individuals taking medication for dyslipidemia were excluded. Statistical

analyses were conducted using SPSS 15.0 software (SPSS Inc., Chicago, IL). Due to the abnormal distribution of blood pressure values, a kernel non-parametric regression test was performed using the software XLSTAT version 2010 (Addinsoft 2010) to estimate the conditional expectation of *ACE*/*NOS3* genotype vs systolic and diastolic blood pressure, using additional parameters (sex, age, BMI, smoking status, myocardial infarction status, levels of TC, LDL-C, HDL-C, TG (mg/dL). Individuals taking anti-hypertensive medication were excluded from the previous analysis.

In all of the analyses a p-value lower than 0.05 was considered statistically significant.

RESULTS

Individual and life-style risk factors of AT

One hundred and sixty-seven of the 305 samples genotyped in this study related to women (54.7%) and 138 to men (45.3%), with a mean age of 44 ± 11 years. The distribution of risk factors for the studied Azorean sample, stratified by gender and age, is shown in Table I. The total hypertensive individuals in the Azorean sample is 39.3% and the mean values for blood pressure are 129 ± 25 mm Hg for systolic BP and 78 ± 25 mm Hg for diastolic BP. Hypertension frequency displays a similar distribution between men and women (26.0% and 20.0% for men and women, respectively), in the first age group (≤44 years). In the older age group (>44 years), the percentage of men with hypertension doubles (53.8%) and that of women triples (59.8%), maintaining the similar differences between genders. The total hypercholestrolemic individuals in this sample is 63.6%, the mean value of TC being 208 ± 50 mg/dL and the mean value for LDL-C being 127 ± 35 mg/dL. In the first age group (≤44 years), approximately half the population (49.4% women and 52.1% men) is hypercholesterolemic, these values rise with age, reaching in the >44 years old group 76.8% for women and 78.5% for men.

The individuals with hypertriglyceridemia represent ~ 30% of this population, for both gender and age groups; mean TG value is 119 ± 66 mg/dL.

The total individuals with obesity are 23.3% for this population and the average value of BMI is 27 ± 5. Neither underweight nor morbidly obese individuals are present in men. The percentage of individuals with normal weight decreases with age in both genders; furthermore, the amount of overweight men increases by 1.6-fold (becoming 58.5%). In the first age group (≤44 years), obesity presents very similar values for men and women (23.4% and 23.1% for men and women, respectively). Age has a strong impact on obesity frequency in women, almost doubling its value (45.2%); men maintain a similar value (23%). Concerning smoking habits, in the ≤44 age group twice as many men, compared to women, were smokers (31.5% and 15.3% of smokers, respectively). Age stratification shows no impact on the smoking habits of men, but is substantial in women at age >44 years, decreasing by 76%. Family history of infarction presented similar values for both genders and age groups.

Table I. Characterization of the Azorean population in study for several atherosclerosis risk factors.

		≤ 44 years		+45 years	
		♀ ($n = 85$) %	♂ ($n = 73$) %	♀ ($n = 82$) %	♂ ($n = 65$) %
Hypertension					
No		80.0	68.5	39.0	43.1
Yes		20.0	26.0	59.8	53.8
Not determined		0	5.5	1.2	3.1
Hypercholesterolemia					
No		50.6	47.9	23.2	21.5
Yes		49.4	52.1	76.8	78.5
Hypertriglyceridemia					
No		77.7	68.5	69.5	63.1
Yes		22.3	31.5	30.5	36.9
BMI					
<18.5	Underweight	2.4	0	0	0
18.5–24.9	Normal	43.5	41.0	17.0	18.5
25.0–29.9	Overweight	30.6	35.6	37.8	58.5
30.0–34.9	Obese	16.5	22.0	29.3	20.0
35.0–39.9	Severely obese	5.8	1.4	12.2	3.0
>40	Morbidly obese	1.2	0	3.7	0
Smoking					
No		83.5	63.0	94.0	66.2
Yes		15.3	31.5	3.6	30.7
Not determined		1.2	5.5	2.4	3.1
Family history of infarction					
No		56.5	65.7	51.2	67.7
Yes		34.1	23.3	39.0	27.7
Did not know		9.4	11.0	9.8	4.6

Population analysis of AT susceptibility loci

Since no differences were detected between samples from the three islands, we pooled the data and used it as a single Azorean sample. Corroborating the results from the differentiation tests, AMOVA showed that genetic variation within populations was significantly higher than that observed among populations (99.7% and 0.3%, respectively), providing no evidence of significant genetic differences between the islands. Allele and genotype frequencies for all analysed markers are presented in Table II. All loci were in conformity with Hardy-Weinberg equilibrium expectations. The levels of expected heterozygosity are similar to those reported for other European populations, with the highest value being registered for *PAI1* (51.52%) and the lowest for *APOE* (22.10%).

When comparing the allele frequencies obtained for the present sample with those available for mainland Portugal samples (relative to loci *ACE*, *PAI1*, *NOS3*, *FGB*, *PON1* and *APOE*) no significant differences were observed. For the analysed polymorphism at the *PON1*, although the most frequent allele in the Azorean sample is the 55-M allele as opposed to mainland Portugal, where the 55-L allele is the most frequent, the differentiation test between the populations for this locus did not reach statistical significance ($p = 0.116$).

Table II. Allele and genotype frequencies of the polymorphisms for the seven genes for atherosclerosis among 305 Azorean.

Risk factors	Position	Allele	%	Genotype	%
ACE	intron 16	D	61.5	DD	39.0
		I	38.5	DI	44.9
				II	16.1
PAI1	− 675	5G	49.2	5G/5G	21.0
	4G > 5G	4G	50.8	5G/4G	56.4
				4G/4G	22.6
NOS3	894 G > T	G	60.0	GG	35.4
		T	40.0	GT	49.2
				TT	15.4
LTA	804 C > A	C	73.4	CC	54.8
		A	26.6	CA	37.4
				AA	7.8
FBG	− 455 G > A	G	79.0	GG	61.0
		A	21.0	GA	36.0
				AA	3.0
ITGB3	176 T > C	T	79.7	TT	63.9
		C	20.3	TC	31.5
				CC	4.6
PON1	55 L > M	L	46.2	LL	16.7
		M	53.8	ML	59.0
				MM	24.3
APOE	471 T > C	ε2	7.0	ε2*ε2	0.0
	609 C > T	ε3	83.0	ε2*ε3	11.8
		ε4	10.0	ε2*ε4	2.3
				ε3*ε3	68.5
				ε3*ε4	17.1
				ε4*ε4	0.3

The only differences to a European population were observed with the Spanish sample, for the *ITGB3* polymorphism ($p = 0.0107$). Furthermore, the exact test of population differentiation revealed significant differences between the Azores and samples from the following non-European populations: the Japanese (*ACE*, $p = 0.0000$), sub-Saharan Africans (*LTA*, $p = 0.0039$; *NOS3*, $p = 0.0000$) and African-Americans (*LTA*, $p = 0.0001$; *PAI1*, $p = 0.0002$; *NOS3*, $p = 0.0001$; *FGB*, $p = 0.0030$).

Genotype/phenotype correlations

The results of the analysis between *APOE* and *PON1* 55 genotypes and lipid profile are shown in Table III. A statistically significant difference was observed between LDL-C values in the three *APOE* genotypes. Individuals with the ε3*ε4 genotype had significantly higher LDL-C values (220 ± 7 mg/dl) than the individuals with the ε3*ε3 (207 ± 4 mg/dl) and ε2*ε3 (198 ± 8 mg/dl) genotype. No significant effect of the *APOE* genotypes on triglycerides, TC and HDL-C levels was detected.

For *PON1*, individuals with the 55-LL genotype had significantly higher levels of TC (226 ± 7 mg/dl) than individuals with the 55-ML genotype (202 ± 4 mg/dl). No significant differences were observed between triglycerides, LDL-C and HDL-C levels among *PON1* genotypes.

The kernel regression test showed a slight correlation between genotype and blood pressure (both for *ACE* I/D gene polymorphism and *NOS3* 894G > T gene polymorphism). R^2 was 0.0252 (systolic BP) and 0.0259 (diastolic BP) for *ACE* and 0.0073 (systolic BP) and 0.0147 (diastolic BP) for *NOS3*. Therefore, the probability of blood pressure depending on *ACE* genotype is 2.52% for systolic BP and 2.59% for diastolic BP. For the *NOS3* locus, the probability is 0.73% and 1.47% for systolic BP and diastolic BP, respectively.

DISCUSSION

We have genotyped eight polymorphisms in established susceptibility genes for atherosclerosis in 305 Azorean samples. The genetic profile disclosed was similar to that observed in other European populations, namely on the mainland of Portugal. No over-representation of the respective risk alleles at the loci studied was shown in our sample.

Results from this study indicate that serum total cholesterol is a major risk factor for atherosclerosis in the Azorean population, followed by hypertension and obesity. The BMI value found in this study, although high, has been previously reported for other Portuguese populations (Mendonça et al. 2008). These latter authors, studying a sample from mainland Portugal, found a mean value of BMI (27.4 ± 4.1) which is very similar to the result obtained for the Azorean sample. Interestingly, the frequency of hypercholesterolemia reported by Mendonça et al. (2008) (30.3%) is much lower than the one in the present sample (63.6%). The high frequency of hypercholesterolemic individuals in the Azorean population clearly sets the background for an increased pre-disposition for atherosclerosis. Since we failed to detect any increased frequency of the risk alleles in the loci putatively associated with serum cholesterol compared with the Portuguese population, the genetic basis for the observed tendency for hypercholesterolemia remains to be identified.

Table III. Plasma lipid profile according to *APOE* and *PON1* genotypes more represented in Azores population (mean ± SE).[a]

	APOE genotypes			*PON1* genotypes				
Variable	ε2*ε3 ($n = 36$)	ε3*ε3 ($n = 180$)	ε3*ε4 ($n = 46$)	MM ($n = 67$)	ML ($n = 156$)	LL ($n = 46$)	Multiple comparison	*p*-value
CT (mg/dl)	198 ± 8	207 ± 4	220 ± 7	209 ± 6	202 ± 4	226 ± 7	ML ≠ LL	0.008
LDL-C (mg/dl)	117 ± 6	126 ± 2	142 ± 5	133 ± 4	123 ± 3	135 ± 5	ε2*ε3 ≠ ε3*ε4	0.011
							ε3*ε3 ≠ ε3*ε4	0.035
HDL-C (mg/dl)	57 ± 3	57 ± 1	53 ± 2	54 ± 2	56 ± 1	57 ± 2		ns
TG (mg/dl)	119 ± 11	155 ± 5	135 ± 9	122 ± 8	121 ± 5	121 ± 9		ns

CT, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high- density lipoprotein cholesterol; TG, triglycerides; ≠, two groups are different; Adjustments for multiple comparisons: Bonferroni; ns, non-significant ($p > 0.05$). [a] Concentrations adjusted for age, sex and BMI.

It is noteworthy that the frequency of family history of infarction reported in Mendonça et al. (2008) is almost half the value (17.4%) presented in this work. The presence of a higher familial aggregation for infarction in the Azorean population could either reflect the shared environmental factors or variation in loci other than those analysed in this study. The possibility that familial forms of hypercholesterolemia could explain, at least in part, some of this aggregation is currently being investigated.

Numerous studies (see among others, Sing and Davignon 1985; Hanis et al. 1991; Howard et al. 1998) have previously reported associations between variation at the *APOE* locus and cholesterolemia, which we were able to confirm in the present study. Thus, the $\epsilon 2$ allele lowers the concentration of TC and LDL-C levels and the $\epsilon 4$ allele raises them. The $\epsilon 2$ cholesterol-lowering effect is 2–3-times that of $\epsilon 4$ cholesterol-raising effect. In several studies, $\epsilon 4$ carriers have been associated with an increased risk of coronary heart disease (Eichner et al. 2002). On the other hand, $\epsilon 2$ carriage may confer cardioprotective effects (Bennet et al. 2007). A correlation has previously been reported between blood pressure and variation at *ACE* (Henskens et al. 2003; Di Pasquale et al. 2004) and *NOS3* (Jia et al. 2003; Sawada et al. 2008); in accordance with these findings a slight correlation between genotypes in the previous loci and BP was reported in the Azorean correlation.

In conclusion, the fact that no over-representation of the respective risk alleles at the loci studied was evidenced in our sample does not preclude the possibility that variation at other loci could influence the pre-disposition for atherosclerosis in the Azorean population. Given the fact that AT is a multi-factoral disorder, with several genetic and environmental factors involved, we have to consider the possibility that an important non-genetic influence is playing a role in the high cholesterolemia present in the Azorean population. Diet is the most important life-style risk factor for dyslipidemia; studies to evaluate the dietary characteristics of this population are currently ongoing.

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