Research Article

Population variability in some genes involving the haemostatic system: data on the general population of Corsica (France), Sardinia and Sicily (Italy)

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Abstract

Three different population samples from Corsica (France), Sardinia and Sicily (Italy) were studied using nine genetic markers. For the first time, allele distributions of FGA TaqI, FGB Bcl I, FGB Hind III, PAI-1 Hind III, PLAT TPA-25, GPIIIa Taq I, GPIIb I/D 9bp, FVII HVR4 and FVII -323 10 bp markers, which are thought to be associated with cardiovascular disease risk, were studied in the general population of the three islands. The frequencies of the markers analysed in the present work show some peculiarities: the locus FVII HVR4 is characterized by the presence of a rare allele (H5), found in Corsicans and in Sardinians; the locus FBG BclI shows a low frequency of the B1 allele and the absence of the B1B1 genotype. The frequencies of some alleles have a distribution that is in agreement with the low risk for cardiovascular diseases in south European countries. The results highlight a genetic differentiation between the three Mediterranean islands and the other European populations.

Key words: haemostatic genes, genetic markers, Mediterranean populations.

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Introduction

Genes involved in haemostasis are relevant candidate genes for susceptibility to cardiovascular diseases. During the last decades, a growing number of common polymorphisms in the genes that encode coagulation, as well as fibrinolytic and adhesive platelet proteins, have been associated with coronary heart disease (Reiner *et al*., 2001). In spite of the great interest in these polymorphisms for epidemiological studies, little is known about their variation in the general population. On the other hand, several previous studies of these genetic markers related to risk and disease resistance have proved useful for the study of human populations (Bernardi *et al.,* 1996; Tishkoff *et al.,* 2000).

We have explored, for the first time, the frequency distributions of the nine markers in seven haemostatic genes of three general population samples from Corsica (France), Sardinia and Sicily (Italy). We studied: alpha and beta fibrinogen (FGA: OMIM 134820, and FGB: OMIM 134830, respectively, at 4q28), factor VII (F7: OMIM 227500, 13q34), plasminogen activator inhibitor-1 (PAI1: OMIM 173360, 7q21.3-q22), plasminogen activator tissue (PLAT: OMIM 173370, 8p12), and alpha and beta subunits of the platelet GPIIb/IIIa integrin complex (GPIIb or ITGA2B: OMIM 273800, and GpIIIa or ITGB3: OMIM 173470, respectively, at 17q21.32). Some of these polymorphic sites have been reported as being associated with the risk for cardiovascular disease (de Maat *et al.,* 1995; de Maat *et al.,* 1997; van der Bom *et al.,* 1997; Weiss *et al.,* 1996; Zito *et al.,* 1999; Di Castelnuovo *et al.,* 2000).

The fibrinogen gene cluster contains three of the RFLP markers examined, which include HindIII (C/T^{-148} base change) at the promoter, the BclI restriction site in the 3' region of the FGB gene, and RFLP/TaqI in the 3' region of the FGA gene. Allele variants of these markers have shown to be associated with an increased plasma fibrinogen level, which is an independent risk indicator for cardiovascular disease (de Maat *et al.,* 1995; Iso *et al.,* 1995; Zito *et al.,* 1997, Donati *et al.,* 2000). Genetic variation of the Factor VII gene (F7) was tested by means of the Insertion/Deletion (at the -323 site in the promoter region) and the HVR4 (VNTR of 37bp repeat core next to intron G) markers. The I (Insertion) and H7 (HVR4) alleles have been reported to be associated with low Factor VII plasma concentration, which is interpreted as conferring protection against the risk of cardiovascular disease (Iacoviello *et al.,*

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1998; Girelli *et al.,* 2000; Geng *et al.,* 2003). Moreover, the typed RFLP/HindIII polymorphism in the 3' region of the fibrinolytic protein plasminogen activator inhibitor (PAI1) influences the plasma levels (PAI-1) and has been associated with the development of cardiovascular disease (Benza *et al.,* 1998; Lee *et al.,* 1999). The only exception to the lack of population studies is the Alu insertion polymorphism, located within intron 8 of the PLAT gene, the geographic distribution of which has been extensively studied (Ludwig *et al.,* 1992; Tishkoff *et al.,* 2000). Contrasting results of association studies on thrombosis and myocardial infarction risk suggest that this polymorphism is not a major risk factor in cardiovascular disease (van der Bom *et al.,* 1997; Steeds *et al.,* 1998). Lastly we typed the RFLP/TaqI (A_C base change at the 20500 site in exon 9) of the GPIIIa gene and the Ins/Del (9bp) at intron 21 of the GpIIb gene (Zimrin *et al.,* 1990; Peyruchaud *et al.,* 1995). The proteins encoded by these genes influence the interaction of the platelet vessel walls or thrombus formation and therefore they may also be candidate genes for coronary heart disease risk.

This paper analyses the distribution of the previously mentioned markers in three autochthonous population samples from Corsica, Sardinia and Sicily. Our main purpose was to determine the degree of population variability of these candidate genes for CAD (coronary artery disease) in a set of geographically related populations who present certain genetic peculiarities (Ghiani *et al.,* 2002; Varesi *et al.,* 2000; Vona, 1997; Vona *et al.,* 2001). These may include aspects such as degrees of isolation and different incidences of malaria epidemics from other Mediterranean regions, as well as lower cardiovascular prevalences, as compared to north and central Europe (Iacoviello *et al.,* 1998).

Material and Methods

Blood samples were obtained from 292 apparently healthy, unrelated blood bank donors (157 males and 135 females). The donors, as well as their parents and grandparents, were born and residing in some areas of Corsica (N = 92) (France), Sardinia (N = 100) and Sicily (N = 100) (Italy). All individuals were aged between 25 and 65 years (x = 46.9 ± 0.25) and gave their informed agreement to be included in this research. DNA was extracted from the blood samples by the phenol-chloroform method. The samples were then amplified with oligonucleotides, under conditions previously described by the authors listed in Table 1.

Allele frequencies were determined by gene counting. Genotype frequencies were tested to see if they were in agreement with the Hardy-Weinberg equilibrium expectations, using Fisher's Exact Test (Guo and Thompson, 1992) helped by the statistical software package Arlequin (Schneider *et al.,* 2000). The Bonferroni correction was applied (Weir, 1991), and unbiased estimates of expected heterozygosity were computed as proposed by Nei (1978). Fisher's Exact Test was used to calculate heterogeneity for single markers and for the overall total of seven markers in our three samples. Genetic distances were calculated according to Nei's method (Nei, 1972), and a neighbor-joining tree (Saitou and Nei, 1987) was constructed from the matrix of genetic distances using the PHYLIP 3.5 package (Felsenstein, 1993). The tree topology was assessed through 1000 bootstrap iterations.

Results

Genotype and allele frequencies for the nine polymorphisms studied in the Corsican (France), Sardinian and

Table 1 - Chromosomal localizations, oligonucleotides and type of the studied genetic markers.

Markers	Localiz.	Primer	Type	Reference
FGA TaqI	4q28	5'-AGCCGTGCCTATCTTTG-3' 5'-TGTCTCAGGTACATTTAGC-3'	RFLP	Thomas *et al.*, 1994
FGB Bcl I	4q28	5'-ACCTGGTTTTCTTGCCACAAG-3' 5'-AATAGTTCTCATACCACAGTGT-3'	RFLP	Thomas *et al.*, 1994
FGB Hind III	4q28	5'-TGGTTGCCTTGTGAGTAGG-3' 5'-ATTGTCGTTGACACCTTGGG-3'	RFLP	GenBank (M64983)
PAI-1 Hind III	7q21.3-22	5'-AGCAATCCACCTGTCTCGGC-3' 5'-TCCTGACCTCAGGTGATCCG-3'	RFLP	Grenett *et al.*, 2000
PLAT TPA-25	8p12-q11.2	5'-GTGAAAAGCAAGGTCTACCAG-3' 5'-GACACCGAGTTCATCTTGAC-3'	Ins.Alu	Tishkoff *et al.*, 1996
FVII HVR4	13q34	5'-AATGTGACTTCCACACCTCC-3' 5'-GATGTCTGTCTGTCTGTGGA-3'	VNTR	Marchetti *et al.*, 1991
FVII –323 10bp	13q34	5'-AGGCTCTCTTCAAATAATTACATC-3' 5'-CGGGCTGGCTCCTGGATTT-3'	I/D	Marchetti *et al.*, 1993
GPIIIa Taq I	17q21.32	5'-TTTCTTGTCTTCTTGTGCCC-3' 5'-TGTCTCCAATCTTGAGTCCC-3'	RFLP	GenBank (M32674)
GPIIb 9bp	17q21.32	5'-CAGACCTTCCAAGGGAGCTT-3' 5'-GTGAGGACCAAGATTCTGGC-3'	I/D	Peyruchaud *et al.*, 1995

Sicilian (Italy) populations are reported in Tables 2 and 3, respectively; values of p for the Hardy-Weinberg equilibrium, the observed (H^o) and the expected (H^e) heterozygosity are also shown in these tables. The genotype distributions were found in general accordance with the Hardy-Weinberg equilibrium expectations, with the exceptions of PAI-1 Hind III and PLAT TPA-25 in Corsicans, and PLAT TPA-25, FVII HVR4 and GPIIIa TaqI in Sardinians. The significant departure from the Hardy-Weinberg equilibrium found for PLAT TPA-25 in the Corsican sample was due to a great reduction of the heterozygote frequencies. On the other hand, a significant excess of heterozygotes was observed in the Corsican sample for the PAI-1 HindIII marker, and for the PLAT TPA-25 and GPIIIa TaqI loci in the Sardinian sample. As for the FVII

HVR4 marker in the Sardinian sample, the divergence was due to the presence of a rare H5-H5 homozygote. Using the Bonferroni correction for multiple comparisons, however, the significance of these values disappears.

The expected heterozygosity levels ranged from a minimum of 0.188 in FGB BclI in the Sardinian sample to a maximum of 0.503 for the FVII HVR4 locus in the Sicilians (Table 3). The overall mean heterozygosity values for the nine loci for the Corsican, Sardinian and Sicilian samples were 0.338, 0.363 and 0.402, respectively.

Fisher's Exact Test among the three populations and for all the markers examined showed a significant genetic differentiation value ($\chi^2 = 137.4$; d.f = 54; p < 0.0001). The values of this test for the genetic differentiation by locus showed that the three populations were heterogeneous with

Table 2 - Genotype frequencies and probability of test for the Hardy Weinberg equilibrium at nine loci in the three Mediterranean populations.

FGA TaqI				GPIIIa Taq I			
Genotype freq.	Corsica	Sardinia	Sicily	Genotype freq.	Corsica	Sardinia	Sicily
T1/T1	14 (15.7)	4 (4.1)	8 (8)	T1/T1	0	0	6 (6)
T1/T2	28 (30)	26 (26.3)	44 (44)	T1/T2	32 (34.8)	54 (54.5)	28 (28)
T2/T2	50 (54.3)	69 (69.6)	48 (48)	T2/T2	60 (65.2)	45 (45.5)	66 (66)
HW (p)	0.077	0.476	1	HW (p)	0.317	0.0001	0.379

FGB Bcl I				GPIIb I/D			
Genotype freq.	Corsica	Sardinia	Sicily	Genotype freq.	Corsica	Sardinia	Sicily
B1/B1	0	0	0	I/I	30 (32.6)	53 (53)	34 (34)
B1/B2	24 (26)	19 (20.8)	24 (24)	I/D	50 (54.4)	44 (44)	50 (50)
B2/B2	68 (74)	72 (79.2)	76 (76)	D/D	12 (13)	3 (3)	16 (16)
HW (p)	1	0.589	1	HW (p)	0.54	0.119	1

FGB Hind III				FVII -323 10bp			
Genotype freq.	Corsica	Sardinia	Sicily	Genotype freq.	Corsica	Sardinia	Sicily
Hd1/Hd1	70 (76)	59 (59)	66 (66)	I/I	4 (4.3)	5 (5)	10 (10)
Hd1/Hd2	22 (24)	36 (36)	28 (28)	I/D	44 (47.8)	35 (35.4)	24 (24)
Hd2/Hd2	0	5 (5)	6 (6)	D/D	44 (47.9)	59 (59.6)	66 (66)
HW (p)	1	1	0.379	HW (p)	0.299	0.787	0.041

PAI-1 Hind III				FVII -HVR4			
Genotype freq.	Corsica	Sardinia	Sicily	Genotype freq.	Corsica	Sardinia	Sicily
Hd1/Hd1	2 (2.1)	30 (30.3)	26 (26)	H5/H5	0	1 (1.03)	0
Hd1/Hd2	72 (78.2)	49 (49.5)	62 (62)	H5/H6	2 (2.17)	0	0
Hd2/Hd2	18 (19.7)	20 (20.2)	12 (12)	H6/H6	50 (54.33)	58 (59.8)	30 (30)
HW (p)	0.001	1	0.088	H7/H6	32 (34.8)	30 (30.9)	46 (46)
				H7/H7	8 (8.7)	8 (8.25)	24 (24)
PLAT TPA-25				HW (p)	0.779	0.001	0.579

PLAT TPA-25			
Genotype freq.	Corsica	Sardinia	Sicily
I/I	26 (28.3)	3 (3)	24 (24)
I/D	32 (34.8)	80 (80)	44 (44)
D/D	34 (36.9)	17 (17)	32 (32)
HW (p)	0.042	0.001	0.406

Table 3 - The allele frequencies of the haemostasis markers, heterozygosity values and p values of genic differentiation in the Corsican, Sardinian and Sicilian populations (SE = Standard Error, Ho = observed Heterozygosity and He = Heterozygosity Nei 1978, p = values of genic differentiation between populations for locus).

FGA TaqI							GPIIIa Taq I						
Allele freq.	Corsica	SE[a]	Sardinia	SE	Sicily	SE	Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE
T2	0.695	± 0.053	0.828	± 0.028	0.700	± 0.050	T2	0.826	± 0.042	0.727	± 0.034	0.8	± 0.042
H^o	0.300		0.263		0.440		H^o	0.348		0.545		0.280	
H^e	0.428		0.286		0.424		H^e	0.290		0.399		0.323	
p = 0.01							p = 0.12						
FGB Bcl I							**GPIIb I/D**						
Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE	Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE
B2	0.869	± 0.036	0.896	± 0.023	0.880	± 0.034	I	0.598	± 0.059	0.750	± 0.033	0.590	± 0.057
H^o	0.260		0.208		0.240		H^o	0.544		0.440		0.500	
H^e	0.230		0.188		0.213		H^e	0.486		0.377		0.489	
p = 0.74							p = 0.00						
FGB Hind III							**FVII -323 10bp**						
Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE	Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE
Hd1	0.880	± 0.035	0.770	± 0.032	0.800	± 0.042	D	0.717	± 0.051	0.773	± 0.032	0.780	± 0.044
H^o	0.240		0.360		0.280		H^o	0.478		0.354		0.240	
H^e	0.213		0.356		0.323		H^e	0.410		0.353		0.347	
p = 0.08							p = 0.50						
PAI-1 Hind III							**FVII-HVR4**						
Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE	Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE
Hd2	0.587	± 0.067	0.449	± 0.042	0.430	± 0.058	H5	0.011	± 0.074	0.010	± 0.050	0.000	± 0.070
H^o	0.782		0.495		0.620		H6	0.728	± 0.050	0.753	± 0.033	0.530	± 0.060
H^e	0.490		0.497		0.495		H7	0.261	± 0.071	0.237	± 0.049	0.470	± 0.062
p = 0.05							H^o	0.370		0.310		0.460	
PLAT TPA-25							H^e	0.406		0.379		0.503	
Allele freq.	Corsica	SE	Sardinia	SE	Sicily	SE	p = 0.00						
D	0.543	± 0.062	0.570	± 0.041	0.540	± 0.059							
H^o	0.348		0.800		0.440								
H^e	0.502		0.493		0.502								
p = 0.84													

respect to FGA TaqI, PAI-1 HindIII, GpIIb 9 bp I/D and FVII HVR4. FVII HVR4 showed the highest variability, whereas TPA-25 showed the lowest variability, as compared to the other loci.

There are few available data in the related literature on the general European population and therefore only some comparisons among frequencies could be made (Table 4). For FGA Taq I, the frequency in the Sardinian sample was similar to those of other European populations, whose T2 allele frequencies range from 0.710 (Netherlands) to 0.849 (Basque Country), while those of the Corsican and Sicilian populations were lower.

Only the Italian samples showed a lower frequency (0.720) for the B2 allele of FGB Bcl I, the three Mediterranean islands presenting frequencies similar to all the other populations listed in Table 4. The Corsican sample showed a higher (0.880) frequency for the Hd1 allele of the FGB HindIII locus, while the allele frequencies of the Sardinian and Sicilian samples were similar to those of the other populations. Regarding the PAI-1 HindIII locus, the allele frequencies of our samples were within the range of the populations listed in Table 4. The Corsican and Sicilian samples showed the higher frequencies for the allele T2 of the GpIIIa locus and lower frequencies for the allele I of the GpIIb locus, respectively. The FVII -323 10bp deletion showed a lower frequency on the three islands than in any other European population. Notably, the high frequency found for the H7 allele of the FVII HVR4 polymorphism in

the Sicilian sample occurred simultaneously with the absence of H5. Our three Mediterranean populations were found to have high frequencies for the PLAT TPA-25 locus, as compared to the other European populations (Table 4).

The neighbour-joining tree shown in Fig. 1 is based on genetic distances computed according to Nei (1972), using the gene frequencies of 6 loci (FGA TaqI, FGB HindIII, FGB BclI, FVII -323 10bp, FVII HVR4, and PLAT TPA25) from Tables 1 and 4. The gene frequencies of European populations were pooled in two samples: North Europeans (Denmark, England, Ireland, the Netherlands, Norway and Sweden) and Central-South Europeans (Austria, Basque Country, France, Italy and Central Spain). The tree clearly shows a great genetic differentiation among the studied populations, with strong bootstrap support.

Discussion

We investigated the frequencies of nine DNA genetic markers and, since their variation is not well known, we studied them in the general population of three west Mediterranean islands: Corsica (France), Sardinia and Sicily (Italy). The frequencies obtained showed some peculiarities: 1) T2 (FGA TaqI) and I (GPIIb I/D) were remarkably higher in Sardinians than in Sicilians or Corsicans; 2)

Corsicans were characterised by a higher frequency of Hd1 (FGB HindIII) and Hd2 (PAI-1 HindIII); 3) Corsicans and Sardinians presented a rare allele (H5) in the FVII HVR4 marker; 4) The three islands were characterised by a low frequency of the allele B1 and total absence of the genotype B1B1 (FGB BclI). This allele variant has shown to be associated with an increased plasma fibrinogen level, which is an independent risk indicator for cardiovascular disease (de Maat *et al.,* 1995; Iso *et al.,* 1995; Zito *et al.,* 1997; Donati *et al.,* 2000).

The global heterogeneity found among the three island populations proved to be very significant. The genetic tree indicated a certain degree of differentiation among the compared populations. The results obtained using classical genetic markers (Vona, 1997; Memmì *et al.*, 1998; Ghiani *et al.*, 2002) and sequences of mtDNA indicated a sharp differentiation between Corsicans and Sardinians with respect to Sicilians (Vona *et al.,* 2001) and the other Mediterranean populations (Vona *et al.,* 2003, Falchi *et al.,* 2003). The genetic differentiation between Corsicans and Sardinians appeared to be lower than the one among the other European populations. This might be explained by the reciprocal exchange that has taken place between the two populations ever since prehistoric times. For instance, during the late Neolithic period, north Sardinia saw the «Megalithic Circle» culture, that was in strong correspondence with the

Table 4 - Allele frequencies in different ethnic groups.

| Population	FGA Taq I	FGB Bcl I	FGB Hind III	PAI-1 Hind III	GPIIIa Taq I	GPIIb I/D	FVII -323 10 bp	FVII-HVR4				PLAT TPA25	References
	T2	B2	Hd1	Hd2	T2	I	D	H5	H6	H7	H8	D	
Corsica	0.695	0.869	0.880	0.587	0.826	0.598	0.717	0.011	0.728	0.261	0.000	0.544	Present study
Sardinia	0.828	0.896	0.770	0.449	0.727	0.750	0.773	0.010	0.753	0.237	0.000	0.570	Present study
Sicily	0.700	0.880	0.800	0.430	0.800	0.590	0.780	0.000	0.530	0.470	0.000	0.540	Present study
Italy		0.720					0.873	0.007	0.638	0.356	0.000		Zito *et al.,* 1997; Dell'Acqua *et.al.,* 1997; Iacoviello *et al.,* 1998
North Italy								0.019	0.658	0.323	0.000		Girelli *et al.,* 2000
Italy (North, Center, South)							0.835	0.010	0.679	0.320	0.000		Di Castelnuovo *et al.,* 2000
Austria			0.752										Schmidt *et al.,* 1998
Central Spain	0.790	0.837	0.780	0.404	0.740	0.700	0.840	0.010	0.720	0.270	0.000	0.411	Comas *et al.,* 2000; Batalla *et al.,* 2001; Lopez Alomar
Basques	0.849	0.841	0.806	0.346	0.718	0.560	0.972	0.000	0.632	0.368	0.000	0.412	Lopez Alomar
France	0.764	0.829				0.700						0.275	Behague *et al.,* 1996; Peyruchaud *et al.,* 1995; Ruan *et al.,* 1993
England	0.730	0.850	0.810				0.883	0.046	0.672	0.282	0.000		Thomas *et al.,* 1994; Humphries *et al.,* 1996; de Knijff *et al.,* 1994
Netherland	0.710	0.830					0.861						de Maat *et al.,* 1997; de Knijff *et al.,* 1994; Doggen *et al.,* 2000
Sweden				0.463									Dawson *et al.,* 1991
Ireland				0.632									Lee *et al.,* 1999
Norway	0.734	0.826											Berg and Kierulf
Denmark								0.024	0.690	0.274	0.012	0.500	de Knijff *et al.,* 1994; Tishkoff *et al.,* 2000
Northen Europe	0.732	0.838	0.810	0.548			0.872	0.035	0.681	0.278	0.006	0.447	pooled data
Southern Europe	0.764	0.775	0.820	0.375	0.729	0.653	0.854	0.011	0.670	0.317		0.343	pooled data
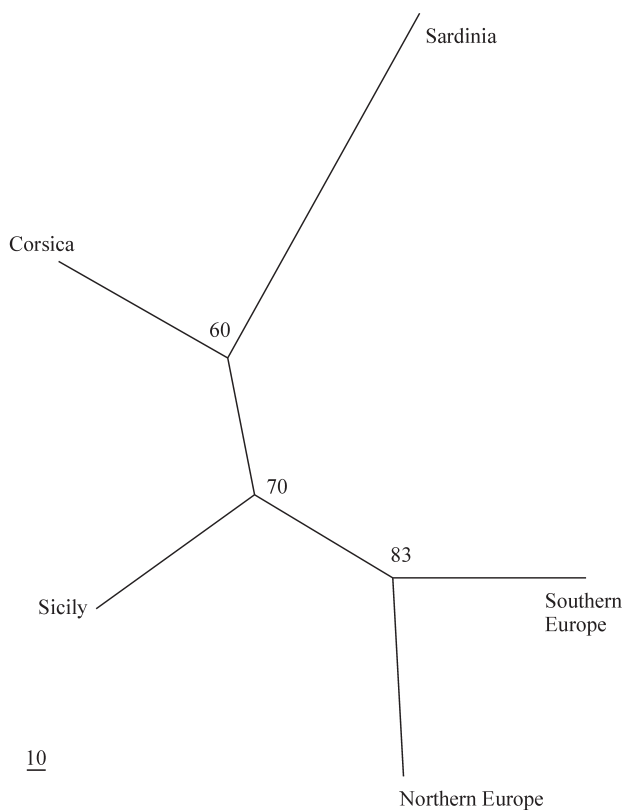
Figure 1 - Neighbour-joinig tree for 6 of the 9 loci analysed in the studied populations.

contemporaneous «Torreana» culture in Corsica. The contact between the two islands continued throughout both the Bronze and Iron Ages. The relationship between Corsica and southern Sardinia is also obvious in their dialectal origins (Blasco-Ferrer, 1984; Lilliu, 1988). The classical genetic markers and mtDNA (Varesi *et al.,* 2000; Vona *et al.,* 2002; Vona *et al.,* 2003) confirm the hypothesis of a common origin shared by the two populations and lead to the conclusion that Corsicans and Sardinians are genetically similar, although not identical. Demographic events may have contributed to this differentiation, triggering a series of phenomena which produced the effects of genetic drift. It is sufficient to recall: 1) the low population density and the serious epidemics which caused bottleneck effects; 2) the long periods of internal and external isolation experienced by many Corsican and Sardinian villages (Vona, 1997). The analysis of the genetic structure of Sicily has been pursued by several authors using different sets of genetic markers, mtDNA (Piazza *et al.,* 1988; Rickards *et al.,* 1998; Vona *et al.,* 2001), and names and surnames (Guglielmino *et al.,* 1991; Zei *et al.,* 1993). Their results show that the Sicilian gene pool has been influenced by other Mediterranean populations because of the various immigrations which took place in Sicily (Vona *et al.,* 2001). Both the analyses made previously by several authors and our present results indicate that genetically Sicily occupies a mid-

way position between Corsica and Sardinia and other European populations.

Several works highlighted the fact that some of the genes analysed in the present study seem to show a correlation with cardiovascular diseases. In our research, we have found that the frequency distribution of the allele B1 of the locus FGB BclI, of the allele T1 of the locus FGA TaqI and of the allele I of the FVII -323 10bp in Sardinia, and the frequency distribution of the allele H7 (FVII HVR4) in Sicily are in agreement with the low risk for cardiovascular diseases of the south European countries (Iacoviello *et al.,* 1998). Furthermore, the population heterogeneity could be extremely interesting to clarify the distribution process of cardiovascular diseases, as there is such a diversity of patterns of different pathologies between the other European and Mediterranean populations (Tunstall-Pedoe *et al.,* 1994).

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