TNFA *locus* is associated with β°39 thalassemia in Corsica and Sardinia

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ABSTRACT. Malaria causes more than one million deaths annually, worldwide. Understanding the genetic defenses against this disease is an important challenge for science. We know that the long-term presence of endemic malaria has led to a prevalence of the β°39 heterozygous thalassemia mutation in the two islands of Corsica and Sardinia. The populations of both islands are isolated, which could make it easier to find other genetic traits selected by disease pressure. We chose to investigate genes implicated in the primary defenses against *Plasmodium falciparum*: oxidative metabolism and the immune response. We indeed selected genes coding for nitric oxide synthase 2 (NOS2 promoter, polymorphisms NOS2(AAAT) I/D and NOS2(CCTTT)n) and genes coding for tumor necrosis factor-α (TNFA 3'UTR, polymorphisms TNFd(GA)n and TNFe(GA)n). Some associations of TNFA alleles or haplotypes were found either with or without the β°39 mutation, suggesting a complex link originally between TNF-α and resistance or susceptibility to infection.

Keywords: tumour necrosis factor, nitric oxide synthase, polymorphism, microsatellite, thalassemia, malaria

Malaria is the most common parasitic disease in tropical and subtropical regions and is on the increase [1, 2]. Every year, there are 300 to 500 million clinical cases of malaria and it causes more than one million deaths [3]. Disease occurrence and severity depend on both parasite and host genetic factors. Candidate genes studies are performed in order to find those human genes involved in the disease and susceptibility to infection.

In the Corsican and Sardinian populations, the incidence of the β-thalassemia trait is reported to be respectively 3% and 12% [4], and β-thalassaemias are known to give a protective effect with respect to *Plasmodium* [5]. Despite the sporadic incidence of the disease today, the presence of endemic malaria over millenia has led to a prevalence of the β°39 heterozygous thalassemia mutation, a mutation associated with the β-globin haplotype II, amongst the inhabitants of the two islands of Corsica and Sardinia [4, 6]. The aim of this study was to investigate β°39 heterozygous carriers and controls, genetic polymorphisms in tumor necrosis factor A (TNFA) and nitric oxide synthase 2A (NOS2A) genes. Two microsatellites in TNFA, TNFd(GA)n and TNFe(GA)n located in 3'UTR and two polymorphisms in NOS2A promoter, NOS2(AAAT) I/D and NOS2(CCTTT)n, were selected.

TNF has been widely studied because genetic variants of immunity-related genes have been postulated to influence susceptibility to malaria [7]. Indeed, TNF-α is a pro-inflammatory cytokine essential in the protection against many infectious diseases[8, 9]. TNF-α is involved in the killing of *Plasmodium falciparum* mediated by neutrophils and monocytes [10, 11]. High serum levels of TNF-α and polymorphisms in the TNFA promoter have been associated with increased susceptibility to severe malaria [12-21]. TNF-α is a critical mediator of malaria fever [22, 23], and Knight *et al*. [9] have shown that polymorphisms in the TNF-α promoter change the binding of some nuclear factors, up- or down- regulating transcription.

NOS were investigated because, on the one hand, nitric oxide (NO) is a major component of the defensive response against protozoan infections [24, 25], and on the other hand, TNF-α generally increases NOS2-derived NO synthesis [26]. Indeed, during a malarial infection, NO synthesis is regulated by a balance of pro- and anti-inflammatory cytokines and reciprocally, NO can influence cytokine secretion [27-30]. Polymorphisms in the NOS2A gene have been linked to malaria, but the consequences of genotype linking are still debated. Indeed, the NOS2A –954 (G/C) polymorphism has been associated with protection against malaria in Gabon [31, 32] and in Ghana [33]. Some alleles of the (CCTTT)n polymorphism appeared to be linked to protection against malaria in Ghana (181pb associated with –954C [32]), in Gambia ($n \geq 196$pb [34]) and generally in the African continent (186pb [35]), but other alleles seemed to be associated with disease severity in Ghana (206pb associated with –1173T [33]), in Thailand ($n \geq 216$pb [36]) and in Gambia ($n \leq 196$pb [34]]).

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DONORS AND METHODS

Patients and controls

The sample was composed of 160 unrelated adults (72 males and 88 females, mean age 47 ± 0.04 years): 42 Sardinians heterozygous for β°39 and 42 healthy Sardinians, 38 Corsicans heterozygous for β°39 and 38 healthy Corsicans (experiments carried out with 101 Corsican controls and 90 Sardinian controls, showed the same results). β-thalassemia carriers were selected on the basis of the following hematological parameters: MCV < 84 fL, MCH < 27 pg, HbA_2 > 3.5% and were genotyped for the β°39 mutation in previous studies [4, 6]. All the subjects were born and reside respectively on the two islands, as did their ancestors for at least three generations. The study received ethics committee approval and each blood donor provided informed consent to participate in this research study.

DNA extraction and genotyping

DNA was extracted from blood samples using the QIAmp DNA blood mini kit (Qiagen SA, France). Fluorescent oligonucleotide source (Applied Biosystem SA, France), Genbank accession number and PCR programs are listed in *table 1*.

PCR reactions were conducted in a solution of 20 µL containing 250 ng of DNA, 10 µL (1 U) of Taq Polymerase Master Mix (with 400 µM of each dNTP and 3 mM of $MgCl_2$, Qiagen®) and 10 pmoles of each primer. PCR products were analyzed by an ABI 3730 DNA Analyzer (Applied Biosystems, SA France). Genotypes were identified using GENESCAN® and GENOTYPER® softwares (Applied Biosystems).

Statistical analysis

Allelic frequencies were determined using Genetix 4.05 software [37], haplotypic frequencies via E.M. algorithm [38-41] and the Hardy-Weinberg equilibrium using Arlequin 3.01 software [42].

Fisher's exact test was performed using Graphpad Instat 3.01 [43] (Graphpad software Inc., San Diego, CA, USA).

RESULTS

Allelic frequencies

For NOS2(AAAT)I/D, no significant difference was found between β°39 carriers and controls, either in Corsica or in Sardinia (p > 0.05; results are not shown).

For NOS2(CCTTT)n, 191pb and 206pb alleles showed significant differences between β°39 carriers and controls, but not in the same way *(table 2)*. Indeed, in Corsica, 191pb was more frequent in β°39 carriers with an individual ratio n/n of 5/1 (13.64% *versus* 3.57%, p = 0.0238) and 206pb in controls with a ratio of 12/6 (30.95% *versus* 15.15%, p = 0.0112). We noted for this allele an odds ratio (OR) of 2.067, which indicates a probability of presence in the healthy status 2-fold higher than in the β°39 heterozygote population (95% confidence interval (CI) = 1.1910-3.5850). An converse situation was found in Sardinia (respectively for β°39 carriers and controls, for 191pb allele: 2/13, 5.17% *versus* 30.49%, OR = 6, 95% CI = 2.4260-14.8390, p < 0.0001 and for 206pb allele: 8/2, 18.97% *versus* 4.88%, p = 0.0390). In addition, the 211pb allele was more represented in Sardinian β°39 carriers than in controls, with an individual ratio of 4/0 (8.62% *versus* 1.22%, p = 0.0185).

No significant difference was found between groups for TNFe(GA)n, either in Corsican and Sardinian β°39 carriers or in controls (p > 0.05; results are not shown).

Three TNFd(GA)n alleles showed significant differences in allelic frequencies between groups, but only in one of the two islands *(table 3)*. In Corsica, 132pb was more prevalent in controls than in β°39 carriers, with an individual ratio of 6/2 (14.77% *versus* 4.29%, OR = 3.75, 95% CI = 1.289-10.909, p = 0.0140). In Sardinia, 134pb was more frequent in β°39 heterozygotes than in controls, with a ratio of 10/2 (25% *versus* 4.88%, p = 0.0001) and 138pb was more prevalent in controls, with a ratio of 18/8 (41.46% *versus* 18.33%, OR = 2.278, 95% CI = 1.409-3.681, p = 0.0006). The 136pb and 140pb alleles had the same frequency pattern in the two islands *(table 3)* with a prevalence of 136pb in β°39 carriers (Corsica: 22/15, 57.14% *versus* 38.64%, p = 0.0159; Sardinia: 24/15, 56.67% *versus* 35.37%, p = 0.0028) and of

Table 1
Genotyping information (oligonucleotide sequences, source, Genbank accession number and PCR programs)

Polymorphism	Primers	Source	Genbank accession number	PCR program
NOS2(AAAT) I/D	5'tggtgcatgcctgtagtcc3' 5'gaggcctctgagatgttggtc3'	[52]	AF017634	95°C 15 min, 30 cycles (94°C 1 min ; 62°C 1 min ; 72°C 1 min), 72°C 15 min
NOS2(CCTTT)n	5'acccctggaagcctacaactgcat3' 5'gccactgcaccctagcctgtctca3'	[53]	AF017634	95°C 15 min, 10 cycles (95°C 1 min ; 60°C 1 min ; 72°C 1 min), 10 cycles (95°C 1 min ; 58°C 1 min ; 72°C 1 min), 10 cycles (95°C 1 min ; 56°C 1 min ; 72°C 1 min), 72°C 5 min
TNFd(GA)n	5'agatccttccctgtgaggttctgct3' 5'catagtgggactctgtctccaaa3'	[54]	Y14768	95°C 15 min, 30 cycles (94°C 1 min ; 60°C 1 min ; 72°C 1 min), 72°C 5 min
TNFe(GA)n	5'gtgcctggttctggagcctctc3' 5'tgagacagaggataggagagacag3'	[54]	Y14768	95°C 15 min, 30 cycles (94°C 1 min ; 60°C 1 min ; 72°C 1 min), 72°C 5 min

Table 2
NOS2(CCTTT)n allele frequencies for the 76 Corsicans (38 β°39 carriers and 38 healthy individuals) and 84 Sardinians (42 β°39 carriers and 42 healthy individuals) studied

	Corsica								Sardinia								
	Controls			β°39 carriers			P (Fisher)	OR	95% CI	Controls			β°39 carriers			P (Fisher)	OR	95% CI
	Frequency	n	%	Frequency	n	%				Frequency	n	%	Frequency	n	%			
181	0.0000	0	0	0.0000	0	0				0.0366	1	3.66	0.0000	0	0			
186	0.0238	1	2.38	0.0303	1	3.03				0.0976	4	9.76	0.0517	2	5.17			
191	0.0357	1	3.57	0.1364	5	13.64	0.0238	0.2857	0.0974-0.8383	0.3049	13	30.49	0.0517	2	5.17	< 0.0001	6	2.4260-14.8390
196	0.1429	5	14.29	0.1515	6	15.15				0.2317	10	23.17	0.2069	9	20.69			
201	0.1905	7	19.05	0.3030	12	30.30				0.2073	9	20.73	0.2759	11	27.59			
206	0.3095	12	30.95	0.1515	6	15.15	0.0112	2.067	1.1910-3.5850	0.0488	2	4.88	0.1897	8	18.97	0.0390	0.2632	0.1022-0.6774
211	0.1786	7	17.86	0.1364	5	13.64				0.0122	0	1.22	0.0862	4	8.62	0.0185	0.1111	0.0143-0.8612
216	0.1190	5	11.90	0.0909	3	9.09				0.0488	2	4.88	0.0862	4	8.62			
221	0.0000	0	0	0.0000	0	0				0.0122	0	1.22	0.0517	2	5.17			

n: number of individuals; OR: odds ratio; CI: confidence interval.

Table 3
TNFd(GA)n allele frequencies for the 76 Corsicans (38 β°39 carriers and 38 healthy individuals) and 84 Sardinians (42 β°39 carriers and 42 healthy individuals) studied

	Corsica								Sardinia								
	Controls			β°39 carriers			P (Fisher)	OR	95% CI	Controls			β°39 carriers			P (Fisher)	OR	95% CI
	Frequency	n	%	Frequency	n	%				Frequency	n	%	Frequency	n	%			
130	0.0227	1	2.27	0.0143	0	1.43				0.0000	0	0	0.0000	0	0			
132	0.1477	6	14.77	0.0429	2	4.29	0.0140	3.7500	1.2890-10.9090	0.0000	0	0	0.0000	0	0			
134	0.0114	0	1.14	0.0571	2	5.71				0.0488	2	4.88	0.2500	10	25	0.0001	0.2000	0.0797-0.5016
136	0.3864	15	38.64	0.5714	22	57.14	0.0159	0.6842	0.5076-0.9222	0.3537	15	35.37	0.5667	24	56.67	0.0028	0.6140	0.4473-0.8429
138	0.2045	8	20.45	0.2714	10	27.14				0.4146	18	41.46	0.1833	8	18.33	0.0006	2.2780	1.4090-3.6810
140	0.2159	8	21.59	0.0429	2	4.29	0.0004	5.2500	1.869-14.7490	0.1463	6	14.63	0.0000	0	0	< 0.0001	Infinite	
142	0.0114	0	1.14	0.0000	0	0				0.0244	1	2.44	0.0000	0	0			
144	0.0000	0	0	0.0000	0	0				0.0122	0	0	0.0000	0	0			

n: number of individuals; OR: odds ratio; CI: confidence interval.

140pb in controls (Corsica: 8/2, 21.59% *versus* 4.29%, OR = 5.25, 95% CI = 1.869-14.749, p = 0.0004; Sardinia: 6/0, 14.63% *versus* 0%, infinite OR, p < 0.0001).

Haplotype frequencies

Considering the NOS2A gene, three haplotypes showed significant differences in haplotype frequencies, but not in the same way for the two islands *(table 4A* and *B)*. Haplotype 318pb-206pb (NOS2(AAAT)I/D-NOS2(CCTTT)n) was more frequent in Corsican controls than in β°39 carriers, with an individual ratio of 9 ± 2/4 ± 1.5 (24.56% *versus* 10.02%, p = 0.0136) and an OR of 2.4 (95% CI = 1.211-4.755), but more frequent in Sardinian β°39 carriers than in controls (8 ± 2.1/0, 18.96% *versus* 1.57%, p < 0.0001). Haplotype 318pb-191pb showed a significant difference between β°39 carriers and controls in Corsica, with a ratio of 5 ± 1.6/1 ± 0.8 (respectively 13.63% *versus* 3.57%, p = 0.0237) and between controls and β°39 carriers in Sardinia with a ratio of 9 ± 2.1/2 ± 1.2 (respectively 5.17% *versus* 34.28%, OR = 6.8, 95% CI = 2.773-16.677, p < 0.0001). Haplotype 322pb-216pb showed a significant difference between controls and β°39 carriers only in Corsica, with a ratio of 2 ± 1.1/0 (respectively 0% *versus* 6.41%, infinite OR, p = 0.0289).

The TNFA gene was also characterised by significant differences in five haplotype frequencies between groups with various patterns in the two islands *(table 5A* and *B)*. In Corsica, haplotypes 132pb-108pb and 136pb-104pb (TNFd(GA)n-TNFe(GA)n) were more frequent in controls than in β°39 carriers (respectively 6 ± 1.4/2 ± 0.9, 14.%77 *versus* 4.28%, OR = 3.75, 95% CI = 1.289-10.909, p = 0.0140; 3 ± 1.3/0, 7.51% *versus* 0%, infinite OR, p = 0.0140). In Sardinia, haplotype 134pb-108pb was prevalent in β°39 heterozygotes with a ratio of 8 ± 2.2/0 (20% *versus* 2.56%, p < 0.0001), and haplotype 138pb-108pb in controls with a ratio of 13 ± 2.2/5± 1.8 (30% *versus* 11.67%, OR = 2.5, 95% CI = 1.359-4.599, p = 0.0029).

Two TNFA haplotypes had a peculiar distribution for β°39 carriers and controls in the two islands *(table 5A* and *B)*, *(figure 1)*. 136pb-108pb was prevalent in β°39 carriers (Corsica: 22 ± 2.2/12 ± 2, 57.14% *versus* 31.12%, p = 0.0003; Sardinia: 23 ± 2.7/13 ± 2.4, 55% *versus* 31.53%, p = 0.0010) and 140pb-108pb in controls

Table 4
NOS2A haplotype frequencies for Corsican (**A**) and (**B**) Sardinian populations

		Controls				β°39 carriers				P (Fisher)	OR	95% CI
		Frequency	s.d.	n	%	Frequency	s.d.	n	%			
A)												
318	181	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
318	186	0.0119	0.0123	0	1.19	0.0303	0.0217	1 ± 0.8	3.03			
318	191	0.0357	0.0207	1 ± 0.8	3.57	0.1363	0.0416	5 ± 1.6	13.63	0.0237	0.2857	0.0974-0.8383
318	196	0.1428	0.0399	5 ± 1.5	14.28	0.1096	0.0403	4 ± 1.5	10.96			
318	201	0.1904	0.0437	7 ± 1.7	19.04	0.3030	0.0581	11 ± 2.2	30.30			
318	206	0.2456	0.0530	9 ± 2	24.56	0.1002	0.0406	4 ± 1.5	10.02	0.0136	2.4000	1.2110-4.7550
318	211	0.1279	0.0414	5 ± 1.6	12.79	0.0780	0.0358	3 ± 1.4	7.80			
318	216	0.0549	0.0275	2±1	5.49	0.0909	0.0363	3 ± 1.4	9.09			
318	221	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
322	186	0.0119	0.0123	0	1.19	0.0419	0.0278	2 ± 1	4.19			
322	196	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
322	201	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
322	206	0.0638	0.0324	2 ± 1.2	6.38	0.0512	0.0290	2 ± 1.1	5.12			
322	211	0.0506	0.0280	2 ± 1	5.06	0.0583	0.0327	2 ± 1.2	5.83			
322	216	0.0640	0.0289	2 ± 1.1	6.40	0.0000	0.0000	0	0	0.0289	Infinite	
B)												
318	181	0.0428	0.0247	2 ± 1	4.28	0.0000	0.0000	0	0			
318	186	0.1142	0.0378	5 ± 1.6	11.42	0.0517	0.0288	2 ± 1.2	5.17			
318	191	0.3428	0.0567	14 ± 2.4	34.28	0.0517	0.0285	2 ± 1.2	5.17	< 0.0001	6.8	2.7730-16.6770
318	196	0.2091	0.0513	9 ± 2.1	20.91	0.2068	0.0543	9 ± 2.3	20.68			
318	201	0.1465	0.0461	6 ± 1.9	14.65	0.2568	0.0587	11 ± 2.5	25.68			
318	206	0.0157	0.0161	0	1.57	0.1896	0.0506	8 ± 2.1	18.96	< 0.0001	0.0526	0.0072-0.3859
318	211	0.0000	0.0000	0	0	0.0344	0.0268	1 ± 1	3.44			
318	216	0.0428	0.0246	2 ± 1	4.28	0.0362	0.0250	1 ± 1	3.62			
318	221	0.0142	0.0137	0	0	0.0517	0.0287	2 ± 1.2	5.17			
322	186	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
322	196	0.0194	0.0190	1 ± 0.8	1.94	0.0000	0.0000	0	0			
322	201	0.0391	0.0258	2 ± 1.1	3.91	0.0189	0.0179	0	1.89			
322	206	0.0128	0.0134	0	1.28	0.0000	0.0000	0	0			
322	211	0.0000	0.0000	0	0	0.0517	0.0301	2 ± 1.3	5.17			
322	216	0.0000	0.0000	0	0	0.0499	0.0303	2 ± 1.3	4.99			

s.d.: sdandard deviation; n: number of individuals; OR: odds ratio; CI: confidence interval.

Table 5
TNFA haplotype frequencies for Corsican (**A**) and (**B**) Sardinian populations

		Controls				β°39 carriers				P (Fisher)	OR	95% CI
		Frequency	s.d.	n	%	Frequency	s.d.	n	%			
A)												
130	104	0.0000	0.0000	0	0	0.0143	0.0137	0	1.43			
130	108	0.0227	0.0159	1 ± 0.6	2.27	0.0000	0.0000	0	0			
132	108	0.1477	0.0376	6 ± 1.4	14.77	0.0429	0.0243	2 ± 0.9	4.29	0.0140	3.7500	1.2890-10.9090
134	104	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
134	108	0.0114	0.0113	0	1.14	0.0571	0.0276	2 ± 1	5.71			
136	102	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
136	104	0.0751	0.0333	3 ± 1.3	7.51	0.0000	0.0000	0	0	0.0140	Infinite	
136	106	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
136	108	0.3113	0.0527	12 ± 2	31.13	0.5714	0.0572	22 ± 2.2	57.14	0.0003	0.5439	0.3877-0.7629
138	102	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
138	104	0.0726	0.0299	3 ± 1.1	7.26	0.0714	0.0313	3 ± 1.2	7.14			
138	106	0.0000	0.0000	0	0	0.0143	0.0131	0	1.43			
138	108	0.1319	0.0384	5 ± 1.4	13.19	0.1857	0.0482	7 ± 1.8	18.57			
140	106	0.0114	0.0118	0	1.14	0.0000	0.0000	0	0			
140	108	0.2045	0.0464	8 ± 1.8	20.45	0.0429	0.0249	2 ± 0.9	4.29	0.0008	5.0000	1.7720-14.1090
142	104	0.0114	0.0115	0	1.14	0.0000	0.0000	0	0			
144	104	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
B)												
130	104	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
130	108	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
132	108	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
134	104	0.0128	0.0140	0	1.28	0.0500	0.0306	2 ± 1.3	5.00			
134	108	0.0256	0.0189	1 ± 0.8	2.56	0.2000	0.0534	8 ± 2.2	20.00	< 0.0001	0.1000	0.02400-0.4167
136	102	0.0000	0.0000	0	0	0.0167	0.0171	0	1.67			
136	104	0.0180	0.0195	0	1.80	0.0000	0.0000	0	0			
136	106	0.0128	0.0151	0	1.28	0.0000	0.0000	0	0			
136	108	0.3153	0.0575	13 ± 2.4	31.53	0.5500	0.0635	23 ± 2.7	55.00	0.0010	0.5636	0.4004-0.7935
138	102	0.0000	0.0000	0	0	0.0167	0.0174	0	1.67			
138	104	0.1102	0.0381	5 ± 1.6	11.02	0.0500	0.0294	2 ± 1.2	5.00			
138	106	0.0256	0.0209	1 ± 0.9	2.56	0.0000	0.0000	0	0			
138	108	0.3000	0.0532	13 ± 2.2	30.00	0.1167	0.0440	5 ± 1.8	11.67	0.0029	2.5000	1.3590-4.5990
140	106	0.0000	0.0000	0	0	0.0000	0.0000	0	0			
140	108	0.1410	0.0398	6 ± 1.7	14.10	0.0000	0.0000	0	0	< 0.0001	Infinite	
142	104	0.0256	0.0179	1 ± 0.7	2.56	0.0000	0.0000	0	0			
144	104	0.0128	0.0125	0	1.28	0.0000	0.0000	0	0			

s.d.: sdandard deviation; n: number of individuals; OR: odds ratio; CI: confidence interval.

(Corsica: 8 ± 1.8/2 ± 0.9, 20.45% *versus* 4.28%, OR = 5, 95% CI = 1.772-14.109, p = 0.0008; Sardinia: 6 ± 1.7/0, 14,10% *versus* 0%, infinite OR, p < 0.0001).

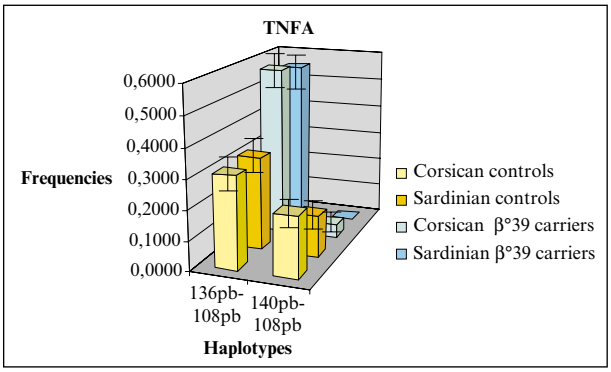

Figure 1
TNFA 136pb-108pb and 140pb-108pb haplotype frequencies.

Hardy-Weinberg equilibrium

Most parts of the *loci* studied were in Hardy-Weinberg equilibrium for the four populations. Only three exceptions emerged: NOS2(CCTTT)n in Corsican controls (P < 0.001), TNFd(GA)n and TNFe(GA)n in Sardinian β°39 carriers (respectively p < 0.001 and p < 0.05) *(table 6)*.

DISCUSSION

Population structure and history have influenced genetic diversity, and natural selection may have played an important role. Indeed, infectious diseases, such as malaria, have exerted a big selective pressure in mediterranean populations over millenia. Genetic traces have already been highlighted, such as the prevalence of the β°39 mutation in the islands of Corsica and Sardinia [4, 6, 44]. Against

Table 6
Observed heterozygosity (Hobs), expected heterozygosity (Hexp), Hardy-Weinberg P value (P)

	Corsicans				Corsican β°39 carriers				Sardinians				Sardinian β°39 carriers			
Locus	**Hobs**	**Hexp**	**P value**	**s.d.**	**Hobs**	**Hexp**	**P value**	**s.d.**	**Hobs**	**Hexp**	**P value**	**s.d.**	**Hobs**	**Hexp**	**P value**	**s.d.**
TNFd(GA)n	0.70455	0.74817	0.17476	0.00081	0.68571	0.60207	0.13292	0.00118	0.56410	0.68765	0.19781	0.00117	0.86667	0.59266	***0.00029	0.00005
TNFe(GA)n	0.29545	0.28971	1.00000	0.00000	0.17143	0.21077	0.09325	0.00095	0.35897	0.37929	0.79047	0.00126	0.20000	0.27119	*0.03684	0.00054
NOS2(AAATt)n1/D	0.23810	0.33133	0.14231	0.00104	0.30303	0.28671	1.00000	0.00000	0.14286	0.13458	1.00000	0.00000	0.24138	0.24622	1.00000	0.00000
NOS2(CCTTT)n	0.80952	0.80924	***0.00034	0.00006	0.78788	0.84522	0.95886	0.00065	0.68571	0.78923	0.41328	0.00122	0.86207	0.83666	0.09839	0.00074

s.d.: standard deviation; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ***** $p < 0,00001$.

this background, we focused our research on genetic polymorphisms which may have been influenced by selective pressure. Results for four polymorphisms located in the TNFA and NOS2A genes in Corsican and Sardinian β°39 carriers and controls are presented here. These two islands were chosen because of the long-term presence of infectious disease pressure, and for their genetic similarity, also highlighted by studies on neutral *loci* [45-49]. The principal limitation of our study is the number of individuals included (160 individuals in four groups of 42; 42; 38 and 38), but the significance of results reported here is attested by and related to similarity and homogeneity of the Corsican and Sardinian populations [45-49].

We found only three deviations from Hardy-Weinberg equilibrium, (NOS2(CCTTT)n in Corsican controls, and TNFd(GA)n and TNFe(GA)n in Sardinian β°39 carriers), due to excess or loss of heterozygotes, probably correlated respectively with balanced and directional selection of *loci*.

For the NOS2A gene, at allelic or haplotype level, the significant differences between β°39 carriers and controls are not the same for Sardinia and Corsica. We can hypothetise that this variability between islands depends on local phenomena. In spite of the similarities between Corsica and Sardinia, some genetic differences do exist, probably due to genetic drift or other local adaptation phenomena. Some studies have shown various patterns in the two islands, such as a peculiar Y haplotype in Sardinia [47]. The same phenomena have probably led to analogous differences observed for some alleles or haplotypes at TNFA *loci* locally between groups.

Some genetic selections were found in the two islands. *Loci* showing significantly different allelic or haplotype distribution between β°39 carriers and controls in the two islands could reflect the impact of infectious pressure. Two TNFA haplotypes have apparently been impacted: 136pb-108pb (1) and 140pb-108pb (2): 1 was mainly found in β°39 carriers and 2 in controls, in Corsica and in Sardinia. So 1 has probably been selected because it confers some advantage or resistance in the case of malaria infection, such as the β°39 mutation. Indeed, TNFα is a cytokine which can directly modulate NOS2 activity and consequently, NO concentration, acting on oxidative stress and resistance to the parasite. So β°39 and haplotype 1 could participate in the resistance to parasites, both at the allelic (β°39 and 136pb) and haplotype level. Haplotype 2 was more frequent in controls, suggesting that allele 140pb could be linked to susceptibility to infection.

So, in these subjects, selective pressures have not impacted the first level of constitutive NO production, but they have impacted the regulation of TNF levels.

The two microsatellites studied in TNFA gene are located in 3'UTR. Some microsatellite variants in the 3'UTR region of other genes are linked with regulation of expression. Indeed, variants of a 3'UTR microsatellite in the CD154 gene are associated with regulation of mRNA and protein expression [50]. The 3'UTR microsatellite of the FGF9 gene is a functional polymorphism that plays roles in FGF9 expression (strongest promoter activity and longest mRNA activity) [51]. So, TNFd and TNFe polymorphisms could be linked to regulation of

TNF-α production, with implications for susceptibility or resistance to malaria.

To conclude, this work highlights, for the first time, selection of two TNFA haplotypes, 136pb-108pb and 140-108pb, correlated respectively with the presence or absence of the β°39 mutation, in Corsican and Sardinian populations. These patterns could be as consequence of infectious pressure and could have implications in TNF-α production. This protein appears to be implicated in a complex mechanism of resistance or susceptibility for pathogens such as.

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