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# Multiple sclerosis and anti-*Plasmodium falciparum* innate immune response

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## Abstract

Several epidemiological investigations conducted in Sardinia, insular Italy, indicate that the strong selective pressure of malaria along the centuries may have concurred to the elevated genetic MS-risk in this region. To test such hypothesis in an experimental setting, we have compared the immune response to *P. falciparum* (the causative agent of malaria) in Sardinian MS patients relative to their ethnic healthy controls and control MS patients of different ethnicity. To this purpose, the *P. falciparum*-driven peripheral mononuclear cell proliferation, the production of pro-inflammatory cytokines of the innate immunity such as TNF- $\alpha$ , IL-6 and IL-12 and the ability to inhibit the parasite growth have been tested in relation to HLA-DR alleles and TNF promoter polymorphisms known of being associated to MS.

We found that *P. falciparum*-induced proliferation, cytokine production and parasite killing are significantly augmented in Sardinian MS patients as compared to controls ( $p < 0.01$ ). Additionally, a correlation is found with genes associated to Sardinian MS, namely the TNF<sub>-376A</sub> promoter polymorphism and the class II HLA-DRB1\*0405 allele. In conclusion, we have found evidences that some genetic traits formerly selected to confer a protective responses to *P. falciparum* now partially contribute to the elevated MS susceptibility amongst Sardinians.

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**Keywords:** Multiple sclerosis; Malaria; *Plasmodium falciparum*; TNF; HLA

## 1. Introduction

It is known that malaria exerts a strong selective pressure which can result in an enrichment of alleles that may increase the susceptibility to other diseases. Since the first “malaria hypothesis” (Haldane, 1949), several haemoglobinopathies (Modiano et al., 2001), thalassaemias (Flint et al., 1986; Allen et al., 1997), HLA and TNF polymorphisms (Hill et al., 1991; Knight et al., 1999; McGuire et al., 1999) as well as red-cell enzyme deficiencies (Ruwende et al., 1995) have been con-

sidered efficient genetic weapons to protect many populations from severe forms of *P. falciparum* malaria. Some examples have been clearly documented in the population of Sardinia, insular Italy, which include the selection of alleles determining the glucose-6-phosphate dehydrogenase deficiency, also known as favism, and the  $\beta$ -thalassaemia in response to the heavy environmental burden of the century-lasting malaria (Siniscalco et al., 1961; Brown, 1981).

Multiple sclerosis (MS) is an immune-mediated disease of the central myelin with a putative (auto)immune-mediated pathogenesis and unknown aetiology (Hohlfeld and Wekerle, 2004). A number of predisposing genes are believed to act in concert with precipitating environmental events to determine MS appearance, although there is no consensus as to how many

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and which of either one is the causal factor(s) (Lincoln et al., 2005). The geographical distribution of MS describes areas of high (northern Europe), medium (Mediterranean basin) and low prevalence rates (Africa; Pugliatti et al., 2002). However, the prevalence of MS has raised in Sardinia during the last four decades resulting in one of the highest worldwide despite its Mediterranean location (Pugliatti et al., 2001).

Several studies conducted on red cells, HLA, mitochondrial and Y chromosome DNA gene-frequency clearly indicate that Sardinians have a different phylogeny from Europeans, including mainland Italians, along with a high value of consanguinity due to their geographical isolation (reviewed in Sotgiu et al., 2004).

In 1951, the WHO-coordinated Sardinian campaign eradicated malaria from the island (Brown, 1998). Perhaps not coincidentally, and certainly not fully justified by an improved diagnostic accuracy, a 3-fold increase of MS incidence has been recorded in Sardinia starting from 1960 (Rosati et al., 1986; Pugliatti et al., 2005). This “MS epidemics” represents a rare event and still an unsolved enigma. However, because 50 years is too short a span for a substantial Sardinians’ genetic change, an environmental modification becomes a likely determinant (Sotgiu et al., 2003; Pugliatti et al., 2006).

Compared to other Caucasians living in the same Mediterranean area, Sardinians have had among the highest mortality rate for malaria (Kaneko et al., 2000; Brown, 1998). Curiously, the “Sardinian” MS-associated HLA haplotype has the highest odds ratios in the same former highest-rate malarial areas (Bitti et al., 2001) and the frequency of some TNF polymorphisms, associated with both MS and malaria (Knight et al., 1999; McGuire et al., 1999; Fernandez-Arquero et al., 1999), are 10-fold elevated amongst Sardinians as compared to any neighbor populations (Wirz et al., 2004).

Despite these circumstantially good suggestions, a clear-cut experimental association between MS and malaria has never been demonstrated to date. With the present study, we aim at disclosing evidences of an abnormal immunological response to *P. falciparum* in Sardinian MS patients (sMS) as compared to their ethnic unrelated healthy controls (sHC) and MS patients from continental Italy (iMS). Monocytes and lymphocytes of the peripheral blood (MNC) were used to this purpose as they are protagonists of the immune response against *P. falciparum* and of the pathogenesis of MS (Lassmann et al., 2001; Deininger et al., 2002; Hendriks et al., 2005).

## 2. Materials and methods

### 2.1. Patients and controls

Twenty-eight patients of Sardinian ancestry (18 females and 10 males, mean age  $29 \pm 5$  years) with definite MS (Poser et al., 1983) (sMS) and 28 age and sex-matched ethnic healthy controls (sHC, mean age  $28 \pm 2$ ) were recruited for this study. An additional series of 16 age and sex-matched MS patients (11 females and 5 males, mean age  $31 \pm 4$ ) of mainland Italian phylogeny (iMS) were also selected as control of sMS. All donors gave a written consent to participate to this study.

### 2.2. HLA-DR and TNF-promoter genotyping

Polymorphic DRB1 gene and dot blot analysis with oligonucleotide probes were carried out according to published methods (Marrosu et al., 1997). SNPs of the TNF promoter region were determined as previously described (Wirz et al., 2004).

### 2.3. *P. falciparum* cultures and antigen preparation

*P. falciparum* clone 3D7A (Walliker et al., 1987) were maintained *in vitro* according to the method described by Trager and Jensen (1976). Parasites were grown in O<sup>+</sup> red blood cells in RPMI 1640 plus hypoxanthine 50 µg/ml, supplemented with 10% defibrinated human plasma at 37 °C, in a 2% O<sub>2</sub> and 5% CO<sub>2</sub> atmosphere. The culture medium was refreshed daily and the parasitaemia monitored using thin blood smears (Giemsa stain). *P. falciparum* trophozoites were used for antigen preparation. To obtain parasite synchronization, 8–10% parasitaemia cultures were centrifuged at 1500 rpm. The pellet was resuspended in incomplete RPMI medium supplemented with 0.75% Gelatine (Merck) to make an overall 10% parasite suspension, and incubated at 37 °C for 45 min. Top layer was washed in phosphate-buffered saline (PBS). Morphological analysis of Giemsa-stained slides indicated that resulting cultures typically contained >80% trophozoite stages. Parasitized erythrocytes were lysed with 0.05% saponin solution in 1 × PBS (Wallach, 1982), pelleted and flash frozen in liquid nitrogen. The pellet of *P. falciparum* was repeatedly (six times) sonicated on ice for 30 s, Bandelin Sonopuls, Berlin. A lysate composed of a mixture of *P. falciparum* antigens was obtained.

### 2.4. *P. falciparum*-driven MNC proliferation test

Peripheral blood was collected in EDTA-containing tubes. Mononuclear cells (MNC) were isolated by centrifugation on a discontinuous density gradient (Lymphoprep; 1.077 g/ml; Nycomed, Oslo, Norway). The optimal concentration of *Plasmodium* to be used in the MNC proliferation experiments was preliminarily determined with the use of a dose-dependent assay, as follows:  $2 \times 10^5$  MNC/well from 4 MS patients and 4 HC were plated in triplicate on a microtiter plate alone or in the presence of either 10 µg/ml LPS, or *P. falciparum* lysate at concentrations of 0.1, 1 and 10 *Plasmodium*/MNC. After 48 h, cell cultures were harvested, transferred in another 96-well plate and incubated with BrdU. After an additional 3 h culture a proliferation assays was performed, in triplicate, following instructions from the manufacturer (Amersham Biosciences). Optical density (OD) was measured using an ELISA reader. According with the manufacturer indications, OD value of 1 is equivalent to  $20 [^3\text{H}]\text{-thymidine cpm} \times 10^{-3}$  in a proliferation test of 24 h duration and 500 L929 cell/well concentration.

At the highest *P. falciparum* concentration (10 *Plasmodium*/MNC) the proliferative response was the lowest, possibly due to a toxic effect. The concentration of 1 *Plasmodium*/MNC gave the highest response and was therefore used for the remaining tests. MS patients and controls were all rhesus+ to exclude an

unspecific MNC proliferative response. The inter-assay coefficient of variation was lower than 10%.

### 2.5. Inhibition of *P. falciparum* growth by macrophages

Peripheral blood was collected in EDTA-containing tubes. MNC from sMS patients and sHC were isolated by centrifugation on a discontinuous density gradient (Lymphoprep, as above). MNC were subsequently centrifuged on a Percoll gradient (Pharmacia, Uppsala, Sweden) consisting of three density layers (1.076, 1.059 and 1.045 g/ml). The upper fraction, containing predominantly monocytes, was allowed to adhere to Nunclon culture flasks (Nunc, Roskilde, Denmark) in RPMI 1640 medium. Monocytes were identified on day 3 of culture by flow cytometry as positive for CD14 and HLA-DR, but lacking the markers for CD3 (T cells), CD19-CD20 (B cells), CD16-CD56 (NK cells), and CD123 (plasmacytoid dendritic cells). Adherent monocytes were collected, washed twice with PBS and resuspended in T-25 flasks containing 10 ml RPMI 1640 supplemented with 50 µg/ml gentamicine sulfate and 20% fetal calf serum (FCS). Cells were cultured for 5 days at 37 °C in 5% CO<sub>2</sub> atmosphere. Adherent cells (monocyte-derived macrophages) from each group were collected by centrifugation at 1200 rpm for 10 min and resuspended at a concentration of  $1 \times 10^6$  cells/ml in complete RPMI 1640; 100 µl of such cell suspension plus 100 µl of *P. falciparum* synchronous cultures were dispensed on a 96-well microtiter plate, yielding a final parasitaemia of 1% and a final haematocrite of 1% in each well. The plates were incubated at 37 °C, in 2% O<sub>2</sub> and 5% CO<sub>2</sub> atmosphere for 48 h. For each series of macrophages the experiment was made in eight replicates.

Parasite growth was determined by measuring the activity of its lactate dehydrogenase (pLDH), according to a modified version of the method described by Makler and Hinrichs (1993). pLDH test is based on the principle that the plasmodial lactate dehydrogenase (LDH) can use 3-acetylpyridine NAD (APAD) as coenzyme, which is converted to APADH during the lactate oxidation (Makler and Hinrichs, 1993).

After incubation, cultures were resuspended; 20 µl from each well were transferred into another 96-well plate containing 100 µl Malstat reagent: L-(+)-lactic acid (110 mM), Trizma base (0.034 mM), Triton X-100 (0.125%), 3-acetylpyridine adenine dinucleotide (APAD, 0.62 mM), 25 µl nitroblue tetrazolium (NBT, 1.96 mM) and phenazine ethosulfate solution (PES, 0.24 mM) (all reagents from Sigma). The plate was incubated at room temperature for 15 min. The pLDH activity was measured through a 650 nm OD spectrophotometer. Data were expressed as percentage of parasite growth and the different macrophage groups were compared to the untreated controls.

### 2.6. Soluble MNC products

Enzyme-linked immunosorbent assays (ELISA) tests for human IL-6, TNF-α and IL-12p40 (all from Euroclone, Switzerland) were performed on the supernatant of MNC proliferation test, according with indications and suggestions

from the manufacturer. Measurements were carried out on triplicate MNC wells, plated alone and with LPS (10 µg/ml) and *P. falciparum* lysate (1 *Plasmodium*/MNC). Values were expressed as pg/ml.

### 2.7. Statistical analysis

The differences of MNC proliferation and macrophage-mediated *P. falciparum* killing were analysed by *T*-test. The level of cytokine production was compared by using median, interquartile range—IQR: 25 (1st) and 75 (3rd) percentile, and the Mann–Whitney Rank Sum Test. sMS and sHC were subgrouped based on the distinct DRB1 and TNF-promoter polymorphisms; their TNF production was analysed by using mean, standard deviation (SD) and *T*-test. The frequency of HLA and TNF polymorphisms was analysed through the  $\chi^2$ . Significance was set at <0.05. The power of samples was calculated with Statmate 2 program for Windows (GraphPad Prism version 4, USA).

## 3. Results

### 3.1. MNC proliferation in the presence of *P. falciparum* antigens

The mean proliferation (OD) of untreated MNC was  $2.4 \pm 0.5$  in sMS patients,  $2.7 \pm 0.3$  in iMS patients and  $2.9 \pm 0.9$  in sHC (*p* not significant). Compared to these untreated MNC, the average proliferation was significantly higher (*p*=0.01) following the LPS-driven stimulation ( $4.6 \pm 1.6$  in sMS patients,  $4.8 \pm 1.2$  in iMS patients and  $5.4 \pm 2.0$  in HC), with no significant difference among the three groups.

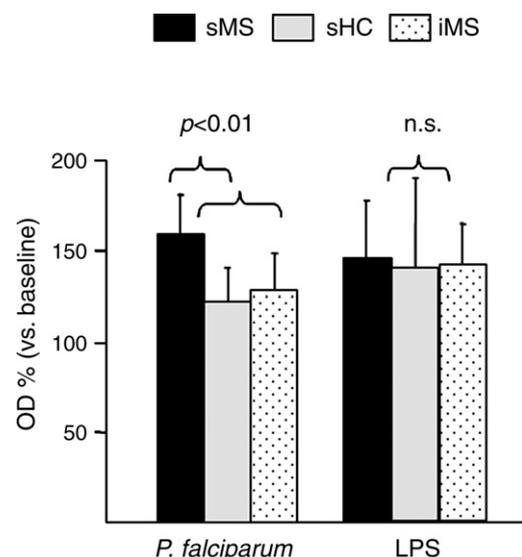


Fig. 1. Proportion of the proliferation (% OD on Y axis) of *P. falciparum*- and LPS-stimulated as compared to non stimulated (untreated, white bars) peripheral blood mononuclear cells from 28 Sardinian MS patients (sMS), 28 Sardinian healthy donors (HC) and 16 mainland Italian MS patients (iMS); statistical *p* values of the different LPS- and *P. falciparum*-driven proliferation in the three groups are reported.

The average proliferation of the *P. falciparum*-stimulated MNC was  $5.1 \pm 1.1$  in sMS patients  $4.9 \pm 1$  in iMS patients and  $4.8 \pm 1$  in HC, also significantly different from the relative untreated MNC proliferation ( $p=0.01$  for sHC,  $p=0.0001$  for sMS and  $p<0.01$  for iMS). When analysing the percentage levels of *P. falciparum*-driven proliferation as compared to the background level of the untreated MNC (%OD on Fig. 1, Y axis), we found a highly significant difference between the three groups:  $158.3 \pm 23\%$  in sMS,  $125 \pm 19\%$  in iMS and  $121.7 \pm 19\%$  in sHC, respectively (sMS vs. HC  $p=0.002$ ; sMS vs. iMS  $p<0.01$ ).

### 3.2. *P. falciparum* growth inhibition

The *P. falciparum* inhibition test, measuring the activity of pLDH after 48 h incubation with macrophages, showed that the *P. falciparum* growth is inhibited on average by  $8.8 \pm 6\%$  with macrophages from the 12 sHC and by  $36 \pm 7\%$  with macrophages from the 12 sMS patients. The different killing capacity is highly significant ( $p<0.001$ ; Fig. 2).

### 3.3. Cytokine production

Unstimulated MNC produced low levels of TNF (median 95 pg/ml, interquartile range—IQR 89–121 in sMS; 99, IQR 68–153 in sHC and 89, IQR 57–123 in iMS) which did not differ between the three groups. LPS-stimulated MNC produced higher amounts of TNF (median 411 pg/ml, IQR 334–453 in sHC; 383, IQR 339–501 in sMS and 323, IQR 289–479 in

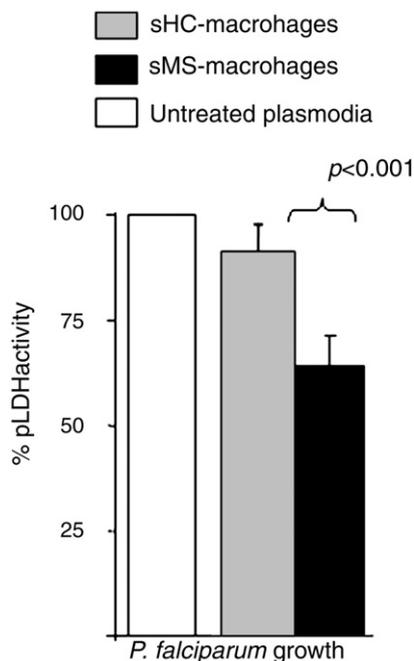


Fig. 2. Proportion (percentage on Y axis) of pLDH activity of *P. falciparum* in three different conditions: untreated (white bar, 100%), treated with macrophages from healthy controls (sHC, grey bar) and treated with macrophages from sMS patients (black bar). As compared to the untreated condition there is a significant reduction of *P. falciparum* growth due to sMS-derived macrophages as compared to HC-derived macrophages.

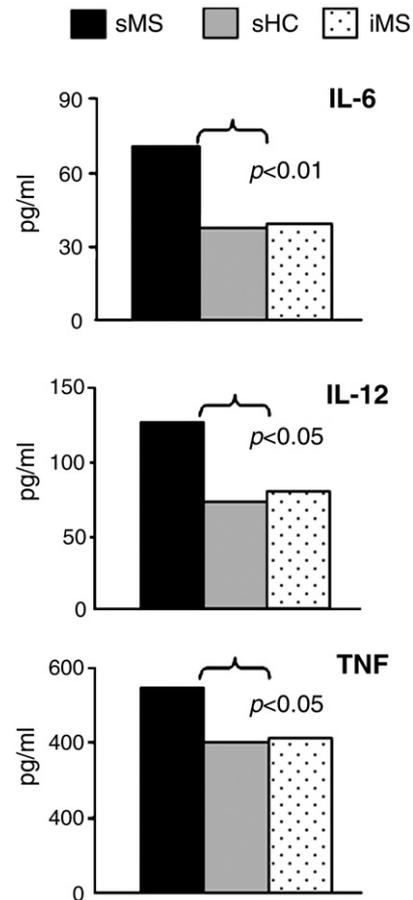


Fig. 3. Median TNF, IL-6 and IL-12 production (pg/ml on Y axis) in the supernatant of *P. falciparum*-treated MNC from 28 Sardinian MS patients (sMS, black bars), 28 Sardinian healthy donors (sHC, grey bars) and 16 mainland Italian MS patients (iMS, dotted bars). The *P. falciparum*-driven production of the three cytokines is significantly different higher in sMS as compared to iMS and HC.

iMS) which also did not differ between groups. On the contrary, *P. falciparum*-driven TNF production significantly differed between sMS, iMS and sHC: median TNF level was 547 pg/ml (IQR 347–642) in sMS, 399 pg/ml (IQR 265–430) in iMS and 410 pg/ml (IQR 329–450) in sHC (Mann–Whitney  $p<0.05$ ; Fig. 3). The highest TNF production was found in the sMS group associated with the presence of both the HLA-DRB1\*0405 allele and the TNF<sub>-376A</sub> polymorphism (Table 1).

LPS-stimulated MNC from sHC, sMS and iMS also produced high levels of IL-6 (median 25 pg/ml, IQR 12–32 in sMS; 17, IQR 6–23 in sHC and 15, IQR 4–19 in iMS) and IL-12 (median 154 pg/ml, IQR 60–252 in sMS; 123, IQR 80–203 in sHC and 135, IQR 71–201 in iMS) which did not significantly differ between the three groups. On the contrary, the *P. falciparum*-driven IL-6 and IL-12 production significantly differed between the groups, as indicated on Fig. 3. In details, median IL-6 level was 71 pg/ml (IQR 36–96) in sMS, 38 (18–52) in sHC and 40 (11–59) in iMS (sMS vs. HC and iMS  $p<0.01$ , for both). Median IL-12 level was 126 pg/ml (IQR 36–201) in sMS, 74 (36–104) in sHC and 80 (39–122) in iMS (sMS vs. HC and iMS  $p<0.05$ , for both).

Table 1  
HLA and TNF genotyping and the anti-*P. falciparum* TNF production from sMS and sHC (analysed with mean value±SD)

DRB1	TNF promoter												TNF (mean±SD)		
	376/w		376/376		238/w		238/238		308/w		w/w		t	MS	HC
	MS	HC	MS	HC	MS	HC	MS	HC	MS	HC	MS	HC			
0405=0405		1	1		1								3	759±214 <sup>§</sup>	567±222
0405/other		1			2		1	2	1	2	2		13	665±233 <sup>§</sup>	532±243
0301=0301	2				1						1		4	600±321	465±327
0301/other	2	1			1	1	1		1	2	1	1	11	612±243	433±324
1601=1601					1	1					1	1	4	289±211	302±256
1601/other		2					1				2	3	8	290±221	270±243
1104/other	2				1						1	1	5	334±132	302±249
1501/other	1											2	3	342±278	–
Other		1			1						1	2	5	367±251	421±319
Total	7	6	1	–	7	5	2	1	3	3	8	13	56		
TNF	712 <sup>#</sup> ±	356±	709 <sup>#</sup> ±	–	584±	562±	624±	548±	312±	329±	333±	318±			
(mean±SD)	345	259	301		286	312	273	279	222	232	269	222			

<sup>§</sup>=TNF production in MNC from DRB1\*0405 vs. DRB1\*0301 MS patients:  $p=0.057$ ; DRB1\*0405 vs DRB1\*1601 and others MS patients:  $p<0.001$ .  $t$ =total of cases, <sup>#</sup>=TNF production in TNF<sub>-376A</sub>-bearing MS patients vs. all others:  $p<0.01$ .

### 3.4. HLA and TNF polymorphisms

Of the 28 sMS patients, 9 (32.1%) carried at least one “Sardinian MS-predisposing” DRB1\*0405 allele, 8 (28.6%) the Sardinian MS-predisposing DRB1\*0301 allele and 1 (3.6%) the “Caucasian MS-predisposing” DRB1\*1501 allele (Marrosu et al., 1997). The remaining patients carried “non MS-associated” DRB1 alleles. The 28 sHC had a similar frequency of the “MS-predisposing” DRB1\*0405 (8 subjects, 28.6%), and the DRB1\*0301 allele (7 subjects, 25%). Seven (43.7%) iMS carried the “Caucasian MS-predisposing” DRB1\*1501 allele. The remaining iMS carried other non MS-predisposing HLA-alleles.

As for TNF<sub>-376A</sub>, TNF<sub>-308A</sub> and TNF<sub>-238A</sub> polymorphisms of the TNF- $\alpha$  promoter region, the TNF<sub>-376A</sub> was present in 8 sMS patients (28.6%), in 6 sHC (21.4%); not determined in iMS. TNF<sub>-308A</sub> was found in 3 sMS (10.7%), in 3 sHC (10.7%) and 3 iMS (18.7%). TNF<sub>-376A</sub> was found in 9 sMS patients (32.1%), 6 sHC (21.4%) and 1 iMS (6.2%). Neither TNF<sub>-376A</sub> nor TNF<sub>-238A</sub> were preferentially associated with DRB1\*0405 or DRB1\*0301 MS-predisposing alleles in our study. See Table 2.

## 4. Discussion

The recent increased incidence of autoimmune and allergic diseases in the so called “developed” world has been significantly correlated to the reduced rate of childhood infections and the rapid amelioration of the hygienic conditions (Yazdanbakhsh et al., 2002). In fact, whilst immune-mediated

diseases, including MS, are rarely seen in “developing” and malarial regions of Africa, they show a significant increase in African migrants living in “western” countries (Butcher, 1991; Delasnerie-Laupretre and Alperovitch, 1990; Kurtzke et al., 1998).

The heavy segregational load induced by the selective pressure of malaria in Sardinia ( $\beta$ -thalassaemia and favism; Cavalli Sforza and Bodmer, 1971; Siniscalco et al., 1961; Brown, 1981), the similarity of polymorphic genes associated to MS susceptibility and protection against malaria (Wirz et al., 2004) and the genetic “footprints” that link MS to malaria (Bitti et al., 2001) allow us to hypothesize that some genetic traits that had conferred increased resistance to *P. falciparum* might now contribute to increase the susceptibility toward MS.

The eradication of the *anopheles* mosquito, the vector of *P. falciparum*, has been a dramatic, abrupt and radical environmental change as compared to the rather slow adaptation of the Sardinian genome against malaria. As a consequence, some formerly selected genes may have become hardly compatible with a full healthy status (Haldane, 1949; Butcher, 1991; Yazdanbakhsh et al., 2002; Sotgiu et al., 2003).

Therefore, we have measured the MNC response to *P. falciparum* in Sardinian individuals affected by MS, in healthy ethnic controls (sMS and sHC) and mainland Italian control MS patients (iMS). Two different immune patterns have been clearly seen. While the anti-LPS response was mounted to similar extents in the three MNC subgroups, the response driven by *P. falciparum* antigens was significantly

Table 2  
Percentage of the MS-predisposing alleles of the HLA-class III TNF promoter region (TNF<sub>-376A</sub>, TNF<sub>-308A</sub> and TNF<sub>-238A</sub>) and of the HLA-class II region (HLA-DR2,-DR3 and -DR4) in the three series of subjects: 28 Sardinian MS (sMS), 28 Sardinian healthy controls (HC) and 16 mainland, continental Italian MS (iMS)

Population	TNF <sub>-376A</sub>	TNF <sub>-308A</sub>	TNF <sub>-238A</sub>	HLA-DR4 (DRB1*0405)	HLA-DR3 (DRB1*0301)	HLA-DR2 (DRB1*1501)
sMS (n=28)	28.6% (8)	10.7% (3)	32.1% (9)	32.1% (9)	28.6% (8)	3.6% (1)
sHC (n=28)	21.4% (6)	10.7% (3)	21.4% (6)	28.6% (8)	25% (7)	–
iMS (n=16)	No data	18.7% (3)	6.2% (1)	–	–	43.7% (7)

elevated only in sMS patients. Also, killing of the parasite and phagocytosis of infected red blood cells by macrophages was significantly enhanced in sMS patients as compared to sHC. These findings suggest, for the first time, that Sardinian individuals affected by MS have a stronger anti-*P. falciparum* immune response than healthy controls and MS controls of different phylogeny.

To further substantiate this skewed response, we have polarised our investigation on some genes in the context of the HLA region. This region spans on chromosome 6 with HLA class I, II, III and other genes. Among all gene-coded proteins, HLA class I molecules mediate immune responses against “endogenous” antigens and virally infected targets by restricting CD8. T cell function; HLA class II molecules are involved in the presentation of “exogenous” antigens to T helper cells. The HLA class III region contains genes encoding cell-mediated related or unrelated proteins that regulate immune responses, including TNF. Among all these genes we selected the ones which are the most likely associated with both MS and malaria (Knight et al., 1999; McGuire et al., 1999; Fernandez-Arquero et al., 1999; Wirz et al., 2004).

The Common Disease/Common Variant hypothesis (Chakravarti, 1999) predicts that the genetic risk for common diseases will often be due to disease-predisposing alleles with relatively high frequencies. According to this hypothesis, MS is associated with the frequent DRB1\*1501 in continental Italians and with the frequent DRB1\*0405 and DRB1\*0301 HLA-alleles in the genetically distinct population of Sardinia (Marrosu et al., 1997). As for the TNF, TNF<sub>-376A</sub> and TNF<sub>-238A</sub> polymorphic alleles are extremely frequent amongst Sardinians (Wirz et al., 2004), associated with MS and with the evolutionary protection against severe forms of malaria (Knight et al., 1999; McGuire et al., 1999; Fernandez-Arquero et al., 1999). In our study, the abnormal TNF production driven by *P. falciparum* antigens in sMS patients seems to be dependent on the same MS-associated genes, namely DRB1\*0405 and TNF<sub>-376A</sub>, a result which is in agreement with our own former studies (Sotgiu et al., 1999, 2000).

However, while the difference of the anti-*P. falciparum* immune response between the two Sardinian groups (sMS and sHC) was significant, the allelic frequency of DRB1 and TNF was not. Thus, concerns raise on which anti-malaria mechanism actually distinguishes between healthy and MS individuals of Sardinian ancestry. The strategic location of TNF within the HLA region may let us speculating that this MS-specific anti-malaria response resides in strict proximity of such alleles.

We have observed that the anti-*P. falciparum* macrophage *in vitro* response well recalls that occurring during the acute *P. falciparum* malaria *in vivo* (Malaguamera and Musumeci, 2002) and, along with that of TNF, the *P. falciparum*-driven production of IL-6 and IL-12 is also significantly augmented in sMS as compared to sHC and iMS. These *in vitro* results may indicate that IL-6 and IL-12 encoding genes may be contextually involved in our working hypothesis. However, with the exception of some IL-6 gene polymorphisms, not associated with MS in our patients (Schmidt et al., 2003), we have no further evidences to prove or disprove this idea. A more

detailed genetic analysis is needed which may disclose some gene combinations consistent with the accepted multilocus model of the gene-encoded susceptibility to MS (Lincoln et al., 2005).

In conclusion, by relying on the current model of MS pathogenesis and on principles of evolutionary biology, we have found some experimental evidences favoring the view of a link between MS and malaria in Sardinia. On a mechanistic view, some monocyte gene-products (e.g. TNF) were once positively selected by strong and long-lasting environmental pressure forces such as malaria. In pre-hygienic conditions, some Sardinian individuals, favoured by the positive selection, were able to survive from fatal forms of malaria by virtue of their strong anti-*P. falciparum* response. Soon after the World War II, the radical hygienic change may have counterbalanced the positive selection in a way that the former “positive” genes now induce strong monocyte pathogenic reactions in response to some triggering environmental, unknown factors. In fact, after the eradication of malaria and the breaking of the geographical isolation, descendants of those positively selected individuals may now abnormally respond to other pathogens having conformational similarity with *P. falciparum*. This overstimulated immunity (IL-6, IL-12 and TNF) may provoke the development of some immune-mediated diseases, such as MS. Much has yet to be done to formally prove our simplistic evolutionary idea and further immuno-genetic studies are planned with this aim.

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