

# A 24-bp duplication in exon 10 of human chitotriosidase gene from the sub-Saharan to the Mediterranean area: role of parasitic diseases and environmental conditions

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*Human chitotriosidase (Chit) is a member of the chitinase family and it is synthesized by activated macrophages. Recently, a genetic polymorphism was found to be responsible for the common deficiency in Chit activity, frequently encountered in different populations. We analyzed the Chit gene in some ethnic groups from the Mediterranean and African areas, to evaluate whether the Chit gene polymorphism correlates with the changes in environmental features and the disappearance of parasitic diseases. We found a heterozygote frequency for the duplication of 24 bp in exon 10 of 44% in Sicily and 32.71% in Sardinia, whereas those homozygous Chit deficient were 5.45 and 3.73%, respectively. In contrast, in Benin and Burkina Faso, both mesoendemic regions for Plasmodium falciparum malaria and other infections due to intestinal parasites, a low incidence of Chit mutation was found (heterozygous 0 and 2%, respectively) and no subject was homozygous for Chit deficiency. Our results provide evidence of the fact that the low frequency or the absence of mutant Chit gene may represent a protective factor in the population still living in disadvantaged environmental conditions. The present study suggests that the disappearance of parasitic diseases and the improved environmental conditions may have ensued the occurrence of a high percentage of 24-bp mutation in Sicily, in Sardinia and in other Mediterranean countries, whereas in the sub-Saharan regions (Benin and Burkina Faso), the widespread parasitic diseases and the poor social status have contributed to maintenance of the wild-type Chit gene.*

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## Introduction

Human chitotriosidase (Chit) is a member of the chitinase family, a group of enzymes with the capability to hydrolyze chitin. Chitin, a glycopolymer, is present as a structural component in the coating of many living species, such as the cell wall of fungi,<sup>1</sup> the sheath of parasitic nematodes<sup>2</sup> and in the lining of gut of many insects.<sup>3</sup> Human Chit exhibits a remarkable sequence homology among other chitinase from plants, bacteria, fungi, nematodes and insects.<sup>4,5</sup>

In humans, Chit is synthesized by activated macrophages. Chit levels have been proposed as a biochemical marker of macrophage activation in several lysosomal diseases,<sup>6</sup> especially in Gaucher diseases or beta-glucocerebrosidase deficiency,<sup>7</sup> where uncleaved membrane glycolipids are stored in the macrophage cells. Moreover, Chit is increased in patients affected by malaria infection and in other hematological disorders where activated macrophages are involved.<sup>6–9</sup> In keeping with the established antiparasitic function of homologous chitinases in plants, it is conceivable that human Chit might

fulfill a role in the degradation of chitin-containing pathogens.

Chit is mainly secreted as a 50 kDa active enzyme containing a C-terminal chitin-binding domain.<sup>10</sup> In macrophages, this protein is proteolytically processed to a C-terminally truncated 39 kDa isoform characterized by hydrolase activity and accumulated in the lysosomes.<sup>10</sup> In addition, the 50 kDa Chit form is synthesized by neutrophilic granulocyte progenitors and stored in their granules.<sup>4</sup>

The Chit gene is localized in chromosome 1q31–q32.<sup>11</sup> The gene consists of 12 exons and spans about 20 kb of genomic DNA.<sup>4,12</sup> Exon 11 can be alternatively spliced. This exon is usually skipped in the splicing process generating the predominant mRNA form encoding the 50 kDa protein. In macrophages, a different form of mRNA is rarely produced as a result of a lack of exon 11 skipping (39 kDa). A recessive inherited deficiency in Chit activity is frequently encountered in different populations. A 24-bp duplication in exon 10 results in the activation of a cryptic 3' splice site, generating an abnormally spliced mRNA with an in-frame deletion of 87 nucleotides.<sup>12</sup> The spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids.<sup>12</sup> This Chit mutant allele has been found in 33–35% of Ashkenazi Jewish and Dutch individuals, respectively, whereas both populations were about 6%

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homozygous for this allele.<sup>12</sup> Additional studies performed in different populations confirmed the presence of the 24-bp duplication in individuals completely deficient in enzymatically active Chit.<sup>12,13</sup>

Based on the variable incidence of Chit activity deficiency in different populations,<sup>12,13</sup> we performed a comparative study of the Chit gene polymorphism in different ethnic groups of the Mediterranean area (Sicilian and Sardinian populations) and African sub-Saharan area (Benin and Burkina Faso), which differ in terms of a distinct environmental feature and incidence of parasitic diseases.

## Materials and methods

### Subjects

We collected blood samples from healthy subjects from different countries:

- In all, 107 Sardinian healthy subjects, 46 males and 61 females, aged 28–67 years (average 48 years), born in Sardinia from Sardinian parents for two generations, submitted to a physical examination and considered clinically healthy.
- In all, 100 Sicilian healthy blood donors, 80 males and 20 females, aged 22–62 years (average 43 years), born in Sicily from Sicilian parents for two generations.
- In all, 99 African subjects, born and living in Burkina Faso, 27 males and 72 females, aged 1–75 years (average 25 years), submitted to a physical examination and to routine blood analysis.
- In all, 100 African subjects, born and living in Benin, 45 males and 55 females, aged 8–50 years (average 24 years), submitted to a physical examination and to routine blood analysis.
- In all, 67 African children, affected by acute *Plasmodium falciparum* malaria, born and living in Burkina Faso, 40 males and 27 females, aged 2–72 months (average 16 months).

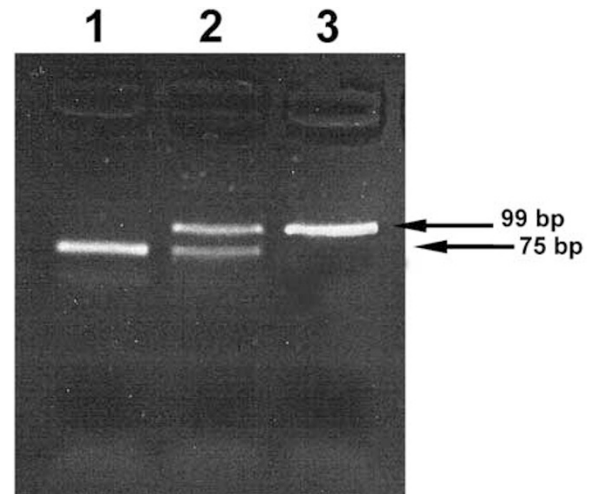
The local Ethical Committees both in Italy and in African countries (Benin and Burkina Faso) approved this study. All individuals participating in the study signed informed consent forms.

### DNA analysis

Genomic DNA was isolated from 5 ml of peripheral blood as previously described.<sup>14</sup> In total, 30 ng of DNA was used as a template in subsequent PCR reactions. The duplication mutation analysis was performed using specific primers (Chs9 (AGCTATCTGAAGCAGAAG) and Chas8 (GGAGAAGCCGGCAAAGTC)) and fragments of 75 and 99 bp were amplified from the normal and mutant Chit genes, respectively. Electrophoresis in Metaphore gel (4%) allowed the detection of both fragments. A mixture of both fragments was detected in heterozygous subjects (Figure 1).

### Chit activity

Chit activity determination was performed as described by Barone *et al.*<sup>8</sup> A measure of 5  $\mu$ l of undiluted plasma was incubated with 100  $\mu$ l of a solution containing 22  $\mu$ mol/l of the artificial substrate 4-methylumbelliferyl- $\beta$ -D-N,N',N''-triacylchitotriose (Sigma Chemical Co) in 0.5 M citrate-phosphate buffer pH 5.2, for 15 min at 37°C.



**Figure 1** Detection of the 24-bp duplication of the chitotriosidase gene detected by PCR of genomic DNA. Amplified fragments were separated on a 4% Metaphore gel and stained with ethidium bromide. Lane 1, homozygote wild type (wt); lane 2, heterozygote wt/mutant and lane 3, homozygote mutant.

The reaction was stopped using 2 ml of 0.5 mol/l  $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer, pH 10.7. The fluorescence was read by a Perkin-Elmer fluorimeter, on 365 nm excitation and 450 nm emission. The Chit activity was measured as nanomoles of substrate hydrolyzed per ml per hour (nmol/ml/h). The plasma Chit was expressed as nmol/ml/h. Samples with Chit levels >110 nmol/ml/h were reassayed after a dilution of 10- or 50-fold with distilled water.

### Statistical analysis

Since the plasma Chit activity was normally distributed both in the homozygote for the wild-type gene and in the heterozygote for the mutated gene, we used Student's *t*-test to compare the plasma Chit activity between different alleles. SPSS 10 was used for analysis and distribution of plasma Chit activity values.

## Results

The results of the Chit polymorphism are summarized in Table 1 and electrophoresis showing detection of the 24-bp duplication in the Chit gene is shown in Figure 1.

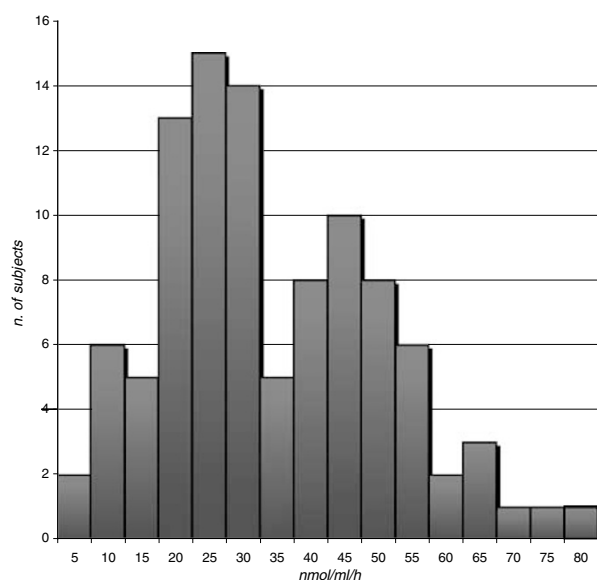
As a whole, a number of 406 Sardinian and Sicilian subjects were genotyped. Sicilian and Sardinian populations were in the Hardy-Weinberg equilibrium with 5.45 and 3.73% homozygous mutant individuals, respectively.

No 24-bp duplication homozygous subject was found among the studied African population. The frequency of this mutant allele was 0.27 and 0.21, while the frequency for the wild-type allele was 0.73 and 0.79 in Sicilian and in Sardinian populations, respectively (Table 1). In the black population living in Benin and Burkina Faso, the mutant allele frequency was 0 and 0.02, respectively.

In Sicilian healthy subjects, the plasma Chit activity levels were higher in homozygous subjects for the wild-type allele than heterozygous subjects for the mutant allele ( $46.87 \pm 16.20$  vs  $29.27 \pm 17.60$  nmol/ml/h).

**Table 1** Frequencies of wild-type and mutant alleles and related plasma Chit activity levels in Sicilian, Sardinian and African subjects

	Sicilian subjects, n=100	Sardinian subjects, n=107	African Benin subjects, n=100	African Burkina Faso subjects, n=100	Chit activity in Sicilian subjects (nmol/ml/h), mean $\pm$ s.d., median (range)	Chit activity in Sardinian subjects (nmol/ml/h), mean $\pm$ s.d., median (range)	Chit activity in African Benin subjects (nmol/ml/h), mean $\pm$ s.d., median (range)	Chit activity in African Burkina Faso subjects (nmol/ml/h), mean $\pm$ s.d., median (range)
Homozygous wild type (wt)	51.01%	63.56%	100%	98%	46.87 $\pm$ 16.20, 44 (0.6–73)	47.92 $\pm$ 20.43, 45 (5–100)	101.3 $\pm$ 89.68, 65 (6–500)	94.71 $\pm$ 93.88, 63 (4–507)
Heterozygous wt/mutant	44.54%	32.71%	0	2%	29.27 $\pm$ 17.60, 24 (4–102)	27.58 $\pm$ 20.04, 23.5 (2–98)	—	45.00, 48.00 nmol/ml/h
Homozygous mutant	5.45%	3.73%	0	0%	1.21 $\pm$ 1.07, 2 (0–2.07)	1.53 $\pm$ 1.15, 3 (0–3.57)	—	—
Wild-type allele frequency	0.73	0.79	1.00	0.98	—	—	—	—
Mutant allele frequency	0.27	0.21	0	0.02	—	—	—	—

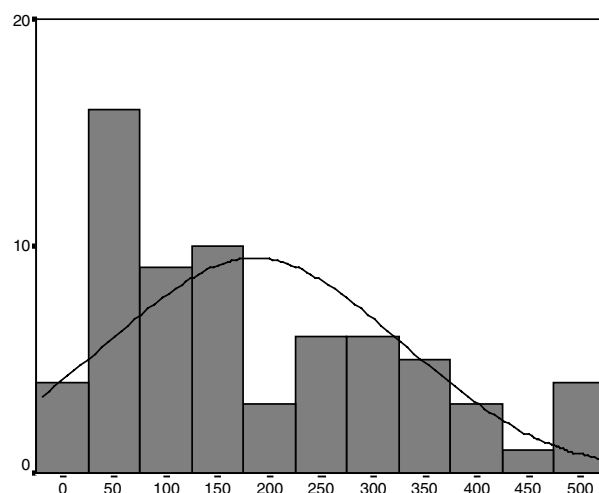
**Figure 2** Distribution of Chit activity levels in Sicilian subjects (value expressed as nmol/ml/h) (s.d.=16.25; mean=34.1 nmol/ml/h; number of subjects analyzed=100).

( $P < 0.0001$ )). The value of plasma Chit activity in subjects homozygous for the mutant allele was  $1.21 \pm 1.07$  (Table 1).

In Sardinian healthy subjects, the levels of plasma Chit activity were comparable to that found in Sicilian subjects in relation to the allele composition (Table 1).

The distribution of plasma Chit activity values in subjects from Sicilian origin is shown in Figure 2 and the curve of the distribution of plasma Chit activity in Sardinian subjects was perfectly comparable to the Sicilian population.

In African subjects (both from Benin and from Burkina Faso), the plasma Chit activity was significantly higher

**Figure 3** Distribution of Chit activity levels in African children with acute malaria (values expressed as nmol/ml/h) (s.d.=141.0; mean 185.0 nmol/ml/h; number of subjects analyzed=67).

than in Sicily and Sardinia ( $P < 0.0001$ ). We found only two heterozygote subjects for mutant allele who showed Chit activity levels of 45 and 48 nmol/ml/h, respectively, only among the subjects from Burkina Faso.

In African children with acute *P. falciparum* malaria, the levels of plasma Chit activity were higher than healthy subjects (mean  $185.0 \pm 141.0$  nmol/ml/h; median 150 nmol/ml/h, range 11–521 vs  $285.65 \pm 73.5$  nmol/ml/h; median 64 nmol/ml/h, range 4–350;  $P < 0.0001$ ) and the distribution of Chit values in these children showed a bimodal behavior (Figure 3). A group of 40 children had a mean of  $84.77 \pm 50.00$  nmol/ml/h and a median 75 nmol/ml/h (range 11–188) and another group of 27 children showed a higher level of plasma Chit activity (mean  $334.14 \pm 91.00$ , median 300, range 220–521;  $P < 0.0001$ ).

## Discussion

The results of our study suggest a relationship between the presence or persistence in Sicily, Sardinia, the Netherlands and other Mediterranean countries (Spain, Israel), where the Chit polymorphism was genotyped, for a high percentage of 24-bp duplication and the improved environmental conditions and/or the disappearance of parasitic diseases. On the other hand, in sub-Saharan regions studied (Benin and Burkina Faso), the widespread parasitic diseases and the poor social status may have contributed to the maintenance of the wild-type Chit gene. These results suggest that subjects bearing the mutant allele might exhibit an elevated susceptibility to infections sustained by parasites. This is in accordance with the findings reported by Choi *et al*,<sup>15</sup> who showed that in a total of 216 individuals from South India genotyped for Chit, the homozygous condition for the defective allele (the HH variant CHIT1 genotype) was associated with the absence of plasma Chit activity and with an elevated susceptibility to human *Wuchereria bancrofti* filarial infection,<sup>15</sup> confirming the importance of Chit in the protection against the chitin-containing pathogens.

The higher levels of plasma Chit activity of African subjects might be surprising if we consider that these levels only reflect the homozygous wild-type allele condition, but the enlarged (up to 507 nmol/ml/h value) distribution curve of plasma Chit activity suggests that among the African population, several subjects may be affected by silent parasite infections, especially in the intestinal apparatus. This observation cannot be excluded since the African population samples were collected during a program of health balance consisting clinical examination and blood sampling for routine analysis.

The study of the Chit activity in children with acute *P. falciparum* malaria showed that in a group of 40 children, the Chit activity was in the range of the African population, whereas in another group of 27 children these levels were significantly higher. In our previous

report,<sup>9-16</sup> a correlation between this parameter of macrophage activation, the level of serum ferritin and platelet count in children with acute malaria was found. This result suggested that Chit could have a role in the immune response and in the outcome of malaria infection.<sup>17</sup>

Moreover, in chimpanzee, it has been demonstrated that IL-12 injection is associated with an enhanced Chit activity,<sup>18</sup> this finding is important since a recent study has shown that protective immunity in malaria is mediated by a cascade of events involving IL-12.<sup>19,20</sup> We cannot rule out the possibility that the elevated levels of IL-12 modulate the macrophage activity stimulating their microbicidal function through a pathway involving Chit activity. This observation and the finding of a very low incidence (0-2%) of heterozygotes for Chit-mutated allele in African subjects (Benin and Burkina Faso) confirms the hypothesis that the persistence of parasitic diseases could have favored the maintenance of the wild-type Chit gene in sub-Saharan regions.

Therefore, the susceptibility to malarial infection and other parasitic disease may be dependent on the Chit allele composition and consequently on the levels of plasma Chit activity. In fact, in Benin where the climatic conditions have favored the *P. falciparum* malaria endemicity, we did not find any subject heterozygote for the mutant allele.

These considerations support the idea that Chit is phylogenetically old as well as the primitive role of macrophages in the defense mechanism against protozoa and helminths. However, the worldwide level diffusion of the Chit mutated allele from Israel to Europe also suggests that this mutation is relatively ancient.

In the populations living in the Mediterranean area and in the Netherlands (where the malaria infection and other parasitic diseases were eradicated after the Second World War),<sup>21-23</sup> plasma Chit activity levels were found to be lower than in the sub-Saharan populations. Moreover, the small differences occurring between Sicilian and Sardinian subjects found in the present and in a previous study<sup>24</sup> could also be related to the diversity of

**Table 2** Comparison between the frequencies of wild-type and mutant Chit alleles in different populations and their correlation with genetic anomalies associated with malaria

Geographical areas	Wild/wild (%)	Wild/mut (%)	Mut/mut (%)	Malaria endemicia	G6PDH deficiency	Hb C hemoglobinopathy	Hb S hemoglobinopathy	Thalassemia syndromes	Ref.
Benin ( <i>n</i> =100)	100	0	0	++++ (actual)	++++	++++	++++	++++ (alpha)	—
Burkina Faso ( <i>n</i> =99)	98	2	0	++++ (actual)	++++	++++	++++	++++ (alpha)	27
Sardinia ( <i>n</i> =107)	65.56	32.71	3.73	+++ (past)	+++	----	----	+++	24
Israel ( <i>n</i> =68)	60.3	33.8	5.9	++ (past)	++	----	----	++	22
Netherlands ( <i>n</i> =171)	58.5	35.1	6.4	+ (past)	+	----	----	+	23
Spain ( <i>n</i> =116)	54.3	39.6	6.03	+ (past)	+	----	----	+	21
Sicily ( <i>n</i> =100)	51.01	44.54	5.45	+ (past)	++	----	+— (restricted)	++	24

<sup>a</sup>*n*: number of subjects analyzed.

environmental factors between these two populations. Sardinian subjects come from a geographical area with a past intermediate malarial endemicity in comparison to East Sicily where malaria was less endemic, and this is confirmed by more elevated frequency of the  $\beta$ -thalassaemia trait and G6PD deficiency in Sardinia.<sup>25,26</sup> The presence of *P. falciparum* should have provided an advantage to the  $\beta$ -thalassaemia and G6PD deficiency genes and at the same time would have favored the wild-type Chit allele. In fact, studies on Sicilian<sup>8</sup> and Sardinian  $\beta$ -thalassaemia patients<sup>24</sup> demonstrated that Chit levels in Sardinia were higher than in Sicily. This finding correlates with the different percentages of mutated alleles found among these two populations (32.71 vs 244.54%) and suggests that the progression in the percentage of heterozygotes for the mutant allele, from Israel to Sicily, is inversely proportional to the disappearance of malaria and the reduction of genetic anomalies associated with malarial infection (see Table 2).

In conclusion, our results provide evidence that the low frequency or the absence of mutant Chit gene may represent a protective factor in the population still living in disadvantaged environmental conditions favoring the parasitic diseases. Nevertheless, we cannot rule out the possibility that the loss of the original action of this enzyme against protozoa- and chitin-coated parasites, as the evolutionary consequence of the parasite's disappearance, could represent a requisite to gain other important physiological roles.<sup>18–27</sup> However, in the absence of evidence of reproductive advantages of the heterozygote carrier for the mutant Chit allele, this last hypothesis needs further investigation.

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