

## Frequency and biochemical expression of C282Y/H63D hemochromatosis (HFE) gene mutations in the healthy adult population in Italy

Stefano Cassanelli<sup>1</sup>, Elisa Pignatti<sup>1</sup>, Giuliana Montosi<sup>1</sup>, Cinzia Garuti<sup>1</sup>, Maria Mariano<sup>2</sup>, Daniele Campioli<sup>3</sup>, Anna Carbonieri<sup>3</sup>, Erasmo Baldini<sup>2</sup>, Antonello Pietrangelo<sup>1,\*</sup>

<sup>1</sup>Department of Internal Medicine, Unit for the Study of Disorders of Iron Metabolism, University of Modena and Reggio Emilia, Policlinico, Via del Pozzo 71, 41100 Modena, Italy

<sup>2</sup>Blood Transfusion Service, Azienda Policlinico di Modena, Modena, Italy

<sup>3</sup>Laboratory of Clinical Chemistry, Azienda Policlinico di Modena, Modena, Italy

**Background/Aims:** The actual prevalence of the main hemochromatosis (HFE) mutations in the Italian adult population and their phenotypic expression have not yet been established. This information is key to advocate a mass-screening program.

**Methods:** Two thousand one hundred adults were tested for the C282Y/H63D HFE gene mutations by an automated genotyping assay as well as transferrin saturation (TS) and serum ferritin levels.

**Results:** No homozygotes for the C282Y mutation were found. Heterozygosity for the C282Y mutation was 3.1%, while heterozygosity and homozygosity for the H63D mutation were 21.5% and 2.5%, respectively. TS was significantly higher in C282Y heterozygotes and H63D homozygotes as compared to wild-type individuals ( $P < 0.01$ ). Interestingly, of the HFE wild-type subjects 5.9% had a TS value above the 45% threshold.

**Conclusions:** This study shows that (i) the predicted prevalence for C282Y homozygosity in Italy is 1:3900; (ii) the C282Y/H63D wild-type population has an increased baseline of iron parameters possibly due to genetic factors not linked to the C282Y/H63D mutations; (iii) since in the latter population the actual tissue iron burden cannot be assessed, phenotypic (TS) screening in Italy is not recommended until the true prevalence of all mutations in the HFE gene and in other hemochromatosis genes will be established.

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**Keywords:** HFE gene; Hemochromatosis; Ferritin; Transferrin saturation; C282Y

### 1. Introduction

Hereditary hemochromatosis (HC) represents one of the most common single-gene hereditary diseases. Studies using genotyping have estimated the prevalence to be 1:100–1:250 [1]. A cornerstone of HC genetics was laid in 1996, when the discovery of the HC gene, called *HFE*, was published [2]. The majority of the HC patients (83–100% in the different series) had the same mutation, changing

cysteine at position 282 to tyrosine (C282Y) in the HFE protein [2]. A second mutation, that changes histidine at position 63 to aspartic acid (H63D) is present in a minority of patients but its role in the pathogenesis of the disease is uncertain [3].

The isolation of the *HFE* gene provided the opportunity to directly analyze the effect of gene mutations on phenotype. The existence of a highly prevalent mutation supports the concept of a ‘founder effect’ (i.e. the genetic mutation causing hemochromatosis has been a unique event originated in a single individual), but it makes difficult to explain the phenotypic variation on the basis of *HFE* genotypes. Recent studies showed that only 64% of Italian patients were homozygous for the C282Y substitution [4]. This

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\* Corresponding author. Tel.: +39-059-4222714; fax: +39-059-4224363.

E-mail address: pietra@unimo.it (A. Pietrangelo).

proportion was similar in other southern European populations [5]. In fact, we showed that at least another form of hemochromatosis may be present in these areas [6].

Although these observations suggest that frequency of the HFE-associated form may be lower in southern Europe, no data are available. This information is a prerequisite for advocating a mass-screening program for HC. To establish the actual frequency of the HFE mutations and the relationship between genotype and phenotype in an adult healthy population in Italy, we carried on a large-scale prospective screening study in a presumably healthy population. We examined 4200 chromosomes from Italian adults and measured serum iron indices in all genotyped subjects, in order to determine the allele frequencies and evaluate the effect of genotype on iron parameters, particularly on transferrin saturation (TS) and serum ferritin (SF).

## 2. Material and methods

### 2.1. Subjects

The study was carried out from September 1997 to June 1999. Fasting blood samples were obtained from 2100 voluntary repeat plasma-platelet-pheresis donors, 1122 men and 978 women, at the of Blood Transfusion Service at the Policlinico of Modena. The study was approved by the Ethics Committee at the University of Modena and all subjects gave written informed consent. Serum iron, transferrin and SF were measured by standard methods in all subjects on morning samples after an overnight fast. TS was also calculated. Blood samples were stored at  $-20^{\circ}\text{C}$  for up 3 months before performing the genetic analyses (see below). All individuals were investigated for any history of blood transfusion, iron medications and daily ethanol consumption. Chronic viral hepatitis infection was investigated by searching for the presence of hepatitis B virus (HBV) surface antigen and hepatitis C virus (HCV) antibodies, and HCV-RNA in the serum by standard laboratory methods.

Hemoglobin electrophoresis, glucose-6-phosphate dehydrogenase and pyruvate kinase, lactate dehydrogenase, haptoglobin and reticulocyte count, were assayed in all subjects.

### 2.2. HFE genotype

Template DNA was extracted from blood samples by using an ion-chelating resin (Chelex-100, Bio-Rad, Richmond, CA) as previously described [7].

Genotyping for the C282Y and H63D mutations was done by using TaqMan-Technology (PE, Apply Biosystem, Foster City, CA). This PCR detection system exploits the  $5' \rightarrow 3'$  endonuclease activity of the Taq DNA polymerase which digests an internal probe labeled with fluorescent reporter dye at the  $5'$  end and a fluorescent quencher dye at the  $3'$  end [8]. In the intact probe, the fluorescence from the reporter is suppressed owing to its spatial proximity to the quencher. During the extension phase of the polymerase chain reaction (PCR) cycle the nucleolytic activity of the DNA polymerase cleaves the probe hybridized to a target sequence within the PCR products. This results in the separation of the reporter and the quencher dyes and in the concomitant increase of reporter fluorescence. The fluorescent emission is monitored using a 96-tube microplate reader (7200 Sequence Detector System, PE, Applied Biosystem) which allows high throughput [9]. The system utilizes more than one reporter dye so that two couples of TaqMan probes and primers were designed for the rapid detection of the C282Y and H63D mutations. Each probe consisted in a specific oligonucleotide labeled with the same  $5'$  end quencher dye TAMRA (6-carboxy-*N,N,N',N'*-tetramethylrhodamine) and a different  $3'$

end reporter dye, FAM (6-carboxyfluorescein) or TET (6-carboxy-4,7,2',7'-tetrachlorofluorescein), to identify wild-type and mutated alleles, respectively. For the detection of the C282Y the following pair of primers and probes were used: forward primer  $5'$ -TCACATACCCAGATCA-CAATGA- $3'$ , reverse primer  $5'$ -AGGGCTGGATAACCTTGGCT- $3'$ , wild-type allele probe  $5'$ -FAM TGCTCCACCTGGTACGTA-TATCTCTGCTCT T TAMRA - $3'$  and mutated allele probe  $5'$ -TET TGCTCCACCTGGCAGGTATATCTCTG TAMRA- $3'$ . To discriminate the presence of the H63D mutation the following pair of primers and probes were designed: forward primer  $5'$ -TTCTACTGGAAACCCATG-GAGTTC- $3'$ , reverse primer  $5'$ -TGTTTGAAGCTTTGGGCTACGTT- $3'$ , wild-type allele probe  $5'$ -FAM CGGCGACTCATGATCATAGAACACG TAMRA- $3'$  and mutated allele probe  $5'$ -TET CGGCGACTCT-CATCATCATAGAACACG TAMRA- $3'$ . The PCR reactions were performed in a final volume of 25  $\mu\text{l}$  containing:  $1 \times$  TaqMan Buffer A, 8% glycerol, 5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dATP, dCTP, dGTP and 400  $\mu\text{M}$  dTTP, 0.05 U/ $\mu\text{l}$  AmpliTaq Gold DNA polymerase (PE, Applied Biosystems), 0.01 U/ $\mu\text{l}$  AmpErase UNG (Uracil-*N*-glycosylase), 3  $\mu\text{l}$  template DNA and a different amount of primers and probes according to the specific mutation. In the case of the discrimination assay for the C282Y mutation 300 nM of each primer, 50 nM of wild-type allele probe and 100 nM of mutated allele probe were used. For the H63D mutation 900 nM of each primer, 50 nM of wild-type allele probe and 200 nM of mutated allele probe were utilized. Each set of reaction included six samples of the following controls: no amplification controls (NAC: reaction samples without DNA polymerase), no template control (NTC: reaction samples without DNA template), negative control reaction (containing 25 ng of genomic DNA extracted from blood samples of normal subjects) and positive control reaction (containing 25 ng of standard genomic DNA extracted from blood samples of subjects homozygous for the specific HFE gene mutation examined). All reactions were done in a 96-well reaction plate using 9700 GeneAmp PCR System (PE, Applied Biosystem,) upon the following thermal profile:  $50^{\circ}\text{C}$ , 2 min;  $94^{\circ}\text{C}$ , 10 min; 40 cycles of  $94^{\circ}\text{C}$ , 15 s and  $62^{\circ}\text{C}$ , 1 min. To assess the ability of the TaqMan assay to identify the wild-type and mutated alleles properly, exons 2 and 4 of the HFE gene were amplified from several DNA samples as previously described [2]. The PCR products were digested with the restriction enzymes *Sna*BI and *Sau*3AI (Promega, Madison, WI) to detect the C282Y and H63D mutations, respectively.

### 2.3. Statistical analysis

The results were expressed as median and range. A non-parametric approach was used to evaluate differences in demographic and biochemical features between groups of subjects: Kruskal–Wallis test (analysis of variance, ANOVA), Mann–Whitney test (pairwise comparison), Dunn's test (multiple comparison), Wilcoxon's rank sum test (to verify differences in biochemical traits after 1 year follow-up), Fisher's exact test (to reveal discrepancies in prevalence). All *P* values are two tailed;  $P < 0.01$  was considered statistically significant. Data analysis was carried out using the statistical package SPSS 7.5 (SPSS Inc., Chicago, IL).

## 3. Results

Table 1 shows the genotypes and allele frequency of the studied population according to gender. No one homozygous for the C282Y mutation was found in the studied groups. Among 1122 men, 3.3% were heterozygous for the C282Y mutation while 20% were heterozygotes and 2.5% homozygotes for the H63D mutation. Within the 978 women, 3.0% were heterozygous for the C282Y mutation while 23.3% were heterozygotes and 2.4% homozygotes for the H63D mutation. Two compound heterozygous, both women, were identified (0.1%). Overall,

**Table 1**  
**HFE genotype frequency<sup>a</sup>**

C282Y/H63D genotype	- - / - - n (%)	- - / + - n (%)	- - / + + n (%)	+ - / - - n (%)	+ - / + - n (%)	Allele C282Y frequency ( $\pm 95\%$ CI)	Allele H63D frequency ( $\pm 95\%$ CI)
Men (n = 1122)	833 (74.2)	224 (20.0)	28 (2.5)	37 (3.3)	0 (0.0)	1.6 $\pm$ 0.5	12.5 $\pm$ 1.4
Women (n = 978)	695 (71.2)	228 (23.3)	24 (2.4)	29 (3.0)	2 (0.1)	1.6 $\pm$ 0.6	14.2 $\pm$ 1.5
Total (n = 2100)	1528 (72.8)	452 (21.5)	52 (2.5)	66 (3.1)	2 (0.1)	1.6 $\pm$ 0.4	13.3 $\pm$ 1.0

<sup>a</sup> n, number of subjects tested; CI, confidence interval; genotype: - indicates the wild-type allele, + indicates the mutated allele.

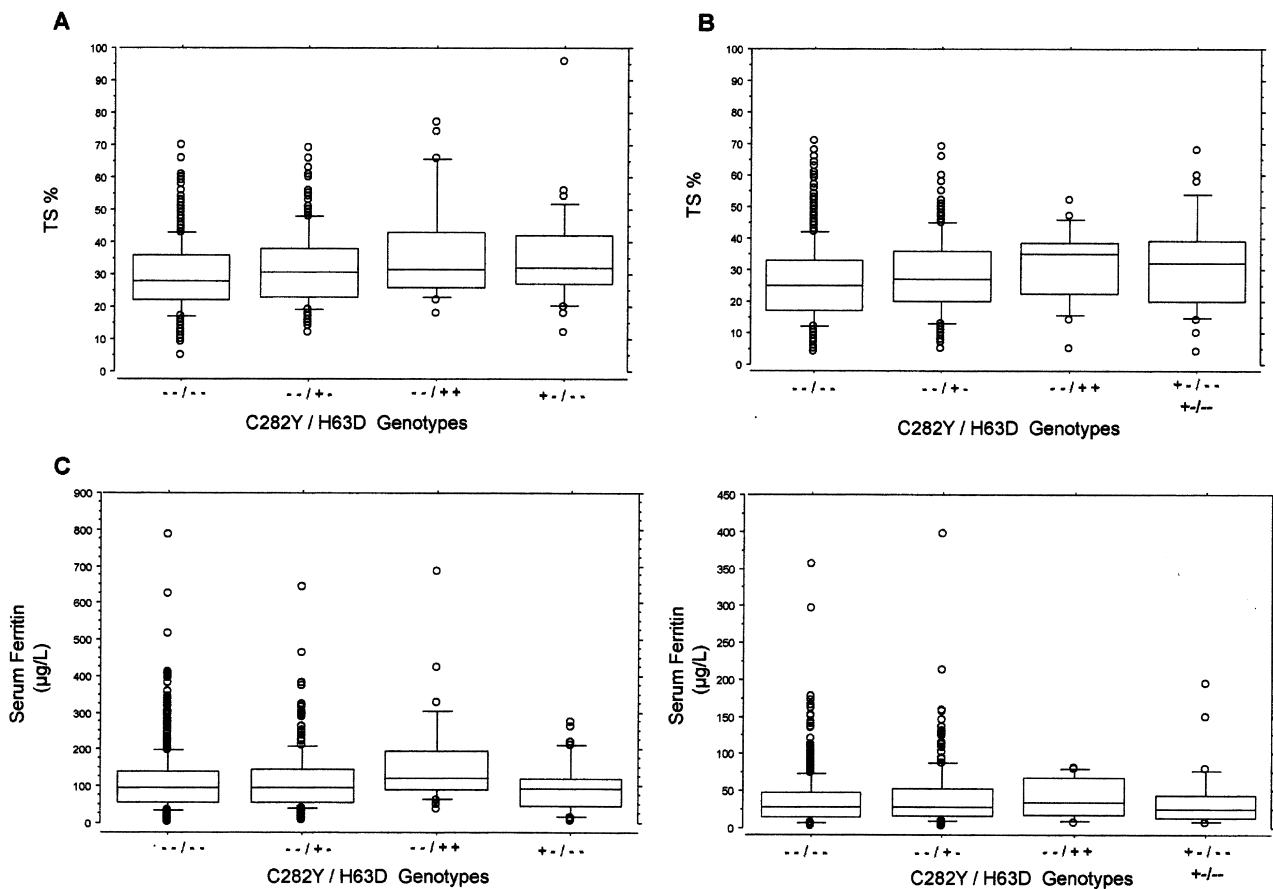
in the studied population, heterozygosity for the C282Y mutation was 3.1%, while heterozygosity and homozygosity for the H63D were 21.5 and 2.5%, respectively.

According to these results, the overall C282Y allele frequency in this population was 1.6% and that of the H63D 13.3%. These figures indicate a 1:3900 prevalence for C282Y homozygosity.

As to the clinical data of the studied population, daily alcohol consumption was less than 10 g/day in all subjects and none had viral hepatitis serological markers. TS levels differed significantly between individuals with different genotype. Significance was  $P < 0.005$ , in men and  $P < 0.003$  in women (Kruskal–Wallis, ANOVA). The TS distri-

bution has been graphically reported in Fig. 1A,B. In both gender groups, the median TS value was significantly higher in C282Y heterozygotes and the H63D homozygotes as compared to individuals carrying the wild-type alleles (Dunn's multiple comparison test,  $P < 0.01$ ) (Table 2). The distribution of SF values was highly skewed in our population (Fig. 1C,D). Differences in SF levels were not statistically significant among subjects with different genotypes, regardless of the gender. However, there was a trend toward higher SF levels in H63D homozygotes (median value 110  $\mu\text{g/l}$  in men and 35  $\mu\text{g/l}$  in women).

In all subjects carrying the C282Y mutation, SF levels fell within the normal range, i.e. below 200  $\mu\text{g/l}$  for women and



**Fig. 1.** Transferrin saturation and serum ferritin distributions. Box plot of transferrin saturation (TS) (%) (A,B) and serum ferritin (SF) ( $\mu\text{g/l}$ ) (C,D) against genotype for men (A,C) and women (B,D). Medians are indicated by a line across the boxes, and the first and third quartiles are indicated by the bottom and top edges of the boxes, respectively. Open circles indicate outlying points. HFE genotype identification as in Table 1.

**Table 2**  
Serum iron tests according to HFE genotypes and gender<sup>a</sup>

Sex	Genotype C282Y	Genotype H63D	Age (years)	TS (%) (range)	SF ( $\mu\text{g/l}$ ) (range)
M	-/-	-/-	39 (20–65)	28 (5–73)	95 (2–786)
M	-/-	+/-	40 (21–62)	30 (12–72)	94 (8–664)
M	-/-	+/+	39 (22–60)	32 (18–77)*	110 (37–686)
M	+/-	-/-	41 (23–61)	33 (12–96)*	94 (4–276)
F	-/-	-/-	37 (19–62)	25 (4–73)	28 (4–351)
F	-/-	+/-	36 (19–63)	27 (5–69)	28 (5–390)
F	-/-	+/+	36 (20–59)	33 (5–52)**	35 (7–81)
F	+/-	-/-	35 (21–59)	32 (4–68)**	27 (6–195)
F <sup>b</sup>	+/-	+/-	(26–36)	(35–58)	(10–33)

<sup>a</sup> Median values for serum ferritin and transferrin are shown. TS, transferrin saturation; SF, serum ferritin; genotype: - indicates the wild-type allele, + indicates the mutated allele. \* $P < 0.01$ , significant difference vs. subjects carrying wild-type alleles; \*\* $P < 0.01$ , significant difference vs. both subjects carrying wild-type alleles and subject carrying one H63D allele (Dunn's multiple comparison test).

<sup>b</sup> The range values for two C282Y/H63D compound heterozygotes are reported.

300  $\mu\text{g/l}$  for men. Also, two compound heterozygotes, aged 26 and 36 years, showed normal SF values, while the older one had TS above 45%. All 68 C282Y carriers were retested for TS and SF 1 year after the first evaluation. No significant differences in these parameters were found as assessed by the Wilcoxon's sum rank test (data not shown).

As to the TS, 17.6% of C282Y carriers (12 out of 68) and 5.9% of C282Y/H63D wild-type subjects (120 out of 2032) had a TS value above the 45% threshold (Fig. 1A,B). In the case of SF, 39 out 2100 subjects (1.8%) had values above the chosen threshold. Most of them (34/39) carried the HFE wild-type (normal) alleles.

Since none of the C282Y carriers showed SF levels above the threshold or abnormal liver enzymes levels, liver biopsy was not considered. The same consideration applied to the C282Y/H63D wild-type individuals.

In this study iron deficiency was defined as SF of 12  $\mu\text{g/l}$  and transferrin saturation  $\leq 15\%$ . In our series 8.6% of women (84/978) and 0.7% of men (8/1122) met these criteria, two of whom were C282Y heterozygotes.

#### 4. Discussion

The total allele frequency for the C282Y mutation of the HFE protein in Europe is 3.8 ( $\pm 0.7$  at 95% CI) according to a recent study in control chromosomes [1]. In particular, C282Y allele frequencies are distributed among a decreasing cline from north to south Europe. This geographical distribution reflects the higher prevalence of C282Y mutation in populations having a heaviest Celtic component [10,11] as compared to other European populations such as Spanish [12–14]. Preliminary reports carried out in small cohorts of Italian subjects, stressed these ethnic differences, showing that the C282Y allele occurred rarely in healthy individuals in Italy (0.5%) [1]. The present study, performed in 4200 chromosomes from Italian presumably healthy individuals, has established the actual C282Y allele and predicted HC homozygous frequency in a southern

European adult population. The reported figures are probably among the lowest reported in Europe. In this context, not surprisingly, this large population screening study, while identifying two female compound heterozygotes, has not detected homozygotes for the C282Y mutation.

As far as the biochemical expression of different genotypes is concerned, it should be pointed out that a study in blood donors may suffer by nature of limitations (i.e. falsely low iron store as a result of regular donation). Nevertheless, in our study, the donors were enrolled in a program of regular plasma-plateletpheresis donations (four donations per year) and did not undergo a whole blood withdrawal. Therefore, no significant perturbations of body iron balance are expected in this population.

Considering the subjects carrying the same genotype, higher TS and SF levels were detected in men vs. women (Table 2), as in other studies [15,16], possibly due to the effect of menstrual blood loss. In both gender groups, the median TS value was significantly higher in the C282Y heterozygotes and in H63D homozygotes as compared to subjects with wild-type genotype ( $P < 0.01$ , Dunn's multiple comparison test), and it was not due to age differences within each gender group (Table 2). To date, homozygosity for the C282Y mutation has been generally considered the predominant factor leading to HC [17]. Previous data on the clinical expression of HC heterozygotes have been collected in pedigree or case-control studies identified by HLA typing [16,18,19]. They found a modest but appreciable elevation in TS, although in most cases the values remained within the normal range. The only studies available that used HFE genotyping were unable to find significant difference in TS levels in C282Y heterozygotes [15,20]. This discrepancy between the latter reports and our study may be due to differences in the study design or in the population investigated. Results from other studies are of limited information since subjects carrying only one C282Y mutation were pooled with compound heterozygotes (C282Y/H63D) [4,21–23].

SF levels in C282Y heterozygotes were not significantly

higher than in wild-type individuals. This is in agreement with the notion that body iron stores are usually normal or only slightly increased in C282Y heterozygotes [24] and with the result of other recent studies showing no increase in SF levels using HFE genotyping [15,20,23]. It is pertinent to mention that 89% of subjects with SF levels above the threshold had a wild-type genotype.

As to the H63D mutation in the HFE protein, initial evidence suggested that its role in the expression of the disease is minor or, at least, confined to the compound heterozygous state (C282Y/H63D) [20,25]. In this report only two compound heterozygotes were detected, both women. Because of the small number, it is not possible to evaluate the relative role of this HFE genotype on phenotype expression. However, these women had normal SF levels while only the oldest showed a TS above 45%. In this study we found that subjects homozygotes for H63D mutation had higher TS levels than subjects carrying wild-type alleles, in both gender groups (Table 2). A similar observation has been reported in other case-report studies [3,4,26] and, more recently, in large screening studies [15,16,27]. Moreover, Fairbanks et al. [28], re-analyzing all the data from ten studies, found that the proportion of H63D homozygotes with HC was nearly three times higher than the proportion of H63D homozygotes in the control group, when the H63D allele frequency was corrected for the number of C282Y negative chromosomes.

Although the exact cut-off test for TS is controversial, it appears now to be decreasing [29] and a threshold of 45% has been recently proposed for both men and women [30,31]. In our study, 16.7% (11/66) of the C282Y heterozygotes had a TS above 45%. In a recent study in hospital employees in Norway, 10.9% of C282Y heterozygotes had a TS above 50% [23]. This may indicate that C282Y heterozygotes from south Europe may express a more severe biochemical phenotype than those from north Europe. On the other hand, factors present in the general population in southern Europe may affect these parameters [32]. In fact, in this study, 7.8% of individuals carrying C282Y/H63D wild-type alleles had TS above 45%. This figure is significantly higher than that found in other populations with northern European ancestry (0.5%) [31]. Seemingly, 62% of subjects with TS above the threshold had a C282Y/H63D wild-type genotype. It must be emphasized that none of the C282Y/H63D HFE wild-type individuals with high TS presented other factors which might affect iron parameters (i.e. alcohol abuse, thalassemia, viral hepatitis). Therefore, iron parameters in the general population in southern European countries appear to be different as compared to northern European countries. Among the iron deficient individuals in this study, only 2.9% (two out of 68 subjects) were C282Y carriers as compared to 6.7% (five out of 75 individuals) in a study from north Europe [23]. Iron deficiency was not uncommon in the past, especially in Northern European countries, where dietary habits led to a lower iron intake [32]. In this vein it has been suggested that the

high frequency of C282Y mutation in north Europe might be the result of a selection advantage for C282Y carriers [22]. In south Europe, environmental factors (i.e. a diet with a rather high meat intake and a richer content in fresh fruits and vegetables) might positively affect the iron balance in the general population. However, it is unlikely that these factors, which may very well decrease the occurrence of 'iron deficiency' (see above), may also account for the high TS documented in the C282Y/H63D HFE wild-type population. Therefore, a more probable explanation is that other HFE mutations or, more likely, genetic determinants non-HFE related (e.g. [6]) in south Europe may be responsible for the difference in the iron status.

In terms of population screening programs, due to the negative results of the present study, it is not possible to evaluate the ability of TS to detect HC homozygotes in countries like Italy. We cannot prove that the C282Y/H63D wild-type individuals with high TS have iron overload, although the normal serum ferritin makes this unlikely. Ideally, a liver biopsy would be indicated to prove iron overload. Since none of these subjects had serum transaminase increase we considered it not ethical to offer a liver biopsy. Only when the true prevalence in Italy of all the mutations in the HFE gene and in other hemochromatosis genes will be established, the utility of the phenotypic screening can be re-evaluated. At the present time, the predictive value of the TS test may still be high even in Italy, when dealing with high-risk individuals such as subjects with an affected family member or patients with HC-associated illness (e.g. diabetes, liver disease, hypogonadism).

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