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Mutations of the hemochromatosis gene in Italian candidate blood donors with increased transferrin saturation

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The aim of this study was to analyze the role of HFE mutations in blood donors with iron parameters suggesting iron overload, taking into account the regional distribution of HFE mutations in Italy. We studied 5880 subjects undergoing evaluation for blood donation eligibility, from different areas of Italy. Abnormal iron parameters were defined as transferrin saturation (TS) $>50\%$ or $>45\%$ and serum ferritin (SF) >300 or $>250\,\mu g/ml$ in males and females, respectively. Subjects with increased TS and/or SF were re-tested and typed for HFE mutations C282Y and H63D. A total of 548 individuals had increased iron parameters at first testing. In total, 179/548 were available for retesting, and in 109 increased TS and/or SF were confirmed. Increased TS was confirmed in 25 individuals, among whom three were C282Y homozygotes and six were compound heterozygotes for C282Y and H63D. Increased TS was more frequent in northern Italy than in southern regions. In individuals with increased TS and/or SF, the frequency of C282Y and H63D was 0.13 and 0.21 in northern-Italy versus 0.05 and 0.45 in southern Italy ($P=0.004$ for H63D). Nine out of 10 individuals carrying hemochromatosis-associated genotypes (including compound heterozygosity for C282Y and H63D) originated from northern regions. Among controls, the allelic frequencies of C282Y and H63D were 0.037 and 0.16 in the northern regions and 0.015 and 0.16 in the southern regions. In conclusion, over one-third of individuals with persistently altered TS carried hemochromatosis-associated genotypes, confirming that a diagnostic approach based on TS and genotyping of selected cases may represent a viable screening procedure.
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Introduction

Hemochromatosis is a common autosomal recessive genetic disorder leading to progressive iron accumulation and parenchymal organ damage due to excessive absorption of dietary iron.[1] The iron loading and subsequent organ damage can be completely prevented by an early diagnosis of the defect and depletion of excessive iron stores by regular venesection.[2] The diagnosis of hemochromatosis has previously been based on the biochemical and histological evaluation of the abnormal iron status in blood and liver. However, this diagnostic approach presents several limitations, the most relevant being the progressive character of iron accumulation and the possible interference of factors, mainly acquired, able to modify the rate of iron accumulation and the clinical manifestation of the disease.[3] The identification of HFE, a gene encoding for a protein involved in cellular iron metabolism involved in hemochromatosis, and of two common mutations tightly associated with the disease,[4] offers the possibility to use diagnostic tools that are unaffected by the iron status of the proband, and therefore is useful for a preclinical diagnosis of the disease. The diagnostic usefulness of a genetic marker in a population, however, is influenced by the range of genetic heterogeneity of the

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disease in that population. In northern European countries, over 90% of the typical hemochromatosis patients are homozygous for C282Y, the main hemochromatosis mutation.[5] In the Mediterranean area, however, the prevalence of this mutation among hemochromatosis patients is lower,[6–9] and the diagnostic use of genotyping for early detection can be questioned. In Italy, for instance, less than 70% of hemochromatosis patients are homozygous for C282Y, and the figure could be as low as 34% in southern and insular regions.[8] In the presence of a heterogeneous geographical distribution, a preliminary evaluation of the regional prevalence of HFE C282Y, and of its association with iron overload, is necessary in order to define a screening strategy for hemochromatosis. The relationship of the second common mutation of HFE, H63D, with iron overload is less defined. Homozygosity for this mutation does not cause hemochromatosis, but its frequency is increased in patients with hemochromatosis,[4] suggesting that, in association with C282Y and other genetic or acquired factors, it can indeed contribute to iron overload.

The aims of this study were to determine the prevalence of the two common HFE mutations among Italian blood donors of different regional origin, and to examine their association with abnormal iron status as detected by standard biochemical tests.

Subjects and methods

In total, 5880 candidate blood donors (age range 18–60 years) from eight transfusional centers were tested for iron parameters before being admitted to blood donation. Among them, 4058 (2404 males and 1654 females) were from northern Italian regions and 1822 (1236 males and 586 females) from southern and insular Italy. Age and sex distributions were similar in the two groups. A control group of 588 subjects provided the allelic frequency of HFE mutations in different regions of Italy. The group of random controls was composed of blood donors from each transfusional center, in the proportion of one subject for every 10 candidate blood donors included in the study.

For each candidate blood donor, age, sex and origin were recorded in addition to routine blood counts and alcohol consumption (>40 mg/kg/day). At first testing subjects were examined for serum iron, transferrin saturation (TS) and serum ferritin (SF). Individuals showing increased iron parameters at first testing were retested within 1–6 months, after an overnight fast. Threshold levels for TS and SF were 50% and 300 ng/ml in males, and 45% and 250 ng/ml in females, respectively. On second testing, in addition to biochemical parameters, HFE genotyping was performed. Informed consent for genetic typing was obtained from subjects undergoing second testing and from subjects belonging to the control group.

Serum iron and serum transferrin were measured by each transfusional service by standard methods and the value of transferrin concentration was converted to total iron binding capacity in order to calculate TS. Serum ferritin was determined by a single laboratory using an immunoturbidimetric method (Ferritin Latex, Parker Lab., Merate, Italy).

The two HFE mutations were detected by polymerase chain reaction (PCR) DNA amplification of the relevant exons and hybridization with allele-specific oligonucleotides using a microplate semiautomated procedure (mDx Hemochromatosis kit, Biorad, Hercules, CA, USA) or restriction analysis with RsaI for C282Y and BclI/MboI for H63D.

The data were analyzed using the statistical package Statview 5.0 (SAS Institute Inc., Cary, NC, USA). Contingency tables were analyzed by the Fisher exact test and two-sided *P*-values were calculated.

Results

At first testing, 548/5880 subjects (9.3%) had values of TS and/or SF above the upper limit. In all, 382 subjects (6.3%) had increased TS, 189 (3.2%) had increased SF and 23 (0.4%) had both parameters increased. Of the 548 individuals showing increased values at first testing, only 179 (153 males and 26 females) were available for retesting iron parameters after an overnight fast and for HFE genotyping within the period of the study. Increased TS and/or SF were confirmed in 109 subjects (100 males and nine females). Of these, 25 had increased TS (with or without increased SF) and 84 had increased SF with normal TS. Increased TS was found in 23/142 (16%) subjects from northern regions and in 2/37 (5%) subjects from the south. In 70 subjects, the altered values of iron parameters were not confirmed at repeated testing.

The overall frequencies of HFE mutations in individuals with confirmed alterations of iron parameters compared with those observed in controls stratified by geographical origin are shown in Table 1. Among controls, the main hemochromatosis mutation C282Y is more common in northern than in southern regions (0.036 versus 0.015), although the difference does not reach statistical significance. H63D frequency in controls is not influenced by geographical origin. Among subjects with increased iron parameters, the allelic frequency of C282Y is significantly increased (0.114 versus 0.031 in controls, $P<0.0001$) and, similar to the

Table 1 Allelic frequency of the two HFE mutations in 109 prospective blood donors with confirmed alterations of iron parameters and in 588 control blood donors originating from northern and southern regions of Italy

	Donors with confirmed alterations of TS and/or SF			*Controls*		
	N	*C282Y*	*H63D*	N	*C282Y*	*H63D*
Northern regions	89	0.129	0.207	406	0.036	0.146
Southern regions	20	0.050	0.450	182	0.015	0.177
	109	0.114	0.252	588	0.030	0.159

control group, is more prevalent in northern regions. Also, H63D is significantly increased in the group with signs of iron overload (0.252 versus 0.162, $P = 0.001$), but in this group the allelic frequency is higher in individuals from southern Italy (0.450 versus 0.207, $P = 0.002$).

Among the 25 subjects with confirmed increased TS, three subjects are homozygous for the C282Y mutation, the genotype commonly associated with genetic hemochromatosis and six subjects are compound heterozygotes for C282Y and H63D, a genotype that can cause severe iron overload. Overall, 68% of subjects in this group carry at least one hemochromatosis mutation and the allelic frequency of C282Y is 0.300, 10 times higher than in controls (0.030). The frequency of H63D in the same group is 0.260 (0.162 in controls). However, considering only chromosomes not bearing the C282Y mutation, H63D was found on 13/35 chromosomes (37%).

Among 84 individuals who had increased values of SF with normal TS the allelic frequency of the two mutations was lower (C282Y: 0.06; H63D: 0.26) and not significantly different from controls. None of these subjects was homozygous for C282Y and only one was a compound heterozygote for the two mutations. Among individuals with isolated increased SF 96% were males, no difference in the geographical distribution was observed, and an increased alcohol intake was reported in 45%. Finally, among the 70 individuals in whom the alteration of iron parameters found at first testing was not confirmed, no genotypes at risk for hemochromatosis were found and the allelic frequency of the two mutations was overlapping with that observed in the control group. Among the 588 controls, none was homozygous for C282Y and eight subjects were compound heterozygotes for the two mutations.

In Table 2, we report the data of 10 individuals bearing hemochromatosis-associated genotypes. Three subjects, all females, are homozygous for C282Y. They are aged 23–38 years, have TS over 80%, but only one has a moderate increase of SF. Seven individuals (six males, one female, age range 24–54 years) are compound heterozygotes for the two mutations. All but one have increased TS (range 36–68%, median 58 years) and mild to moderate increase of SF was observed in four.

Table 2 Prospective blood donors with hemochromatosis-associated genotypes

Origin	*Sex*	*Age (years)*	*TS (%)*	*SF (ng/ml)*	*HFE genotype*
N	F	23	82	435	C282Y homozygote
N	F	38	89	23	C282Y homozygote
N	F	31	94	189	C282Y homozygote
N	M	54	52	325	Compound heterozygote
N	M	47	36	437	Compound heterozygote
N	M	28	65	113	Compound heterozygote
N	F	20	58	70	Compound heterozygote
N	M	32	51	467	Compound heterozygote
N	M	24	59	384	Compound heterozygote
S	M	50	68	734	Compound heterozygote

As for the geographical distribution of genotypes at risk for hemochromatosis, nine of 10 genotypes were found in individuals from northern Italy, while only one subject originating from southern Italy, was a compound heterozygote for the two mutations.

Discussion

Recent studies indicate that the mutation C282Y may be significantly less frequent in Italy as it is in northern Europe.[6] This appears to be in contrast with old estimates, also obtained in blood donors, of heterozygote frequency based on the evaluation of the iron status in blood and liver, which suggested a gene frequency of the main hemochromatosis mutation of 0.09,[10] a value that is similar to the actual frequency of C282Y in northern Europe, and 5–10 times higher than that presently reported in the global population of Italy.[11] An indication of a probable genetic heterogeneity of hemochromatosis in Italy stemmed from the work of Piperno et al.[8]: among Italian patients with a clinical picture meeting the classical criteria for hemochromatosis, only 68% were homozygous for the C282Y HFE mutation. When patients were stratified according to their geographical origin, a significant difference was observed between the northern and southern regions. In the south of Italy, only 34% of patients with a clinical picture of typical hemochromatosis were homozygous for C282Y.

In this heterogeneous context, and in the absence of detailed epidemiological data on the distribution of hemochromatosis mutations in different regions of Italy, it is arduous to define screening and diagnostic criteria based on the use of HFE genotyping.

Our study included a number of genotyped subjects randomly selected among blood donors from the blood banks taking part in the study, in the proportion of one for every 10 subjects included in the main sample, in order to account for geographic heterogeneity. Allelic frequencies for C282Y ranged between 0.014 and 0.057 in northern regions (mean 0.037) and 0.015–0.016 (mean 0.0155) in the south of the country. The highest frequency for C282Y was observed in the north-east and the lowest in the southern regions Puglia and Sicily. The frequency of H63D ranged between 0.11 and 0.21, but the mean frequency (0.16) was identical in northern and southern Italy. In conclusion, the analysis of 588 normal individuals from different Italian regions suggests a relative uniformity of the distribution of H63D along the peninsula, while C282Y is significantly more frequent in north-eastern regions.

While the proportion of individuals with increased values for iron parameters was similar in northern and southern regions, the genetic background related to HFE was different in the two groups. C282Y was more represented in the north than in the south (although the difference between the two values 0.13 and 0.05 did not reach statistical significance) while the frequency of H63D was significantly higher in the south. This finding suggests a geographical difference of the interaction

between genetic and acquired factors. In southern regions, in the presence of a lower frequency of the main hemochromatosis mutation, the interaction of H63D with other genetic or acquired modifiers appears to acquire a more relevant role in the determination of the iron status and particularly of TS.[12] Interestingly, an association between H63D and iron overload was also described in the south of France.[13]

Over 90% of individuals with confirmed alterations of TS were from northern regions and over one-third (9/25, 36%) carried genotypes potentially at risk for hemochromatosis. Three females aged 23–38 years were homozygous for C282Y and six subjects (five males and one female) were compound heterozygous for the two common mutations of HFE. Compound heterozygosity for the two HFE mutations can cause iron overload which, in a minority of cases, can be severe.[14] The prevalence of male individuals among compound heterozygotes for C282Y and H63D confirms the increased penetrance of this genotype in the male sex. As for the C282Y homozygous genotype, the design of our study cannot add elements of discussion to the issue of the penetrance of this genotype.[15–17] Among 588 control blood donors, none carried the C282Y homozygous genotype, while none of the three individuals with increased TS carrying this genotype showed evidence of clinically relevant iron overload. The young age and female sex, however, do not allow a prognostic evaluation of iron loading in these individuals.

Among 84 subjects with normal TS and an increased SF, only one genotype at risk for hemochromatosis was observed and this was a compound heterozygosity. This group was mainly composed of male subjects (96%) and had a significantly increased frequency of alcohol consumption. Frequencies of HFE mutations in this group were slightly higher but not significantly different from controls. SF is increased in subjects with hemochromatosis, but since it is related to the amount of accumulated iron, its specificity is poor in younger individuals and in females. Moreover, SF behaves as an acute-phase reactant with levels increased in inflammation and infection.[18]

Finally, individuals with alterations of iron parameters not confirmed by the second testing had demographical features and allelic frequencies of HFE mutations identical to those of the normal population studied.

Our study adds some information on the geographical distribution of HFE mutations in Italy. The presence of a significant north–south frequency gradient for the main hemochromatosis mutation indicates that other factors, genetic or acquired, may be implicated in a fraction of iron overload cases observed in southern Italy.

The heterogeneity of genetic background is a limitation for the use of HFE genotyping, not only in the context of hemochromatosis screening but also for the identification of blood donors with potential alterations of the iron status. Therefore, genetic testing must be combined, and usually should follow, biochemical tests. The data of this study confirm TS as the most specific test to identify genetically determined alterations of iron status in blood donors, independently from the sex and age of the subject, since a significant proportion of individuals with increased TS carried genotypes at risk for hemochromatosis. SF is less specific, and the majority of alterations of SF observed in this study appeared to be unrelated to HFE genotype and likely to be caused by environmental factors able to influence iron status or to be unrelated to the iron status. However, in donors with increased TS, SF provides a useful indication of the extent of iron loading.

This results of this study suggest that even on a background of genetic heterogeneity and in the presence of environmental factors affecting the iron status, completing the evaluation of candidate blood donors showing increased TS with HFE genotyping may be cost-effective for the prevention of organ damage secondary to iron overload and for the identification of families at risk for hemochromatosis. Moreover, an early genetic diagnosis in blood donors may be useful to identify young individuals carrying iron loading genotypes, in whom frequent donations could be suggested as a means to prevent iron accumulation with a mutual benefit for these subjects and blood banks.

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